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Population Dynamics and Fitness Comparison of Sensitive and Resistant Phenotypes of *Botrytis cinerea* to Benzimidazole, Dicarboximide, and *N*-phenylcarbamate Fungicides

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A total of 2109 isolates of *Botrytis cinerea* were collected from infected plants of strawberry, tomato, and cucumber in Korea from 1994 to 1996. Based on *in vitro* tests for mycelial growth on potato-dextrose agar containing fungicides, the isolates were classified into six phenotypic groups: SSR, SRR, RSS, RRS, RSR, and RRR, representing sensitvity (S) or resistance (R) to carbendazim, procymidone, and diethofencarb. In that order the isolation frequencies of the SSR, SRR, RSS, RRS, RSR, and RRR phenotypes were 28.7, 1.1, 28.8, 39.4, 1.0, and 0.9%, respectively. Three isolates from each SSR, SRR, RSS, RRS, and RSR and an isolate of RRR phenotype were selected and evaluated for their fitness-related characteristics such as pathogenic aggressiveness, mycelial growth rate, sporulation, and sclerotial formation. Competitive abilities of the SSR, SRR, RSS, RRS, and RSR phenotypes were also compared by inoculating mixtures of conidial suspensions of two phenotypes to cucumber plant, and then determining re-isolation frequencies from lesions. In general, significant differences in fitness-related characteristics, except pathogenic aggressiveness, were found not only between but also within phenotype groups. In the competitiveness tests, carbendazim-sensitive phenotypes (SSR and SRR) were found to be more competitive than the resistant ones (RSS and RSR), whereas, the procymidone-resistant phenotypes (SRR and RRS) appeared to be more competitive than the sensitive ones (SSR, RSS, and RSR). There was no consistent dominance in competitiveness between the diethofencarb-resistant and sensitive phenotypes. The RSR phenotype was the least competitive among the five phenotypes.

Keywords : *Botrytis cinerea*, competitive ability, fitness, fungicide resistance.

Botrytis cinerea Pers. is a ubiquitous pathogen that causes severe losses in many fruit, vegetable, and ornamental crops in Korea. Many of the strategies currently being used aim to control gray mold. These strategies involve the use of organic fungicides, especially benzimidazoles and dicarboximides, which were introduced to Korea in 1973 and 1977, respectively. Benzimidazole fungicides such as carbendazim, benomyl, and thiophanate-methyl were widely used in the early 1980s. However, the use of these fungicides eventually decreased because of the occurrence of resistant populations of *B. cinerea* (Kim and Kwon, 1993; Park et al., 1992). Dicarboximide fungicides such as iprodione, vinclozolin, and procymidone became popular slightly later than benzimidazole fungicides, and are still being used extensively to control gray mold in vegetable crops in spite of the reported, fungicide resistance (Kim et al., 1993; Kim and Kwon, 1993; Park et al., 1992).

Benzimidazole and dicarboximide fungicides are known to exert their fungicidal actions by inhibiting beta-tubulin polymerization and by probably causing membrane lipid peroxidation, respectively (Edlich and Lyr, 1992; Ishii, 1992). The resistant strains to both fungicides could be controlled by either compounds with different mode of actions or by fungicides exhibiting negatively correlated cross resistance to benzimidazoles (Delp, 1988; Elad et al., 1988; Fujimura, 1993). Diethofencarb, a *N*-phenylcarbamate fungicide, was introduced to Korea in 1992, and its use has increased rapidly since 1994 because of its effectiveness against *B. cinerea* populations resistant to benzimidazole fungicides.

Currently, fungicide resistance is a major problem to the vegetable crop industry in Korea. In order to establish proper strategies for fungicide management in the field, information on population dynamics of fungicide resistance of pathogens is necessary. This study was conducted to characterize fungicide resistant populations of *B. cinerea* in Korea and to investigate biological fitness of various resistant phenotypes as compared with other phenotypes.

