Survey of Fungicide Resistance for Chemical Control of *Botrytis cinerea* on Paprika

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Four hundred and sixty six isolates of *B. cinerea* were obtained from infected leaves, stems and fruits of paprika grown in greenhouses or plastic film houses in Gangwon province, Korea, between August and November in 2006 and 2007. These isolates were classified into five representative phenotypes of resistant (R) and sensitive (S) reactions as SSR, SRR, RSS, RRS and RSR according to the responses of isolates against benzimidazole, dicarboximide and *N*-phenylcarbamate fungicide in order. The percentage of five phenotypes were 51.3, 2.4, 35.6, 8.1 and 2.6%, respectively. The SSR phenotype (51.3%) was the most common. Among the nineteen fungicide products evaluated to compare their efficacy against gray mold pathogen on the paprika fruit inoculated with fungal mycelia, the mixture of diethofencarb and carbendazim was the most effective followed by iprodione, boscalid, the mixture of iprodione and thiophanate-methyl, fludioxonil, polyoxin-B, fluazinam, the mixture of tebuconazole and tolyfluanid and procymidone; while in the assay methods inoculated with fungal spores, the mixture of tebuconazole and tolyfluanid was the most effective in controlling gray mold followed by boscalid, fludioxonil, the mixture of diethofencarb and carbendazim and the mixture of pyrimethanil and chlorothalonil.

**Keywords**: *Botrytis cinerea*, fungicide resistance, fungicide screening, paprika

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Paprika cultivated in an area of 335 ha, yielded 28,145 tons during the year 2006 was one of the most economically important fruit crops. The gray mold pathogen *B. cinerea* develops mainly on fruits, leaves or petals (Dik & Elad, 1999), threatens the greenhouse paprika productivity throughout the world, and is usually managed by frequent use of prophylactic fungicides (Braun and Sutton, 1984; Bulger et al., 1987; de Visser, 1996; Raposo et al., 1996), which may lead to the development of multiple resistant pathogen population (Moorman and Lease, 1992; Raposo et al., 1996). While the new generation of fungicides like azoles and strobilurins showed limited effectiveness against this pathogen (Vermeulen et al., 2001), many fungicides have been applied routinely on glasshouse crops to control gray mold disease caused by *B. cinerea*. Generally the effectiveness of the commonly applied dicarboximides, benzimidazoles, and the mixture of carbendazim and diethofencarb is much dependent on the accurate and early assessment of the sensitivity of the pathogen population. To investigate it precisely, various bioassay techniques assessing fungal mycelial growth and spore germination on fungicide-amended agar media were used (Pappas, 1997). Benzimidazole and dicarboximide fungicides are known to exert their fungicidal actions by inhibiting beta-tubulin polymerization and by probably causing membrane lipid peroxidation, respectively (Edilich and Lyr, 1992; Ishii, 1992). The resistant strains to both fungicides could be controlled by either compounds with different mode of action or by fungicides exhibiting negatively correlated cross resistance to benzimidazoles (Delp, 1988; Elad et al., 1988; Fujimura, 1993). Diethofencarb, *N*-phenylcarbamate fungicide, was introduced to Korea in 1992 due to its known effectiveness against *B. cinerea* populations resistant to benzimidazole fungicides. Many of the strategies to control gray mold have been used currently. These strategies involved the use of fungicides like 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). Currently, fungicide resistance is a major threat to the vegetable crop industry in the world. In order to establish proper strategies for fungicide management in the field, information on population dynamics of fungicide resistance of pathogens is necessary.

The first objective of this study was to find out the occurrence (%) of gray mold by *B. cinerea* and to recognize changes in sensitivity of *B. cinerea* to benzimidazole, dicarboximide and *N*-phenylcarbamate fungicides. The second objective was to screen nineteen fungicides against...
B. cinerea occurring in paprika and to evaluate the assay methods by using fruits of paprika. The third objective was to evaluate the control efficacy of the selected chemicals among nineteen fungicides by using response against benzimidazole, dicarboximide and N-phenylcarbamate fungicides on amended medium.

