［Research Note］

Fungicide Resistant Profiles of *Botrytis cinerea* in a Vineyard

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Grey mould, caused by *Botrytis cinerea*, is one of the major diseases in grapevine. The use of fungicides has been in common practice for many years against *B. cinerea*, whereas the increasing occurrence of fungicide resistant *B. cinerea* has been becoming a severe problem for disease control. In this study, we collected ninety-three strains of *Botrytis cinerea* from an experimental vineyard over three years and examined *in vitro* fungicide resistance test against three types of fungicide, benzimidazole, dicarboximide and *N*-phenylcarbamate, in an attempt to understand the transition of the fungicide resistant profiles in *B. cinerea* in the vineyard. In three years, *B. cinerea* strains resistant to only benzimidazole were predominant in the experimental vineyard and *B. cinerea* strains resistant to either *N*-phenylcarbamate or dicarboximide fungicides could appear shortly after its application.

Key words: *Botrytis cinerea*, fungicide resistance, benzimidazole, dicarboximide, *N*-phenylcarbamate

Introduction

Grey mould caused by the fungus *Botrytis cinerea* Pers ex Fr. (anamorph of *Botryotinia fuckeliana* (de Bary) Whetz) is one of the major diseases in grapes (2, 5). *B. cinerea* infection occurs at early, mid and late growth stages of grapes (2, 3, 5). The presence of grey mould on ripening grapes results in reduced yield and fruit quality. Therefore, this fungus has qualitative and quantitative effects on wine production.

The use of fungicides is a simple strategy to protect plants against *B. cinerea* disease, although biological control of *B. cinerea* is becoming popular due to environmental concerns (3). Three types of fungicides, benzimidazole, dicarboximide and *N*-phenylcarbamate, which were introduced to Japan in 1971, 1980 and 1990, respectively, have been used to control *B. cinerea* disease for many years. Benzimidazole fungicides such as carbendazim, benomyl and thiophanate-methyl have been widely used since its introduction and are still being used extensively, although fungicide resistance have been reported in many crops (4, 8).

Benzimidazole fungicide and diethofencarb, a *N*-phenylcarbamate fungicide, have the same mode of action, which is to inhibit β-tubulin polymerization (1). Since these two fungicides are in relationship in "negative cross-resistance", the use of diethofencarb has been increased against *B. cinerea* populations resistant to benzimidazole fungicides (8). The fungicidal mixture of a benzimidazole fungicide plus diethofencarb was initially introduced in an attempt to exploit the "negative cross-resistance" phenomenon between benzimidazoles and *N*-phenylcarbamates. However, phenotypes exhibiting resistance to these two fungicides have been detected in *B. cinerea* populations (4, 8, 10). Dicarboximide fungicides such as iprodione and procymidone are thought to interfere with the osmotic signal transduction pathway (11). Dicarboximide fungicides superseded the benzimidazole fungicides in the early 1980s, but also suffer from development of dicarboximide resistant *B. cinerea* strains (8, 11).

In order to establish appropriate strategies for fungicide management in vineyards, information of resistant profiles and its dynamics in *B. cinerea* populations is required. The present study was conducted to identify resistance profiles of *B. cinerea* populations to three fungicides in a vineyard over

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Table 1 Fungicide resistance of *B. cinerea* isolates