Materials and Methods

Pathogen isolation. *Botrytis cinerea*-infected tissues of strawber-

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ries, cucumbers, and tomatoes were collected during the growing seasons of 1994-1996 from greenhouses in five locations (Taejon, Buyo, Gongju, Nonsan, and Kimhae) where carbendazim, procymidone, and mixtures of carbendazim and diethofencarb had been routinely applied. The infected tissues were placed on potato dextrose agar (PDA) containing 100 μg/ml streptomycin and 50 μg/ml chloramphenicol. Tips of mycelia which were grown from infected tissue samples were transferred to a fresh PDA medium and incubated at 20℃ for 5 days. Based on the morphological characteristics of conidiophores and conidia, the identity of *B. cinerea* was confirmed for all isolates used in this study.

Resistance to fungicides. A previous research on mycelial growth of *B. cinerea* on PDA containing 2, 10, 50, 250, 1250, and 5000 μg/ml of benzimidazoles and dicarboximides showed that 10 μg/ml can be used as the cardinal concentration of fungicides for distinguishing. resistant and sensitive isolates of *B. cinerea* population in Korea (Kim et al., 1993). Resistance of *B. cinerea* isolates to fungicides was examined on PDA containing 10 μg/ml carbendazim, procymidone, or diethofencarb, following incubation of the fungus at 20℃ for 5 days. Simultaneous resistance to more than one fungicide was also evaluated on PDA with one of the possible combinations of two or three fungicides. Fungicides dissolved in acetone were added to the treatment cultures. Control contained only the same amount of acetone. The final concentration of acetone in the cultures was less than 1% (v/v). Based on their sensitivity to the fungicides, the fungal isolates were categorized as sensitive (S) or resistant (R) phenotypes.

Fitness-related characteristics. Representative isolates of each phenotype groups were compared based on their fitness-related characteristics such as pathological aggressiveness, mycelial growth, sporulation, sclerotial formation, osmotic sensitivity, and competitive ability.

Pathological aggressiveness. Mycelial discs (5 mm in diameter) from the fungal cultures on PDA were placed on the surface of eggplant fruits wounded by a cork borer (5 mm in diameter) prior to inoculation. The inoculated fruits were placed in a humid chamber at 20℃ for 3 days, and then aggressiveness of isolates was evaluated in terms of the lesion diameter.

Mycelial growth. Discs (5 mm in diameter) placed on eggplant wounded by the cork borer taken from the edge of colonies grown on PDA at 20℃ for 3 days were then placed on fresh PDA. The cultures were incubated at 20℃, and mycelial growth was measured 24, 48, and 72 h later.

Sporulation. In order to induce sporulation, the fungal cultures on PDA were incubated in the dark at 20℃ for 5 days, and subsequently subjected alternately to light/dark conditions at 12/12 h intervals with a light intensity of 4000 lux for an additional 4 days. The spore suspension was prepared by gently scrubbing the surface of culture plates with a small paintbrush after flooding with 10 ml 0.05% Triton X-100 in distilled water. The spore suspension was filtered through three layers of cheesecloth, and the number of spores per Petri-dish plate was calculated using a hemacytometer.

Sclerotial formation. The fungal cultures were incubated on PDA with 9 cm in diameter at 20℃ for 30 days in the dark, and the number of sclerotia formed was counted.

Osmotic sensitivity. Osmotic sensitivities of the fungal isolates were measured according to the method of Beever (1983). The fungal isolates were inoculated on PDA containing 0.68 M NaCl, and then incubated in the dark at 20℃ for 3 days. Mycelial growth of the fungal isolates on the media was compared with that on the control media without NaCl.

Competitive ability. Competitive ability of each phenotype relative to the other phenotypes was evaluated by determining the reisolation frequencies of phenotypes after inoculation on cucumber leaves with a mixture of spore suspensions of two fungal isolates representing two phenotypes. Because subcultures of the isolates belonging to the RRR phenotype did not show consistent responses to the three fungicides, only five phenotypes excluding except the RRR phenotype, were compared for their competitiveness with each other in ten possible combinations. The concentration of conidial suspension of each isolate was adjusted to 1×10^6 conidia/ml. Equal amounts of the conidial suspensions of two phenotypes in various combinations were mixed and used to inoculate the leaves of cucumber plants with 10-14 leaves. Following incubation of the plants in a humid chamber at 20℃ for 3 days, 100 lesions on the leaves were sampled at random. The lesion samples were sterilized by dipping into 1% sodium hypochlorite for 1 min, and then rinsing thoroughly with sterilized distilled water. After removing moisture from the samples using filter papers, the samples were placed on the Kerssies selective medium (1990) for *B. cinerea*, and then incubated at 20℃ for 3 days. Isolates from individual lesions were examined for their resistance to fungicides by culturing on PDA containing 10 μg/ml of carbendazim, procymidone, or diethofencarb.