Materials and Methods

Isolates of B. cinerea from paprika. During gray mold epidemic months between August and November in 2006 and 2007, isolates of B. cinerea were obtained from diseased leaves, stems and fruits of paprika collected from Pyongchanggun, Jungsungun and Gangneungshi of Ganwondo-the major summer paprika production regions in Korea. B. cinerea was isolated by placing fragments of diseased leaves, stems and fruits on potato dextrose agar (PDA) amended with 100 µg/ml of streptomycin. Tips of mycelia which were grown from infected tissue samples were transferred to fresh PDA medium and then incubated at 20°C. Each isolate was maintained at 20°C on PDA slants and fresh subcultures were made when necessary.

Fungicide resistance of B. cinerea. Fungicide resistance of B. cinerea was examined on PDA containing 10 µg/ml of benzimidazole, dicarboximide and N-phenylcarbamate fungicide each. A previous research on mycelial growth of B. cinerea on PDA containing 2, 10, 50, 250, 1,250 and 5,000 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 and 1995). Mycelia discs (diameter; 5 mm) were cut from the margin of colonies and placed at the center of fungicide-amended PDA plates. After the incubation for 5 days, the diameters of the colonies were measured. All tests were replicated three times. Based on their sensitivity to fungicides, the fungal isolates were categorized as sensitive (S) or resistant (R) phenotypes.

Effect of fungicides on paprika fruit. For this experiment, in 2006, paprika plants were grown hydroponically in the greenhouse at Gangneungshi. The nineteen fungicides used for this study were as following; a mixture of iprodione and thiophanate-methyl (70% WP), iprodione (50% WP), fenhexamid (50% WP), dichlofluanid (50% WP), fluazinam (50% WP), a mixture of carbendazim and diethofencarb (50% WP), boscalid (47% WG), fenhexamid (42% SC), a mixture of thiophanate-methyl and Sulfur (42% SC), pyrimethanil (37% SC), a mixture of pyrimethanil and chlorothalonil (43.5% SC), fludioxonil (20% SC), polyoxin-B (50% SP), fluazinam (50% SC), a mixture of tebuconazole and tolyfluanid (65% WP), a mixture of thiophanate-methyl and triflumizole (60% WP), thiophanate-methyl (70% WP), procymidine (50% WP), and triflumizole (30% WP). The concentration of each fungicide adhered to the standard direction (www.koreacpa.org). Discs of 4×4 cm cut from healthy paprika fruits were washed and placed in Petri dishes or chambers spread with filter paper dipped with each fungicide. The mycelia and spore suspension of 1.5×10⁶ conidia/ml concentration of the Jinbu 34 isolate, which showed resistance to benzimidazole and dicarboximide (RRS) were inoculated on the paprika fruit discs. Plates with inoculated fruit discs were kept in a humid chamber at 18°C for one week and then disease development was observed. All the treatments were replicated 5 times.

Data analysis. All of data were analyzed by analysis of variance, and mean separation was done with Duncan's multiple range test (DMRT) at P=0.05 using Statistical Analysis System (SAS, 2003, Institute Inc., Cary, NC, USA).