Origin	Host Plant*	Phenotype**
Hokkaido		
CZ-011	*Phaseolus vulgaris*	SSR
CZ-018	*P. vulgaris*	RSS
CZ-022	*P. vulgaris*	RRS
CZ-023	*P. vulgaris*	SSR
Yamanashi		
YU0503	*Vitis vinifera* (Cha)	RSS
YU0505	*V. vinifera* (Cha)	RSS
YU0506	*V. vinifera* (Cha)	RSS
YU0507	*V. vinifera* x *V. labrusca* (MBA)	RSS
YU0510	*V. vinifera* (CS)	RSS
YU0511	*V. vinifera* (CS)	RSS
YU0512	*V. vinifera* (CS)	RSS
YU0513	*V. vinifera* (CS)	RSS
YU0514	*V. vinifera* (Cha)	RSS
YU0516	*V. vinifera* (Sem)	RSS
YU0518	*V. vinifera* (Sem)	SSR
YU0520	*V. vinifera* (Sem)	RSS
YU0522	*V. vinifera* (Sem)	RSS
YU0523	*V. vinifera* (Sem)	RSS
YU0524	*V. vinifera* (Sem)	RSS
YU0525	*V. vinifera* (Sem)	SSR
YU0526	*V. vinifera* (Sem)	RSS
YU0528	*V. vinifera* (Sem)	SSR
YU0529	*V. vinifera* (Sem)	RSS
YU0530	*V. vinifera* (Sem)	RSS
YU0531	*V. vinifera* (Sem)	RSS
YU0532	*V. vinifera* (Sem)	RSS
YU0533	*V. vinifera* (Sem)	RSS
YU0601	*V. vinifera* (Mer)	SSR
YU0602	*V. vinifera* (Mer)	SSR
YU0603	*V. vinifera* (Cha)	RSR
YU0604	*V. vinifera* (Cha)	RSR
YU0605	*V. vinifera* (CS)	SSR
YU0606	*V. vinifera* (Mer)	SSR
YU0607	*V. vinifera* (CS)	RSS
YU0608	*V. vinifera* (Mer)	RSS
YU0609	*V. vinifera* (Cha)	RSS
YU0610	*V. vinifera* (PN)	SSR
YU0611	*V. vinifera* (Mer)	SSR
YU0612	*V. vinifera* (Sem)	RSR
YU0613	*V. vinifera* (Cha)	RSR
YU0615	*V. vinifera* (Cha)	RSR
YU0616	*V. vinifera* (Cha)	RSR
YU0617	*V. vinifera* (Cha)	SSR
YU0618	*V. vinifera* (CS)	RSR
YU0619	*V. vinifera* (Kos)	RSR
YU0622	*V. vinifera* (Syr)	RSS
YW0603	*V. vinifera* (Kos)	RSS
YW0604	*V. vinifera* (Kos)	RRS
YW0605	*V. vinifera* (Cha)	RRS
YW0606	*V. vinifera* (Cha)	RSR
YW0607	*V. vinifera* (Cha)	SSR
YW0608	*V. vinifera* (CS)	RSS
YU0701	*V. vinifera* (Cha)	RSS
YU0702	*V. vinifera* (Cha)	RSS
YU0703	*V. vinifera* x *V. labrusca* (MBA)	RSS
YU0704	*V. vinifera* (Cha)	RSS
YU0705	*V. vinifera* (CS)	RSS
YU0706	*V. vinifera* (Cha)	RSS
YU0707	*V. vinifera* (Cha)	RSS
YU0708	*V. vinifera* (Sem)	RSS
YU0709	*V. vinifera* (Sem)	RSS
YU0709	*V. vinifera* (Sem)	RSS
YU0710	*V. vinifera* (Sem)	RSS
YU0711	*V. vinifera* (Sem)	RSS
YU0712	*V. vinifera* (Sem)	RSS
YU0713	*V. vinifera* (Cha)	RSS
YU0714	*V. vinifera* (Sem)	RSS
YU0715	*V. vinifera* (Sem)	RSS
YU0716	*V. vinifera* (CS)	RSS
YU0717	*V. vinifera* (CS)	RSS
YU0718	*V. vinifera* (CS)	RSS
YU0719	*V. vinifera* (CS)	RSS
YU0720	*V. vinifera* (CS)	SSR
YU0721	*V. vinifera* (CS)	RSS
YU0722	*V. vinifera* (CS)	RSS
YU0723	*V. vinifera* (CS)	RSS
YU0724	*V. vinifera* (CS)	RSS
YU0725	*V. vinifera* (CS)	RSS
YU0726	*V. vinifera* (CS)	RSS
YU0727	*V. vinifera* (CS)	RSS
YU0728	*V. vinifera* (YS)	RSS
YU0729	*V. vinifera* (Cha)	RSS
YU0730	*V. vinifera* (Cha)	RSS
YU0731	*V. vinifera* (Cha)	RSS
YU0732	*V. vinifera* (CS)	RSS
YU0733	*V. vinifera* (CS)	RSS
YU0734	*V. vinifera* (Sem)	RSS
YU0735	*V. vinifera* (Sem)	RSS
YU0736	*V. vinifera* (Sem)	SRR
YU0737	*V. vinifera* (Sem)	RSS
YU0738	*V. vinifera* (Sem)	RSS
YU0739	*V. vinifera* (Sem)	RSS
YU0740	*V. vinifera* (Cha)	RSS
YU0741	*V. vinifera* (Cha)	RSS
YU0742	*V. vinifera* (Cha)	SSR
YU0743	*V. vinifera* (Cha)	SSR
YU0744	*V. vinifera* (Cha)	RSS
YU0745	*V. vinifera* (Cha)	RSS
YU0746	*V. vinifera* (Cha)	RSS
YU0747	*V. vinifera* (Cha)	RSS
YU0748	*V. vinifera* (Cha)	RSS
YU0749	*V. vinifera* (Cha)	RSS
YU0750	*V. vinifera* (Cha)	SSR
YU0751	*V. vinifera* (Cha)	RSS
Osaka		
CZ-036	*Cumcumis sativus*	RRR
CZ-046	*C. sativus*	RRR
CZ-058	*C. sativus*	RRR
CZ-064	*C. sativus*	RRS
CZ-115	*C. sativus*	RSS
NN001	*C. sativus*	SRR
NN002	*C. sativus*	RSS
NN003	*C. sativus*	SSR
Hyogo		
CZ-116	*C. sativus*	RSS
CZ-120	*C. sativus*	SRR
CZ-122	*C. sativus*	RRR