Statistical analysis. All treatments for each experiment were replicated three times. When the overall F-test in the analysis was significant, mean of all treatments were compared using the least significant difference (LSD) test at 5% level of significance.

Results

Population dynamics of the fungicide resistant phenotypes. Based on the responses of *Botrytis cinerea* isolates to carbendazim, procymidone, and diethofencarb, the fungal isolates collected from greenhouses in Korea during 1994-1996 were classified into six phenotype groups: SSR, SRR, RSS, RRS, RSR, and RRR, representing sensitivity (S) or resistance (R) to benzimidazoles, dicarboximides, and *N*-phenylcarbamates, in that order respectively (Table 1). The isolation frequencies of phenotypes resistant to benzimidazole, dicarboximide, or *N*-phenylcarbamate fungicide were found to be 70.1, 41.4, and 31.7%, respectively. The double resistant RRS phenotype (39.4%) was the most frequently isolated, and the RSS (28.8%) and SSR (28.7%) phenotypes were also commonly isolated. The other three phenotypes (SRR, RSR, and RRR) consisted of extremely minor populations. The fungal isolates resistant to dicarboximides were usually resistant to benzimidazoles, whereas, the benzimidazole resistant isolates were either sensitive or resis-

Table 1. Relative frequency of phenotype groups of *Botrytis cinerea* isolates from greenhouse crops in Korea (1994-1996) examined by their resistance responses to benzimidazole, dicarboximide, and *N*-phenylcarbamate fungicides

Phenotype[a]	Number of isolate[b]	Relative frequency (%)
SSR	606	28.7
SRR	23	1.1
RSS	608	28.8
RRS	831	39.4
RSR	22	1.0
RRR	19	0.9

[a] S and R represent sensitivity and resistance to benzimidazole, dicarboximide, and *N*-phenylcarbamate fungicides, in that order. Isolates were considered as resistance if mycelia grew on a medium containing 10 μg/ml carbendazim, procymidone or diethofencarb, following an incubation of the fungus at 20°C for 5 days.

[b] The total number of isolates examined was 2109.

tant to dicarboximides.

Fitness-related characteristics. Fitness-related characteristics of 16 isolates representing the six phenotype groups were compared (Table 2). In general, significant differences between and within the phenotype groups were found in mycelial growth rate, sporulation, sclerotial formation, and osmotic sensitivity. Pathological aggressiveness measured by lesion size on eggplant fruits did not differ among phenotype groups.

As shown in Table 2, two phenotype groups with different sensitivity to only one fungicide were compared in order to determine effects of resistance to a single fungicide on fitness-related characteristics. The benzimidazolesensitive phenotypes, SSR and SRR, generally showed reduced sporulation and profuse sclerotial formation as compared with the benzimidazoleresistant phenotypes, RSR and RRR, respectively. With regard to resistance to dicarboximides, the sensitive phenotypes, SSR and RSS, produced more sclerotia than the resistant phenotypes, SRR and RRS. However, the relative capacity of sporulation of the dicarboximide resistant and sensitive phenotypes was not consistent: the resistant phenotype seemed to produce a greater number of conidia than the sensitive phenotype based on the comparison between RRS and RSS, but it was not true when RSR and RRR were compared. In the case of resistance to *N*-phenylcarbamate, the resistant phenotype, RSR, showed abundant sporulation but reduced sclerotial formation as compared with the sensitive phenotype, RSS. There were no significant differences in both sporulation and sclerotial formation between RRS and RRR.