Results and Discussion

The symptoms and occurrence of gray mold on paprika. The gray mold pathogen grew densely on withered petals after flowering. Then the fungus invaded the fruit from the fruit apex or a part near the sepals, and caused browning and rotting of the young fruit resulting in fruit falling off. While in immature fruit, small water-soaked and dark-brown round spots progressed on the surface of fruits, the fruit softened and rotted. Leaves and stems were infected by pathogens surviving on infected falling flowers. Also the pathogen was liable to enter through pruning wounds (Fig. 1). During 2006 and 2007, the disease severity (%) of gray mold by B. cinerea in Gangwon province was recorded to be 15.0, 81.4, and 3.3% and 18.0, 8.0, and 3% in Pyongchanggun, Jungsungun and Gangneungshi, respectively (Fig. 2). The main reason why the occurrence of gray mold was so high in Jungsungun in 2006, compared with that of 2007 was because the greenhouses or plastic film houses in Jungsungun in 2006 were somewhat old and poor-suffering from leakage which made the environment in the greenhouses cool and humid and therefore conducive to disease development (caused mostly by B. cinerea during late culture). Another reason was because the farmers working on site did not know how to cope with the disease. After remedying such shortfalls in management, the occurrence of gray mold in 2007 decreased markedly. Three hundred fifty four isolates of B. cinerea were isolated from diseased leaves, stems and fruits of paprika collected from several regions in Ganwondo during 2006 and one hundred twelve isolates of B. cinerea (56, 42 and 14 isolates from Jinbu in Pyongchanggun, Limgae in Jungsungun, and Jibyeundong...
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in Gangneungshi, respectively) were isolated during 2007. *B. cinerea* isolates collected from various locations between 2006 and 2007 showed a little difference in sensitivity to the fungicides tested (Table 1).

**Evaluation of fungicide resistance.** Based on the response of *B. cinerea* isolates to carbendazim, procymidone, and diethofencarb, the fungal isolates collected from paprika greenhouses in Gangwondo during 2006-2007 were classified into five phenotypes; namely SSR, SRR, RSS, RRS and RSR, represented as sensitive (S) or resistant (R) to the fungicides (Fig. 3). The isolation frequencies of phenotypes resistant to benzimidazole, dicarboximide, or *N*-phenylcarbamate fungicide were recorded as 44.0, 10.1 and 54.5%, respectively. The SSR phenotype (51.3%) was the most common followed by RSS phenotype (35.6%) and RRS phenotype (8.1%), while the phenotypes of SRR (2.4%) that show simultaneous resistance to dicarboximide and *N*-phenylcarbamate and RSR (2.6%) that show simultaneous resistance to benzimidazole and *N*-phenylcarbamate were less than any other populations. The phenotype being simultaneously sensitive to three fungicides (SSS) and having resistance only to dicarboximide fungicide (SRS) did not show in this study and the population resistant to

| Year      | Location                | No. of isolates tested | Benzimidazole | Dicarboximide | *N*-phenylcarbamate |
|-----------|-------------------------|------------------------|---------------|---------------|---------------------|
| 2006      | Gangneungshi Jibyeundong| 36                     | 0             | 3(8.3)        | 32(88.9)            |
|           | Gangneungshi Okgye      | 22                     | 1(57.1)       | 2(9.5)        | 11(52.4)            |
|           | Jungsungun Limgye       | 53                     | 20(37.7)      | 6(11.3)       | 30(56.6)            |
|           | Pyongchanggun Jinbu     | 243                    | 108(44.4)     | 37(15.2)      | 132(54.3)           |
| **Subtotal** |                        | **354**               | **143(40.5)** | **45(12.7)**  | **205(58.0)**       |
| 2007      | Gangneungshi Jibyeundong| 14                     | 1(7.1)        | 1(7.1)        | 13(92.9)            |
|           | Jungsungun Limgye       | 42                     | 36(85.7)      | 3(7.1)        | 7(16.7)             |
|           | Pyongchanggun Jinbu     | 56                     | 25(44.6)      | 0             | 37(66.1)            |
| **Subtotal** |                        | **112**               | **62(55.4)**  | **2(3.6)**    | **43(50.9)**        |
| **Total** |                         | **466**               | **205(44.0)** | **47(10.1)**  | **254(54.5)**       |

*a Resistance to benzimidazole, dicarboximide and *N*-phenylcarbamate was examined on PDA media incorporated with 10 µg/ml of each fungicide.