* Cha, Chardonnay; CS, Cabernet sauvignon; Kos, Koshu; MBA, Muscat Bailey A; Mer, Merlot; PN, Pinot noir; Sem, Semillon; Syr, Syrah; YS, Yama sauvignon.
** S and R represent sensitive and resistant in order to benzimidazole, dicarboximide and *N*-phenylcarbamate fungicides, respectively.

three years.

Materials and Methods

B. cinerea strains

One hundred fourteen *B. cinerea* strains were used in this study (Table 1). A total of 93 *B. cinerea* isolates were collected from an experimental vineyard at the University of Yamanashi, Yamanashi, Japan from 2005 to 2007 growing seasons. In these three years, the vineyard was treated with azoxystrobin (Amistar 10%; Syngenta, Tokyo, Japan), iprodione (Robura-ru 50%; Bayer CropScience, Tokyo, Japan), mancozeb (Jimandaisen 75%, Dow AgroSciences, Tokyo, Japan), metalaxyl (RidomiruMZ 10%, Syngenta, Tokyo, Japan), cymoxanil (Horaizun 30%, Nissan Chemical, Tokyo, Japan), famoxadone (Horaizun 22.5%, Nissan Chemical, Tokyo, Japan), thiophanate-methyl (Getta- 52.5%, Nippon Soda, Tokyo, Japan) and diethofencarb (Getta-12.5%, Nippon Soda, Tokyo, Japan) in accordance with pest management programs (Table 2). Six isolates (YW0603 to YW0608) were also collected from three commercial vineyards in Yamanashi, Japan in 2006. All *B. cinerea* isolates were collected by single spore isolation. Briefly, *B. cinerea* spores grown on grape berries from various cultivars of *Vitis vinifera* (Table 1) were collected and spread on potato dextrose agar (PDA, Difco) plates. The plates were incubated at 25ºC for 3 to 4 days. *B. cinerea* colonies were identified according to morphological characteristics, isolated, transferred to new PDA plates, and incubated forincubation at 25ºC. These procedures were repeated at least twice. Sporulation was induced by incubating *B. cinerea* colonies in continuous darkness at 25ºC. Single spore isolation was performed by spore manipulation under a light microscope. Isolated single spores were placed on PDA plates and incubated at 25ºC. Fifteen reference isolates of *B. cinerea* (CZ-011, CZ-018, CZ-022, CZ-023, CZ-036, CZ-046, CZ-58, CZ-064, CZ-115, CZ-116, CZ-120, CZ-122, NN001, NN002 and NN003) were used as controls for *in vitro* fungicide-resistance test. CZ series strains, isolated from common bean or cucumber were gifts from the Central Research Station, Syngenta Japan (Ushiku, Ibaragi, Japan). NN series strains, isolated from cucumber were gifts from the Agricultural Research Division, Mie Prefectural Science and Technology Promotion Center (Tsu, Mie, Japan).

Table 2 Fungicide management in the experimental vineyard.

