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Fitness of Dicarboximide-Resistant and Sensitive *Monilinia fructicola* Isolated from Peach in Korea

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Dicarboximide-resistant isolates of *Monilinia fructicola* grew readily on media amended with dicarboximide fungicides, and showed cross-resistance to pentachloronitrobenzene (PCNB). The fitness of resistant isolates was inferior to that of sensitive isolates. Mycelial growth on fungicide-free medium was not significantly different between the dicarboximide-resistant and sensitive isolates. The originally high EC_{50} values of the resistant isolate decreased after storage for 16 weeks at 4°C. After inoculation with the mixture of spore suspensions of resistant and sensitive isolates, the re-isolation rate of the resistant spores was significantly reduced regardless of the mixing ratio. From the results, it could be concluded that the competitive ability of the resistant isolates is inferior to the sensitive ones.

Keywords : competitive ability, fitness, fungicide resistance, *Monilinia fructicola*.

Brown rot caused by *Monilinia fructicola* is one of the most destructive diseases of stone fruits such as peach, plum, and apricot in Korea. The disease is controlled by the application of fungicides during the flowering and the ripening periods (Agrios, 1997). Before systemic fungicides were introduced, control of brown rot was dependent on the use of protectant fungicides such as captan and thiram.

In the 1970s, benzimidazole fungicides were introduced to control various diseases caused by many plant pathogenic fungi. However, after a few years, fungal isolates resistant to benzimidazole fungicides were reported in several studies (Johnes and Ehert, 1976; Lim et al., 1999; Szkolink and Gilpatrick, 1977; Tate et al., 1974). These fungicides were substituted in many orchard spray programs by dicarboximide fungicides such as iprodione, vinclozolin, and procymidone from late 1970s because of the reduced efficacy by resistant isolates. However, resistance to dicarboximide has been reported worldwide in many pathogenic fungi including *Botrytis cinerea*. Dicarboximide resistance of *Monilinia* sp. has been reported by several authors since the 1980s (Delp, 1988; Lim et al., 1998; Ogawa et al., 1984; Penrose et al., 1985; Sanamuang and Gaunt, 1995). In Australia, a farmer experienced severe production losses in nectarine fruits due to brown rot, in spite of the application of these fungicides in 1984 (Penrose et al., 1985; Sanamuang and Gaunt, 1995). It was reported that these severe losses resulted from the occurrence of *M. fructicola* isolates resistant to vinclozolin.

In 1998, resistant isolates of *Monilinia fructicola* against dicarboximide fungicides were first reported by Lim et al. (1998) from overwintering mummies and peduncles in several locations in Korea. High rates of cross-resistance to the same group of fungicides and double resistance to benzimidazole fungicides were also reported (Lim et al., 1998).

The objective of this study was to examine biological characteristics including mycelial growth, sporulation, spore germination, virulence, stability of resistance, osmotic sensitivity, and competitive ability of dicarboximide-resistant isolates.

Materials and Methods

Fungicides. The following fungicides were used: iprodione (50% WP), procymidone (50% WP), vinclozolin (50% WP), and pentachloronitrobenzene (PCNB, 75% WP). Fungicide suspensions prepared were diluted to respective concentrations, and added to potato-dextrose agar (PDA) immediately before pouring into Petri plates (Zehr et al., 1991).

Fungal isolates. Three dicarboximide-resistant and three dicarboximide-sensitive isolates were obtained from brown rot of peach in the middle of the growing season in 1998 at Chochiwon, Kyongsan, and Youngduk in Korea.

Mycelial growth. The growth of fungicide-resistant and sensitive isolates was tested on fungicide-free PDA and on PDA amended with 0, 0.0033, 0.033, 0.33, 3.3, 33, and 330 μg a.i./ml of iprodione; 0, 0.0028, 0.028, 0.28, 2.8, 28, and 280 μg a.i./ml procymidone; and 0, 0.0029, 0.029, 0.29, 2.9, 29, and 290 μg a.i./ml of vinclozolin. The 5 mm-diameter mycelium plugs were taken from the margin of 7-day-old PDA cultures and transferred to the test media. The diameters of colonies were measured after incubation for 7 days at 25°C.

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Spore germination, sporulation, and virulence of the fungal isolates. After 15 days incubation at 25°C in the dark, the degree of sporulation of resistant and sensitive isolates on PDA was investigated. The number of spores in conidial suspension harvested with 50 ml of sterilized distilled water per plate was counted under a light microscope with a haemacytometer. To determine the germination ratio, spore suspension was adjusted to 1×10^5 conidia/ml. Spore germination was investigated twice for replication and counted from 300 spores in each trial 8 h after incubation at 25°C.