When the competitiveness of five phenotype groups (SSR, RSS, RRS, SRR, and RSR) was compared with each other based on the re-isolation frequencies from lesions produced after the co-inoculations of 10 combinations of

Table 2. Fitness-related characteristics of selected isolates of *Botrytis cinerea*

Phenotype[a]	Isolate	Lesion[b] size (mm)	Mycelial growth rate (mm/day)	Production of conidia (× 10^6/plate)	Formation of sclerotia (#/plate)	Osmotic[c] sensitivity (% inhibition)
SSR	BY-4	31.5	14.1	12.5	140.5	22.3
	BY-9	31.6	13.5	7.0	228.8	34.1
	GA45	26.6	15.5	19.5	184.8	31.1
SRR	P94-2	30.5	8.3	4.6	81.3	91.9
	IC-02	28.5	13.5	15.3	42.5	35.7
	IC2-4	29.5	12.2	8.3	43.0	22.3
RSS	DJ-6	28.3	15.1	3.4	247.0	31.4
	GA-35	34.1	12.3	60.6	55.0	54.1
	GA-54	27.7	14.0	14.1	84.3	34.2
RRS	BY-7	29.8	15.5	85.0	75.5	33.8
	BY-22	30.1	15.2	109.5	17.5	26.3
	JCW-3	34.7	15.3	60.8	76.0	60.5
RSR	SDT-06	29.0	14.7	107.1	22.5	35.0
	SDT-16	27.8	14.0	133.8	28.3	31.2
	SDT-17	23.1	12.9	125.9	67.8	46.4
RRR	YC-44	28.5	14.8	73.4	67.3	28.4
LSD (P=0.05)		5.8	1.0	16.9	41.5	13.7

[a] S and R represent fungicide sensitivity and resistance to benzimidazole, dicarboximide, and *N*-phenylcarbamate fungicides, in that order.

[b] Fruits of eggplant were inoculated with mycelial discs of *B. cinerea* isolates. The average diameters of six lesions measured at 5 days after inoculation were presented.

[c] Osmotic sensitivity was determined by comparing the colony sizes grown on PDA medium with and without 0.68 M NaCl.

Table 3. Re-isolation frequency of *Botrytis cinerea* phenotypes when phenotype pairs in various combinations at 1 : 1 ratio were co-inoculated to cucumber leaves

Counterpart in the co-inoculation	Re-isolation frequency (%) of the phenotype				
	SSR[a]	RSS	RRS	SRR	RSR
SSR	–	12.8	44.1	82.0	11.3
RSS	81.9	–	82.7	81.9	24.4
RRS	55.9	17.3	–	51.4	9.6
SRR	18.0	18.1	48.6	–	17.9
RSR	88.7	75.6	90.4	82.1	–

[a] S and R represent fungicide sensitivity and resistance to benzimidazole, dicarboximide, and *N*-phenylcarbamate fungicides, in that order.

two phenotype groups, the SRR phenotype appeared the most competitive, while the RSR phenotype was the least competitive (Table 3). Re-isolation frequencies of the benzimidazolesensitive phenotypes (SSR and SRR) were much higher than those of the benzimidazoleresistant phenotypes (RSR and RSS). However, the other benzimidazoleresistant phenotype (RRS) appeared to be as competitive as the SSR and SRR phenotype as indicated by the similar re-isolation frequencies of the phenotypes (SSR : RRS = 55.9 : 44.1 and SRR : RRS = 51.4 : 48.6). When the phenotypes resistant and sensitive to dicarboximides were compared, the resistant phenotypes (SRR and RRS) greatly outnumbered the sensitive phenotypes (SSR and RSS), in the re-isolates. The RRS phenotype was also more frequently re-isolated than the other dicarboximidesensitive phenotype (RSR).

With regard to *N*-phenylcarbamate fungicides, dominance in re-isolation frequency of the sensitive and resistant phenotypes seemed to vary depending on the combinations of two phenotypes in the inoculum. The RSR phenotype which was doubly resistant to benzimidazole and *N*-phenylcarbamate fungicides was always less competitive than the other phenotypes. In other comparisons among the phenotype groups, dominance in re-isolation frequencies appeared to be determined by the responses to benzimidazoles or dicarboximides, instead of response to *N*-phenylcarbamates.

Discussion

Considering the history of fungicide introductions to Korea and the existence of negatively correlated cross resistance to benzimidazole and *N*-phenylcarbamate fungicides, the SSR phenotype can be assumed to be the wild type of the original *B. cinerea* population in Korea prior to the introduction of benzimidazole fungicides in 1973. The SSR phenotype was found to be one of the three major phenotypes. Increase in frequencies of the RSS and RRS phenotypes was probably caused by the selection pressure due to extensive use of benzimidazole and dicarboximide fungicides over 20 years. The frequent occurrence of the three major phenotypes under selection pressure of carbendazim, procymidone, and the mixture of carbendazim and diethofencarb was also observed in Spain (Raposo et al., 1994; 1996).