Date	Fungicide*
2005	
Feb. 3	Ben**
May 31	Ben, Pcm
Oct. 18	Ben
2006	
Feb. 28	Ben**
Jun. 19	Ben, Pcm
July 17	Ben, Pcm
2007	
Jan. 29	Ben**
Feb. 28	Ben
July 18	Dic

*Ben, Benzimidazole; Dic, dicarboximides; Pcm, *N*-phenylcarbamate.
**Applied to the cut surface of the shoot after pruning

In vitro fungicide resistance test

Fungicides used in this study were benzimidazole (thiophanate-methyl; Topjin M 70%; Nippon Soda, Tokyo, Japan), *N*-phenylcarbamate (diethofencarb, Wako, Osaka, Japan) and dicarboximide (procymidone; Sumirex 50%; Sumitomo, Tokyo, Japan). For each *B. cinerea* isolate, mycelial disks (6 mm diameter) were excised from the leading edge growing actively on PDA plates and transferred to new PDA plates containing fungicides at the following concentrations: thiophanate-methyl at the discriminatory dose of 10 μg/ml, diethofencarb at the dose of 5 μg/ml and procymidone at the dose of 5 μg/ml.

The plates were incubated at 25ºC for 2 to 4 days to evaluate the response to fungicides. Sensitivity of the isolates to fungicides was classified as follows: sensitive (S) if there was no growth on PDA plates containing fungicides, and resistant (R) if there was growth on PDA plates containing fungicides (Fig. 1).

Results and Discussion

Fungicide resistance in *B. cinerea* populations

A total of 114 *B. cinerea* isolates were used for *in vitro* fungicide resistance test on media containing thiophanate-methyl (benzimidazole), diethofencarb (*N*-phenylcarbamate) or

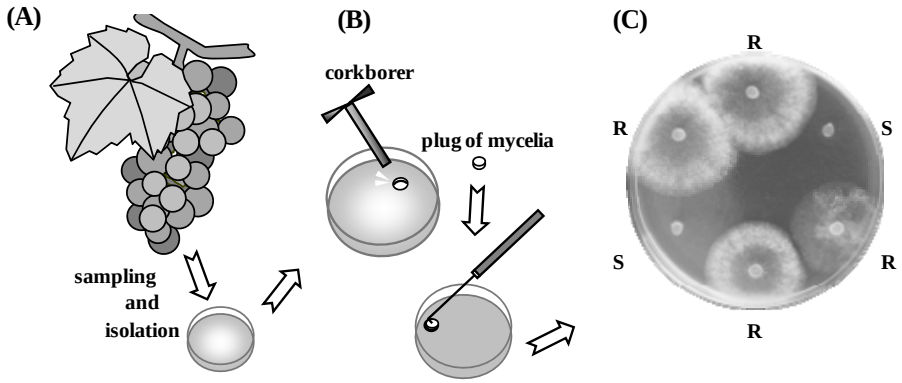

Fig. 1 *In vitro* fungicide-resistance test. (A) *B. cinerea* populations were collected from grapes, followed by isolation of *B. cinerea* isolates in the laboratory as described in Materials and Methods. (B) The edge of the mycelia growing actively in the plate was excised using a corkborer. The plug of mycelia was placed on the plate containing one of the three types of fungicide. (C) The mycelial growth was monitored after incubation at 25ºC for 2-4 days. R, resistant; S, sensitive.

procymidone (dicarboximide). Based on the response of *B. cinerea* strains to the three fungicides, the 93 strains collected from the experimental vineyard, University of Yamanashi, were classified into four phenotypes; RSS, RSR, SRR and SSR, representing sensitivity (S) or resistant (R) to benzimidazoles, dicarboximides or *N*-phenylcarbamates, respectively (Table 1). The frequencies of phenotype resistant to benzimidazoles, dicarboximides and *N*-phenylcarbamates were found to be 81.7, 2.2 and 24.7%, respectively. The frequencies of double resistant phenotype, RSR and SRR were 6.5 and 2.2%, respectively. RRS phenotype was found in commercial vineyards in Yamanashi, but not in the experimental vineyard (Table 1). Among 114 strains, neither SSS nor SRS phenotypes were found, indicating that no strains have developed to maintain sensitivity to both benzimidazoles and *N*-phenylcarbamates, or to exhibit resistance to dicarboximides only. Considering the history of fungicide applications in Japan and the existence of negative cross resistance between benzimidazole and *N*-phenylcarbamate fungicides, the SSR phenotype can be assumed to be the wild type of the original *B. cinerea* population in Japan prior to the introduction of benzimidazole fungicides in 1971. However, our results showed that RSS phenotype was turned out to be the most frequent strain in the experimental vineyard (Fig. 2), indicating that increase in frequency of RSS phenotype has