In vivo sporulation rates of resistant and sensitive isolates were examined on apples treated with and without vinclozolin. Mycelial plugs were taken from the margin of 7-day-old PDA culture and inoculated into the cork-borer wounds (diameter: 5 mm) on apples. The inoculated apples were placed on a vinyl-wrapped plastic tray and kept at 25°C in the dark. Lesion sizes were measured 7 days later (Elmer and Gaunt, 1994), and sporulation rates were examined after 15 days.

Osmotic sensitivity. Sensitivity of the isolates to high osmotic pressure was determined by mycelial growth on the media amended with 1.1 M D-glucose (193 g/l), 1.1 M glycerol (102 g/l), and 0.69 M KCl (51.5 g/l). Mycelium plugs of 5 mm-diameter were taken from the margin of the 7-day-old PDA culture and transferred to the test media. The colony diameters were measured after incubation for 7 days at 25°C.

Stability of resistance. After incubation for 7 days on PDA, mycelial plugs (diameter: 5 mm) were taken from the margin of colony and transferred to PDA slant. After incubation for 7 days at 25°C, the slant cultures were stored at 4°C for 16 weeks. After storage, all isolates were sub-cultured on PDA for 7 days. Using the method mentioned above, the sensitivity of isolates of *M. fructicola* to iprodione was evaluated on PDA amended with 0, 0.0033, 0.033, 0.33, 3.3, 33, and 330 μg a.i./ml of iprodione.

Competitive ability. One dicarboximide-resistant isolate (CH12) and one dicarboximide-sensitive isolate (CH18) were used in this study. The competition between the two isolates in the absence of fungicide was investigated on canned peach fruit. Each conidial suspension of both isolates was adjusted to 1×10^5 conidia/ml, and mixed to give a proportion of the resistant isolate from 0 to 100% at 20 or 30% intervals. The suspension mixtures were inoculated on canned peach fruits by spray, and the treated fruits were incubated on a vinyl-wrapped plastic tray at 25°C in the dark. After incubation for 7 days, conidia were harvested from each fruit with 50 ml of sterilized distilled water. Responses to fungicide were tested on PDA amended with 2.9 μg a.i./ml of vinclozolin. Density of harvested conidia was adjusted to 1×10^5 conidia/ml, and 200 μl of aliquots was pipetted onto PDA plates with or without the fungicide. Conidia in the aliquots were distributed evenly over the surface of the plates by a glass rod, and germinated spores were counted after incubation for 8 h. Germination rates of conidia on the amended medium indicated the ratio of fungicide-resistant spores.

Results

Fungicide response. Of the six isolates used in the first test, three isolates (CH06, CH18, and YO05) were sensitive to dicarboximide fungicides used (Table 1). The isolates had the EC_{50} values of 0.016-0.031 μg a.i./ml to iprodione, 0.019-0.042 μg a.i./ml to procymidone, and 0.022-0.030 μg a.i./ml to vinclozolin (Fig. 1). For the remaining three isolates (CH12, CH26, and KY16), the EC_{50} value of CH12 to the fungicides was over 100 μg a.i./ml, while CH26 and KY16 showed EC_{50} values of 3.49-19.5 μg a.i./ml and 0.23-0.74 μg a.i./ml, respectively (Table 1).

Cross-resistance to PCNB. The EC_{50} values of dicarboximide-sensitive isolates, CH06, CH18, and YO05, to PCNB were 3.9, 5.8, and 2.8 μg a.i./ml, respectively. However, all resistant isolates showed EC_{50} values of more than 500 μg a.i./ml (Table 1). Among the isolates, CH26 had the highest EC_{50} value of 1,418 μg a.i./ml.

On the medium containing 0.33, 0.28, and 0.29 μg a.i./ml

Table 1. Responses of *Monilinia fructicola* to dicarboximides and PCNB

Isolates	Response against fungicides							
	Iprodione		Procymidone		Vinclozolin		PCNB	
	EC_{50}	Rf[a]	EC_{50}	Rf	EC_{50}	Rf	EC_{50}	Rf
DS[b]								
CH06	0.021	1.3	0.030	1.6	0.022	0.6	3.900	0.7
CH18[d]	0.016	1.0	0.019	1.0	0.034	1.0	5.800	1.0
YO05	0.031	1.9	0.042	2.2	0.030	0.9	2.800	0.5
DR[c]								
CH12	399.000	24,937.0	324.300	17,068.0	101.300	2,979.0	620.300	106.9
CH26	3.500	218.1	21.900	1,152.0	19.500	573.5	1,418.000	242.8
KY16	0.200	14.3	0.800	44.2	0.700	21.8	906.300	156.3

[a] Resistance factor: expressed as EC_{50} of a tested isolate/EC_{50} of the standard isolate (Delp and Dekker, 1985).
[b] Dicarboximide-sensitive.
[c] Dicarboximide-resistant.
[d] Standard isolate.