*b Percentages of resistant isolates in parentheses.
three tested fungicides (RRR phenotype) was not found in spite of it being reported in Korea first during 1995 (Kim et al., 1998). The SSR phenotype can be the wild type of the original \textit{B. cinerea} population in Korea prior to the introduction of benzimidazole fungicides in 1973. 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. 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 (Kim et al., 2001). Recent occurrences of the RSR phenotype may have resulted from increased applications of the mixture of carbendazim and diethofencarb to control benzimidazole-resistant \textit{B. cinerea} since 1992. Resistance to benzimidazole fungicides is known to be controlled by one gene in \textit{B. cinerea} contained in \textit{beta-tubulin} polymerization and is negatively correlated with resistance to \textit{N-phenylcarbamate} fungicides (Delp, 1988; Ishii, 1992). Therefore, absence of the negative cross resistance to benzimidazole and \textit{N-phenylcarbamate} in the RSR 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 population of \textit{B. cinerea} (Kim et al., 2001).

**Assay for screening of fungicides on paprika fruit.** Even though the phenotype SSR was the predominant one among the five phenotypes (Fig. 4), it is important to control of RRS phenotype for the sake of gray mold control in paprika. Hence we used the mycelia and spore of the Jinbu 34 (RRS) isolate which was more vigorous than other phenotype isolates for screening of fungicides (data were not shown). The results revealed that the mixture of diethofencarb and carbendazim showed the highest activity against gray mold pathogen of paprika followed by iprodione, boscalid, the mixture of iprodione and thiophanate-methyl, fludioxonil, polyoxin-B, fluazinam, the mixture of tebuconazole and tolylfluanid, and procymidone. In the experiments of assay methods inoculated with fungal spores alone, the mixture of tebuconazole and tolyfluanid was the most effective against gray mold followed by boscalid, fludioxonil, the mixture of diethofencarb and carbendazim and the mixture of pyrimethanil and chlorothalonil. However, the present study was carried out to test the \textit{in vitro} sensitivity of \textit{B. cinerea} obtained from paprika greenhouses. It is necessary for us to use careful management of fungicide applications to achieve effective control against gray mold of paprika. Further long-term monitoring of the shift in sensitivity of \textit{B. cinerea} to fungicides is needed in order to determine the risk of development of practical resistance in greenhouses.

**Response of phenotypes of \textit{B. cinerea} on five selected fungicides.** According to the results, all of the amended media with five selected fungicides mixture of carbendazim and diethofencarb, boscalid, fludioxonil, mixture of tebuconazole and tolyfluanid, and procymidone were effective in controlling all of the four phenotypes (SSR, SRR, RSS, and RRS) of \textit{B. cinerea}. In the amended media with the
mixture of carbendazim and diethofencarb, fludioxonil, the mixture of tebuconazole and tolyfluanid, none of the phenotypes of *B. cinerea* grew. In the media amended with boscalid, the RRS phenotype didn’t grow, whereas the SSR, SRR and RSS phenotypes grew a little (Fig. 5). In the amended media adding procymidone, the SSR and RSS phenotypes were controlled perfectly whereas the phenotypes of SRR and RRS were controlled, but not perfectly.

Benzimidazoles and related compounds have been introduced in the late 1960s, and have enabled the growth of gray mold on various plants to be controlled. However, resistant strains have arisen very rapidly and today there is still a need for novel botryticides that can be applied at veraison and later (Leroux, 1995). Potential candidates may well exist among the experimental fungicides known to inhibit respiratory processes, microtubule assembly, sterol, acetyl-CoA carboxylase and other essential biochemical processes. The results in Fig. 5 confirmed that using the same type of chemicals such as benzimidazoles or dicalboximides can not be recommended throughout the season of growing paprika in order to control gray mold. The present strategy in the world is to alternate the various groups of botryticides with a maximum of one spray per year for each family. Another approach towards durable resistance management against gray mold is the use of mixtures of products with different biochemical modes of action.

Although five phenotype groups of *B. cinerea* populations were determined and selected five fungicides in this study, it was difficult for us to predict that the structural changes of the populations in the future based on its characteristics. However, the results of this study indicate that careful management of fungicide applications is necessary to achieve effective control against gray mold of paprika in Korea.

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