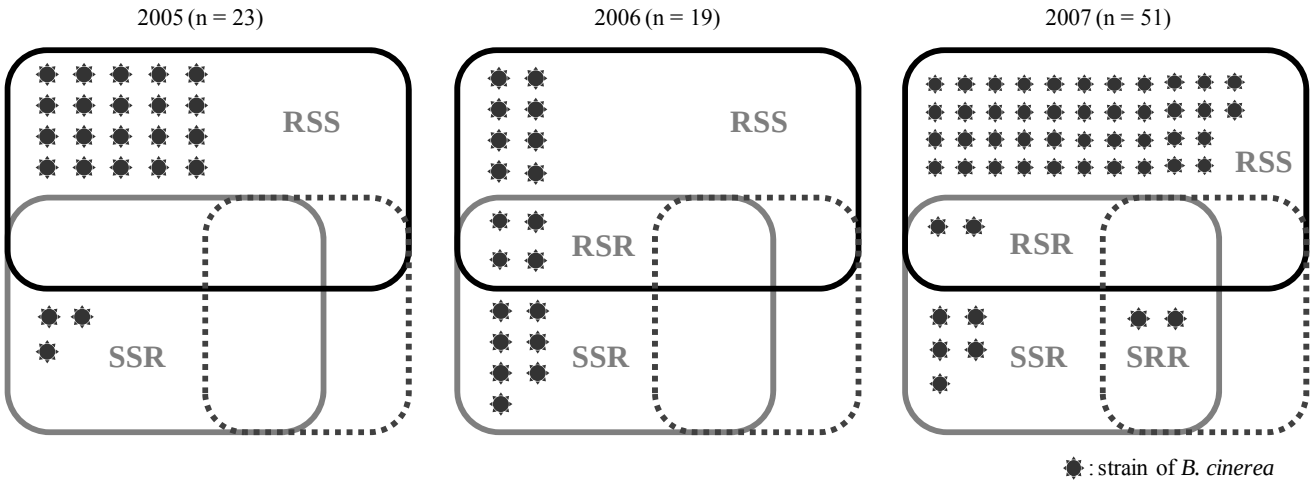

Fig. 2 Fungicide resistant profiles of *B. cinerea* in an experimental vineyard over three years. *B. cinerea* strain enclosed with the solid line in black is resistant to benzimidazole; the solid line in grey to *N*-phenylcarbamate and the dashed line to dicarboximide, respectively. RSS: resistant to benzimidazole and sensitive to both dicarboximide and *N*-phenylcarbamate, SSR: resistant to *N*-phenylcarbamate and sensitive to both benzimidazole and dicarboximide, RSR: resistant to both benzimidazole and *N*-phenylcarbamate, and sensitive to dicarboximide, SRR: resistant to both dicarboximide and *N*-phenylcarbamate, and sensitive to benzimidazole.

probably caused by selection pressure due to the extensive use of benzimidazole fungicides over nearly 4 decades. In fact, benzimidazole fungicides have been applied in the experimental vineyard every year from 2003 to 2007 (data not shown).

Fungicide resistance in a vineyard over three years

Table 2 shows the type of fungicide applied to the vineyard and its application date from 2005 to 2007 and Figure 2 shows fungicide resistant profiles in the experimental vineyard over three years. The isolation frequencies of the strains resistant to benzimidazole were found to be 87.0, 63.2 and 86.3% from 2005 to 2007, respectively, suggesting that benzimidazole fungicides would not be effective for the *B. cinerea* control compared with *N*-phenylcarbamate or dicarboximide fungicides. In fact, the frequencies of the strains resistant to *N*-phenylcarbamate or dicarboximide fungicides were found to be low (24.7%) compared to that of strains resistant to benzimidazole fungicide.