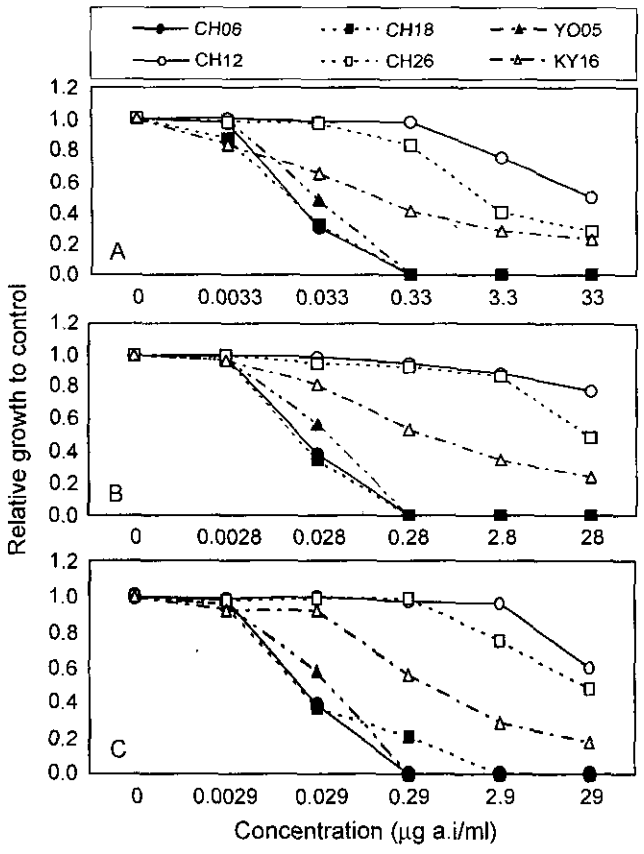

Fig. 1. Relative growth of dicarboximide-resistant (empty symbols) and sensitive (filled symbols) isolates on potato-dextrose agar amended with a fungicide. A: iprodione; B: procymidone; and C: vinclozolin.

of iprodione, procymidone, and vinclozolin, respectively, the relative growth to the fungicide-free medium was 0 for the sensitive isolates, but those of resistant isolates were 0.41-0.98 for iprodione, 0.54-0.95 for procymidone, and 0.56-0.99 for vinclozolin (Fig. 1).

Mycelial growth. Mycelial growth was not significantly different (P=0.05) between dicarboximide-resistant (mean 78.0) and sensitive (mean 78.1) isolates, but it varied significantly among the isolates (P=0.05). The mean mycelial growth of resistant isolates was not lower than that of sensitive isolates (Table 2).

Spore germination. In the fungicide-free potato dextrose-broth, regardless of the responses to fungicide, all isolates showed more than 90% of spore germination. The rates were not significantly different (P=0.05) among the isolates (Fig. 2). In the potato dextrose broth amended with vinclozolin of 0.29 μg a.i./ml, the sensitive isolates showed spore germination rates of 3.8-4.8%, but those of resistant isolates were 82.3-85.6%.

Sporulation. There was a significant difference (P=0.05) in sporulation between fungicide-resistant and sensitive isolates on the fungicide-free PDA (Table 2). The sensitive isolates produced two to five times more spores than the resistant isolates. However, sporulation between isolates in the same group on the fungicide-free medium was not significantly different (P=0.05) (Table 2).

On fungicide-free apples, sensitive isolates formed much more spores than the resistant isolates (Table 2).

Virulence. On fungicide-free apples, fruit rotting and colonization by sensitive isolates were fast and were followed by profuse sporulation. Meanwhile, the resistant isolates caused brown rot and poor sporulation (Table 2).