The six phenotype groups observed in the field survey for fungicide resistance were not significantly different from each other in their pathogenic aggressiveness on eggplant fruits. Furthermore, their fitness-related characteristics varied significantly among the fungal isolates within each phenotype. Therefore, there is limited explanation to relate the differences in occurrence of fungicide resistant phenotypes in the field survey with the differences in their fitness-related characteristics. Based on the results of this study competitiveness and sclerotial formation of *B. cinerea* are particularly important to maintain its population in nature. Because of its strong saprophytic ability, the fungus can avoid fungicide applications and will continue to proliferate. For example, it is easy to find *B. cinerea* growing well on dead plant tissues during intercropping periods when no fungicides are applied. This means that fungicide-sensitive strains also have ecological niches to continue to proliferate based on their biological fitness.

Persistence of the SSR phenotype might have been related with its strong competitiveness and its ability to form abundant sclerotia as compared with other phenotype groups. The abundant sclerotia would provide better chances for the SSR phenotype to survive intercropping periods or unfavorable conditions. On the other hand, the other benzimidazole sensitive phenotype, SRR, did not appear to produce as many sclerotia as the SSR phenotype even if it was quite competitive in the co-inoculation tests. Under strong selection pressure due to extensive applications of benzimidazole fungicides, the SRR phenotype may not be able to maintain its population due to relatively reduced ability to produce sclerotia.

Phenotypes having simultaneous resistance to benzimidazole and *N*-phenylcarbamate fungicides (RSR and RRR) were found for the first time in Korea in 1995. Recent occurrences of the RSR and RRR phenotypes may have resulted from increased applications of the mixture of carbendazim and diethofencarb to control benzimidazole-resistant *B. cinerea* during the last 5 years. Resistance to benzimidazole fungicides is known to be controlled by one gene in *B. cinerea* involved in beta-tubulin polymerization and is negatively correlated with resistance to *N*-phenycarbamate fungicides (Delp, 1988; Ishii, 1992; Schuepp and Kueng, 1981). Therefore, absence of the negative cross resistance to benzimidazole and *N*-phenylcarbamate in the RSR and RRR phenotypes may have been caused by changes in genetic interactions between genes affecting the negative cross resistance. The RSR phenotype was not as competitive as the other phenotypes in the co-inoculation tests, which may have resulted in the low frequency in the field survey for populations of *B. cinerea*. The extreme variations of subcultures of the RRR phenotype might be due to unstable genetic interactions among genes affecting the negative cross resistance.

There are several reports showing resistant isolates of *B. cinerea* dicarboximide fungicides were inferior to sensitive ones in spore production, virulence, and over-summering of sclerotia (Beever, 1983; 1989: Gullino and Garibaldi, 1981; Hisada et al., 1979; Moorman and Lease, 1995). However, no differences in mycelial growth rates, spore production, sclerotial formation, and virulence on cucumbers or strawberries were reported between dicarboximidesensitive and resistant *B. cinerea* isolates in Korea (Kim and Kwon, 1993; Park, 1993). Results in this study showed that dicarboximide-resistant phenotypes (SRR and RRS) were generally inferior to dicarboximide-sensitive phenotypes (SSR and RSS) in their ability to produce sclerotia. However, it was difficult to make conclusions in this study regarding increase or decrease in sporulation capacity in relation to resistance to dicarboximide fungicides. The conflicting results by different authors may be due to the fact that in the ecosystem, *B. cinerea* populations are under selection pressure not from a single fungicide but from various ones. Consequently, well-fitted phenotypes with resistance not only to a single fungicide but also to multiple fungicides are present. In addition, fungicide resistant isolates obtained *in vitro* may not be the same as the ones that evolved naturally in terms of their biological fitness.

Although the structure of *B. cinerea* populations in Korea consisting of six phenotype groups was determined in this study, it was difficult to predict the structural changes of the populations in the future based on the fitness-related characteristics. However, the results of this study indicate that careful management of fungicide applications is necessary to achieve effective control of gray mold in vegetable crops in Korea.

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