Among four resistant phenotypes found in the vineyard, RSS phenotype strains were predominant in the experimental vineyard at the rate of 87.0, 42.1 and 82.4% from 2005 to 2007, respectively. Three strains showed SSR phenotype were found in 2005. This might be due to the fact that *N*-phenylcarbamate fungicide has been sprayed in the vineyard in 2002 and 2003, and in 2005. RSR phenotype strains were not detected in 2005 whereas four and two strains showed RSR phenotype in 2006 and 2007, respectively (Fig. 2). The detection of RSR phenotype strains might result from the benzimidazole and *N*-phenylcarbamate fungicide application for two consecutive years. In 2007, we found two strains resistant to dicarboximide (SRR phenotype), which had not been detected for the previous two years (Fig. 2). Northover (1988) reported that in the absence of dicarboximide fungicides, the frequencies of dicarboximide resistance strains declined from 44% to 9-13% between 1993 and 1994. Dicarboximide fungicide was applied in the vineyard in 2002 and 2003, and then no application of dicarboximide fungicide was made until 2007. Therefore, SRR strains might have existed in the vineyard and emerged by application of dicarboximide in 2007.

Various frequencies of fungicide resistant isolates of *B. cinerea* have been found in surveys of numerous crops throughout the world, and isolates resistant to benzimidazole and/or dicarboximide are common. For instance, 32% of 121 isolates collected from several crops in three European countries and Israel were resistant to both benzimidazole and dicarboximide (13). Of 45 isolates collected from greenhouse-grown crops, 75% were resistant to benzimidazole and 43% were resistant to dicarboximide (7). In Korea, among *B. cinerea* strains collected from various crops during 1994-1996, SSR, RSS and RRS were the major phenotypes at the rate of 28.7, 28.8 and 39.4%, respectively (6).

Taken together, our results suggested that the frequencies of *B. cinerea* strains resistant either to *N*-phenylcarbamate or dicarboximide were relatively low and that, however, resistant strains to these two fungicides could be potentially latent in the population, and could spread rapidly in vineyards within a year.

Management of *B. cinerea* diseases by fungicides

The following conclusions can be led from this study: (a) RSS strains of *B. cinerea* are predominant in the experimental vineyard; (b) The fungicidal mixture of a benzimidazole fungicide plus diethofencarb, or dicarboximide fungicides could be more effective for the *B. cinerea* control than benzimidazole fungicides; (c) *B. cinerea* strains resistant to either *N*-phenylcarbamate or dicarboximide fungicides could appear shortly after its application.

Viticulturists have to understand the distribution of fungicide resistant *B. cinerea* strains in their vineyards for the best integrated pest management against *B. cinerea* diseases. Over the past decade, the DNA based diagnoses have been developed to detect rapidly the resistance of benzimidazole, *N*-phenylcarbamate and/or dicarboximide in *B. cinerea* populations (1, 11, 12). Saito *et al*. (12) reported a novel molecular based method to detect benzimidazole and/or *N*-phenylcarbamate and dicarboximide resistant *B. cinerea* strains at an early growth stage of grapes in vineyards. The method could detect three fungicide-resistant *B. cinerea* strains from grape berries and leaves at Eichorn-Lorenz growth stage 25 to 29. The early diagnosis

of fungicide resistant *B. cinerea* strains may provide the best information to viticulturists for further improvement of integrated pest management programs in their vineyards.

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［Research Note］

同一圃場における灰色かび病菌の薬剤耐性の変遷

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要　約

ブドウ病原菌はブドウの品質・収量に大きな被害を及ぼす要因となるため、一般のブドウ栽培農家は、薬剤散布を行なっている。その一方で、薬剤耐性菌の出現により薬剤散布による防除が困難になってきている。本研究では、ブドウ病原菌の一つである灰色かび病菌(*Botrytis cinerea* Pers ex Fr.)に着目し、山梨大学育種試験地のブドウ畑から3年間にわたり、93株を採集した。同一圃場における灰色かび病菌株の薬剤耐性の現状と変遷を調査することを目的とし、採集した93株に対して、Benzimidazole 系 、 Dicarboximide 系 お よ び *N*-phenylcarbamate系殺菌剤に対する薬剤耐性試験を行った。その結果、3年間にわたりBenzimidazole 耐性株が優勢であり、Dicarboximideおよび*N*-phenylcarbamateに対しては、散布後すぐに耐性菌が出現していることが明らかになった。