Table 2. Biological characteristics of dicarboximide-resistant and sensitive isolates of *Monilinia fructicola* obtained from several locations

Isolates	Mycelial growth (mm)	Sporulation		Lesion diameter[b]
		PDA ($\times 10^4$ spores/ml)	Apple[a]	
DS[c]				
CH06	78.6 Y[e]	26.3	++	30.0 ± 0.83
CH18	78.7 Y	25.9	++	38.9 ± 4.96
YO05	77.1 YZ	20.3	++	30.9 ± 2.05
DR[d]				
CH12	75.3 Z	2.9	+	26.4 ± 3.07
CH26	81.1 X	2.3	+	26.9 ± 0.83
KY16	77.6 YZ	10.6	+	29.1 ± 0.37

[a]Sporulation rates of the fungicide-resistant and sensitive isolates were examined *in vivo* on fungicide-free apple.
[b]Lesion size was measured 7 days after inoculation (Elmer and Gaunt, 1994).
[c]Dicarboximide-sensitive.
[d]Dicarboximide-resistant.
[e]Values followed by the same letters in a column are not significantly different according to Duncan's multiple range test ($P \leq 0.05$)

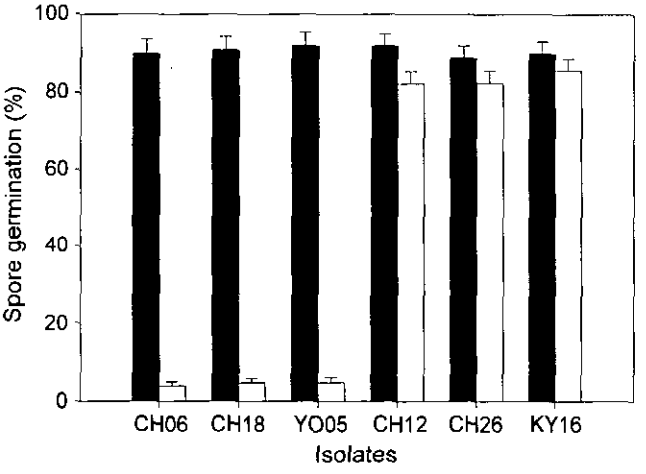

Fig. 2. Spore germination of dicarboximide-resistant and sensitive isolates of *Monilinia fructicola* on potato-dextrose broth without (■) and with vinclozolin (□).

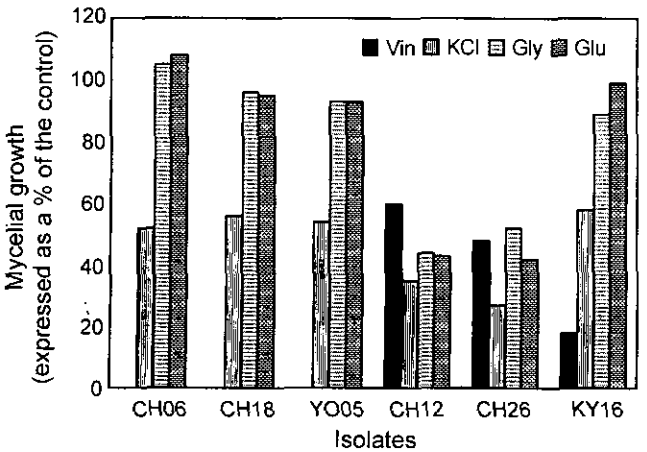

Fig. 3. Effects of various compounds on the mycelial growth of dicarboximide-resistant and sensitive isolates of *Monilinia fructicola* obtained from peach orchards in several locations. Mycelial growth was examined on the media amended with each of the compounds (Vin: Vinclozolin 29 μg a.i./ml, Glu: Glucose 193 g/l, Gly: Glycerol 102 g/l, KCl: 51.5 g/l).

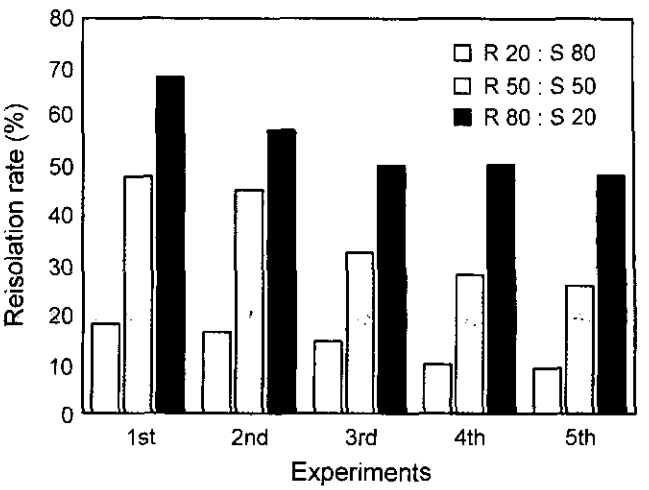

Fig. 4. Reisolation rates of dicarboximide (vinclozolin)-resistant *Monilinia fructicola* isolates from canned peach inoculated with conidial suspension of mixture of resistant and sensitive isolates. Symbols represent mixing ratio of conidial suspension of dicarboximide (vinclozolin)-resistant (R, CH12) and sensitive (S, CH18) isolate.

