Analysis of Fungicide Sensitivity and Genetic Diversity among *Colletotrichum* Species in Sweet Persimmon

Geun-Hye Gang¹, Hyun Ji Cho¹, Hye Sun Kim¹, Yong-Bum Kwack² and Youn-Sig Kwak³*

¹Division of Applied Life Science, Gyeongsang National University, Jinju 660-701, Korea
²Namhae Sub-Station, NIHHS, RDA, Namhae 668-812, Korea
³Department of Plant Medicine and Institute of Agriculture & Life Science, Gyeongsang National University, Jinju 660-701, Korea

(Received on March 11, 2015; Revised on April 12, 2015; Accepted on April 17, 2015)

Anthracnose, caused by *Colletotrichum gloeosporioides* (*C. gloeosporioides*; Teleomorph: *Glomerella cingulata*), is the most destructive disease that affects sweet persimmon production worldwide. However, the biology, ecology, and genetic variations of *C. gloeosporioides* remain largely unknown. Therefore, in this study, the development of fungicide resistance and genetic diversity among an anthracnose pathogen population with different geographical origins and the exposure of this population to different cultivation strategies were investigated. A total of 150 pathogen isolates were tested in fungicide sensitivity assays. Five of the tested fungicides suppressed mycelial pathogen growth effectively. However, there were significant differences in the sensitivities exhibited by the pathogen isolates examined. Interestingly, the isolates obtained from practical management orchards versus organic cultivation orchards showed no differences in sensitivity to the same fungicide. PCR-restriction fragment length polymorphism (RFLP) analyses were performed to detect internal transcribed spacer regions and the β-tubulin and glutamine synthetase genes of the pathogens examined. Both the glutamine synthetase and β-tubulin genes contained a complex set of polymorphisms. Based on these results, the pathogens isolated from organic cultivation orchards were found to have more diversity than the isolates obtained from the practical management orchards.

**Keywords**: anthracnose, *Colletotrichum gloeosporioides*, fungicide sensitivity, PCR-RFLP

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*Corresponding author.
Phone) +82-55-772-1922, FAX) +82-55-772-1929
E-mail) kwak@gnu.ac.kr

Sweet persimmon (*Diospyros kaki* L.) is cultivated as a major economic fruit crop in Korea. The pomiculture of sweet persimmon has been steadily increasing, and currently has reached nearly 29,000 hectares. Correspondingly, sweet persimmon constitutes a large portion of the domestic market in Korea, and it is also steadily increasing as an overseas export. However, anthracnose, caused by *Colletotrichum gloeosporioides* (*C. gloeosporioides*) in sweet persimmon, is a devastating disease that affects fruit production (Kwon and Park, 2004). In general, *Colletotrichum* spp. infect various plants and are responsible for considerable economic losses, including those from sweet persimmon crops. Currently, at least two *Colletotrichum* spp. have been identified as causal anthracnose agents in sweet persimmon, *C. gloeosporioides* and *C. acutatum* (Zhang et al., 2005). Anthracnose occurs on the young twigs, tissues of stems, flowers, and fruits, with minimal effects on leaves (Kim et al., 2004). Anthracnose disease not only reduces fruit yield and quality, but can also cause severe postharvest disease.

Currently, chemical control is considered the best method for treating crop disease. For the cultivation of sweet persimmon, fungicide spray is applied 6–8 times to provide disease control. However, despite the regular application of fungicide spray, many sweet persimmon orchards are still severely damaged by anthracnose disease. This may be due to the development of tolerance by fungicide-resistant pathogen strains (Wharton and Deiguez-Uribeondo, 2004). In a previous study, anthracnose pathogens were found to be effectively controlled by prochloraz and tebuconazole, with up to 91% control achieved (Lim and Choi, 2006). In contrast, propineb only inhibited 46.4% of a hypha growth strain (Lim et al., 2009). Lim and Choi (2006) further demonstrated that the frequency of pathogen isolates that exhibit propineb resistance has increased, and pathogens that were continually exposed to the fungicide developed...
resistance. Moreover, the resistance isolates became more dominant within the pathogen population. For each isolated pathogen, geological separation also contributed to the differences observed in fungicide sensitivity (Kumar et al., 2007).