Osmotic sensitivity. All fungicide-sensitive isolates grew normally on the D-glucose and glycerol rich MEA (Fig. 3). Among the isolates, the growth of CH12 and CH26 with high EC_{50} value to the dicarboximide fungicides was noticeably inhibited by high osmorality (Fig. 3). However, mycelial growth of all isolates was noticeably inhibited by the high concentration of KCl (Fig. 3). CH12 of the highest EC_{50} value to dicarboximide fungicides was the most sensitive isolate to osmorality.

Stability of resistance. After storage at 4°C for 16 weeks, all isolates except for the resistant isolate CH12 had the same EC_{50} values with those before storage (Table 3). In case of isolate CH12 with high EC_{50} (high resistance), the value after storage decreased to 250 μg a.i./ml as compared with the initial EC_{50} value.

Competitive ability. In all combination of spore mixture, re-isolation rate of the dicarboximide-resistant isolate CH12 was reduced. In the mixture with high resistant isolate ratio, reisolation rate of spore of resistant isolate was reduced from 80.0 to 48.3% according to sub-culture. In the mixture with low resistant isolate ratio, re-isolation rate of spore of resistant isolate was reduced from 50.0 to 26.1%, or from 20.0 to 9.3% (Fig. 4).

Table 3. Changes in EC_{50} of iprodione to *Monilinia fructicola*

Isolates	EC_{50} values of iprodione to *M. fructicola*	
	Initial	Final[a]
DS[b]		
CH06	0.021	0.023NS[d]
CH18	0.016	0.020NS
YO05	0.031	0.028NS
DR[c]		
CH12	399.000	250.400*
CH26	3.490	3.500NS
KY16	0.230	0.250NS

[a]Response to fungicide was tested on PDA amended with iprodione after storage for 16 weeks at 4°C.
[b]Dicarboximide-sensitive.
[c]Dicarboximide-resistant.
[d]NS: Treatment mean not significantly different from initital test at 0.05 level, *: Value significantly less than initial test ($P \leq 0.05$).

Discussion

Fitness of dicarboximide-resistant and sensitive isolates was evaluated based on characteristics such as mycelial growth, spore germination, sporulation, virulence, osmotic sensitivity, and competitive ability (Elmer and Gaunt, 1994; Zehr et al., 1991). On the media amended with the fungicides, the resistant isolates grew well, while the sensitive ones showed no mycelial growth. However, mycelial growth on the fungicide-free medium was not significantly different (P=0.05) between dicarboximide-resistant and sensitive isolates (Table 1). The sporulation of the resistant isolates on fungicide-free medium and their virulence to apple fruits were inferior to those of the sensitive isolates (Table 2). These results were similar to those of previous studies (Delp, 1988; Katan and Shabi, 1982). Decrease of the virulence and sporulation of the resistant-isolates on apple suggest that the density of dicarboximide-resistant isolates may decrease under natural condition in which selection pressure is not generated by dicarboximide fungicides (Delp, 1988; Katan and Shabi, 1982). In general, the

frequency (mutation rate) of dicarboximide-resistance in *M. fructicola* was at least 100 times higher than benzimidazole (Katan and Shabi, 1982). However, it seems that the low detection rate of dicarboximide-resistant isolates compared with benzimidazole resistant isolates is associated with reduced fitness of the isolates in the field (Delp, 1988). The negative cross-resistance to osmorality suggests that it is associated with decreased virulence of the resistant isolates on apple containing high concentration of gross sugar (Delp, 1988; Fujimura et al., 2000; Ramesh et al., 2001).

After inoculation with the mixture of spore suspensions of resistant and sensitive isolates, the re-isolation rate of the resistant spores was significantly (P=0.05) reduced regardless of their mixing ratio. From the results, it could be concluded that the competitive ability of the resistant isolates was lower than that of the sensitive isolates.

In this study and based on literature review (Delp, 1985), the fitness of the resistant isolates to dicarboximide seemed to be inferior to that of the sensitive isolates. Results of this study indicate that the presence of dicarboximide-resistant isolates in the fields may be derived from selection pressure generated by application of dicarboximide fungicides. At present, farmers have been using many benzimidazole and dicarboximide fungicides because of several reasons including cost and experience. Therefore, to avoid reduced control of dicarboximide fungicide, it is necessary to reduce the frequency of application, apply dicarboximide by turns with other fungicides possessing different mode of action, and monitor population shift of field isolates which are resistant to dicarboximide fungicide.

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