Morphological characteristics of anthracnose pathogens represent a complicated relationship among the genus. For taxa identification in fungi, sequencing of the internal transcribed spacer (ITS) is most commonly used. Gene variations in both the ITS regions and the β-tubulin gene sequences have been used for the identification of *C. acutatum* versus *C. capsici* and *C. gloeosporioides* species (Phoulivong et al., 2010). However, the genus, *Colletotrichum*, is a genetically variable and complex pathogen, and is considered to be a group species (Sutton, 1992). Therefore, the identification of gene variations is not useful for identifying the *Colletotrichum* species. Recent studies of anthracnose have focused on the complex relationship, or genetic diversity, among pathogen populations (Xie et al., 2010). Molecular markers have been used for characterizing race, phylogenetic relationships, and/or diversity among pathogen populations (Lopez and Lucas, 2010). In particular, restriction fragment length polymorphism (RFLP) patterns of ribosomal DNA have been used to differentiate *Colletotrichum* species (Ramdeen et al., 2013), and often, β-tubulin or glutamine synthetase (GS) gene sequences are employed to identify or determine *Colletotrichum* genetic variations (Guerber et al., 2003).

The present studies were performed to elucidate the relationship between fungicide sensitivity and genetic variations among sweet persimmon anthracnose pathogens. In addition, the effect of different methods of cultivation on the development of fungicide resistance strains among the *Colletotrichum* population was investigated.

### Materials and Methods

**Origin and isolation of the anthracnose pathogens.** During the cultivation season between 2012 and 2013, five sweet persimmon orchards in the Gyeongnam province were visited. Three orchards were maintained with practical management methods (Daeok, Munsan, Yearee) and two orchards (Masan, Masan-2) were maintained with organic farming methods. Typical anthracnose disease symptoms were observed on twigs and fruit, and infected samples were collected and transported to the lab within three hours of collection. Disease lesions were cut from the surface of the fruit, with a depth less than 1 cm. The surface of each lesion was sterilized with 1% sodium hypochlorite followed by 70% ethanol and then the specimens were washed in sterilized distilled water three times. The specimens were dried for 15 min under a fume hood and were placed in water agar media (WA) at 28°C until spores developed. Conidia were observed using a microscope (Olympus BX15). From the five orchards, a total of 485 pathogens were isolated (Table 1).

### Identification of the pathogen isolates with species-specific PCR.** Genomic DNA was extracted from fresh mycelium by the CTAB method (Ford et al., 2000). DNA from each isolate was amplified using the following species-specific primer pairs: Colg1 (5'-AACCTTTGGTAA-CATACC-3') and Colg2 (5'-CCCTCGGATCCCAG-3') (443-bp) for *C. gloeosporioides*; and Colg1 and CT 2 (5'-CTT TAA GGG CCT ACG TCA A-3') (375-bp) for *C. truncatum* (Chen et al., 2006). The PCR conditions included an initial denaturation step at 94°C for 5 min, followed by 34 cycles of 94°C for 30 sec, 63°C for 30 s, and at 72°C for 1 min. A final extension step heated samples to 72°C for 6 min. The primer set, col2 (d5'-TTA CTA CGC AAA GGA GGC T-3') and acut1 (d5'-CCG GAG GAA ACC AAA CTC TAT TTAC-3') (462-bp) were used to detect *C. acutatum*, while the primer set, col1 (d5'-AAC CCT TTG TGA ACR TAC CTA-3') and col2 (318-bp), were employed for all other *Colletotrichum* spp. (Martinez et al., 2003). The same PCR conditions were applied for the identification of *C. acutatum* and *Colletotrichum* spp. samples, except that the annealing temperature was 56°C for the col2 and col2 primers. The PCR mixtures for analysis included 10 µg of template DNA, 1U Taq DNA polymerase, 0.1 mM dNTPs, 2 × PCR reaction buffer, and 10 pmol of each primer. The complete ITS region of the ribosomal DNA were amplified with the primers, ITS1 (5'-GCGTAGGT-GAACCTGCGG-3') and ITS4 (5'-TTCCTCGGTTA-TGATATGC-3) (Kumar et al., 2005). Sequencing was subsequently performed by Solgent Co. (Daejon, Korea). Sequences were compared to previously deposited *Colletotrichum* spp. sequences in GenBank (www.blast.ncbi.nlm.nih.gov/)..

### Table 1. Number of *C. gloeosporioides* isolates that were obtained from each orchard

<table>
<thead>
<tr>
<th>Orchard</th>
<th>Management method</th>
<th>Number of isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Daegok</td>
<td>Practical</td>
<td>122</td>
</tr>
<tr>
<td>Yearee</td>
<td>Practical</td>
<td>49</td>
</tr>
<tr>
<td>Masan</td>
<td>Organic</td>
<td>92</td>
</tr>
<tr>
<td>Masan-2</td>
<td>Organic</td>
<td>82</td>
</tr>
<tr>
<td>Munsan</td>
<td>Practical</td>
<td>140</td>
</tr>
</tbody>
</table>

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