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Monitoring of Benzimidazole Resistance in *Botrytis cinerea* Isolates from Strawberry in Korea and Development of Detection Method for Benzimidazole Resistance

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***Botrytis cinerea* is a major fungal plant pathogen that causes gray mold disease in strawberries, leading to a decrease in strawberry yield. While benzimidazole is widely used as a fungicide for controlling this disease, the increasing prevalence of resistant populations to this fungicide undermines its effectiveness. To investigate benzimidazole resistant *B. cinerea* in South Korea, 78 strains were isolated from strawberries grown in 78 different farms in 2022, and their EC_{50} values for benzimidazole were examined. As a result, 64 strains exhibited resistance to benzimidazole, and experimental tests using detached strawberry leaves and the plants in a greenhouse confirmed the reduced efficacy of benzimidazole to control these strains. The benzimidazole resistant strains identified in this study possessed two types of mutations, E198A or E198V, in the *TUB2* gene. To detect these mutations, TaqMan probes were designed, enabling rapid identification of benzimidazole resistant *B. cinerea* in strawberry and tomato farms. This study utilizes TaqMan real-time polymerase chain reaction analysis to swiftly identify benzimidazole resistant *B. cinerea*, thereby offering the possibility of effective disease management by identifying optimum locations and time of application.**

Keywords **:** benzimidazole, *Botrytis cinerea*, strawberry, TaqMan qPCR analysis

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Strawberry (*Fragaria* × *ananassa*) is a fruiting, herbaceous, cultivated perennial belonging to the Rosaceae family and the genus *Fragaria*. As of 2021, the strawberry cultivation area in South Korea amounted to a total of 6,103 hectares, accounting for 12.7% of the overall fruit cultivation area in the country. Additionally, total production reached 177,480 tons, representing a significant proportion (8.5%) of the total fruit production in South Korea (Ministry of Agriculture, Food and Rural Affairs, 2022). Strawberries are also exported to some extent. In 2021, the export revenue amounted to $64,679,000 highlighting the significant contribution of strawberries to the domestic agricultural industry (Korea Agro-Fisheries and Food Trade Information, 2023). Among the strawberry varieties developed in Korea, the variety 'Sulhyang' accounted for 83.1% of the total surveyed varieties in 2021 (Korea Agro-Fisheries and Food Trade Information, 2023). However, relying heavily on a single variety poses a high risk of significant yield losses in the event of pest and disease outbreaks. For example, gray mold disease can affect 'Sulhyang' strawberries, causing damage of up to 50% during the harvesting season (Nam et al., 2021).

Botrytis cinerea, which causes gray mold in strawberries,

forms conidia and spreads through the air in cool and humid environments, infecting plant tissues (Braun and Sutton, 1987; Strømeng et al., 2009). It is pathogenic in over 200 crops, not limited to strawberries (Williamson et al., 2007). Moreover, throughout post-harvest handling, distribution, and storage processes, it exhibits a high infectivity, resulting in significant negative consequences on the quality and marketability of the crops (Droby and Lichter, 2007). Chemical control using fungicides is known to be the most effective method for managing *B. cinerea* (Mertely et al., 2000). However, continuous use of fungicides poses problems by reducing the effectiveness of disease control measures (Faretra and Pollastro, 1993; Moraes Bazioli et al., 2019). In Korea, it has been reported that 60.7% of *B. cinerea* strains exhibited resistance to benzimidazole fungicides, among various fungicides used for disease control, as of 1993 (Kim et al., 1993).

Benzimidazole fungicides, also known as methyl benzimidazole carbamates, interfere with the formation of the α, β-dimer by binding to the β-tubulin subunit during the microtubule biosynthesis process in fungi (Davidse and Flach, 1977). However, if mutations occur in specific nucleotide sequences of the *TUB2* gene, which encodes the β-tubulin protein, benzimidazole fungicides fail to bind to their target, leading to resistance to the fungicidal effects (Kwak et al., 2017; Leroux et al., 2002; Yarden and Katan, 1993). Specifically, the benzimidazole resistance in *B. cinerea* has been attributed primarily to a mutation at the 198th codon of the β-tubulin (Bardas et al., 2008). Due to this highly specific mode of action, even a single nucleotide mutation can easily confer resistance to benzimidazole fungicides. Therefore, effective management of benzimidazole fungicide usage across all regions in Korea is necessary to ensure their efficacy.

In this study, a total of 78 *B. cinerea* strains were isolated from strawberry farms across regions of South Korea in 2022 to monitor the recent benzimidazole resistance status within the country. The objectives of this study were as follows: (1) to examine the sensitivity of *B. cinerea* strains to the benzimidazole fungicide and sequence the fungicide target gene for determining the level of benzimidazole resistance and mechanism of the resistance, (2) to determine the differences in disease control between resistant and sensitive strains when exposed to benzimidazole fungicide at the concentration commonly used in farms, and (3) to utilize a TaqMan real-time polymerase chain reaction (PCR) analysis for detecting the presence of benzimidazole resistant *B. cinerea* within greenhouses.

Materials and Methods

Fungal strains and plant. In 2022, 78 strains of *B. cinerea* were isolated from 78 different strawberry farms, which were affected by gray mold disease in Korea. Single isolate was collected from each strawberry farm and identified based on ITS and *NEP2* gene sequences (unpublished data). The isolated strains, along with their respective geographic origins, are provided (Supplementary Table 1). They were cultured on potato dextrose agar (PDA; MBcell, Seoul, Korea) at 25°C under dark conditions conducive for mycelial growth. The strains were then stored at –80°C in 25% glycerol solution. To evaluate the fungicide sensitivity of the strains on strawberries, the 'Sulhyang' variety, which has the highest cultivation rate in Korea was used (Jeong et al., 2018). To assess the effectiveness of benzimidazole fungicides against the isolated strains, benomyl (DuPont, Newark, DE, USA) was chosen as a representative agent.

***In vitro* sensitivity assay of *B. cinerea* strains to benzimidazole.** The *B. cinerea* strains were inoculated onto PDA media and incubated at 25°C for 4 days. Agar plugs (5-mm-diameter) from colonies of the isolates were inoculated onto PDA media containing benomyl at concentrations of 0, 0.1, 1, 10, 100, and 1,000 mg/l. After incubation for 3 days at 25°C, the diameter of the colonies was measured using a digital caliper (Mitutoyo, Kawasaki, Japan). Two separate experiments and two replicates (Petri plates) per each experiment were conducted for each strain or concentration. The data were obtained by subtracting the lengths of 5mm agar plugs from mycelial diameters. The EC_{50} (effective concentration for 50% inhibition) values for each strain were calculated using the LL.3 model from the *drc* package (Ritz et al., 2015) in R software (version 4.2.3, R Core Team 2023, R Foundation for Statistical Computing, Vienna, Austria).

PCR amplification and sequencing of β-tubulin (*TUB2*). DNA was extracted from the mycelia grown on a PDA medium using the CTAB method (Cubero et al., 1999). The *TUB2* gene was amplified using nPfu-Forte DNA polymerase (Enzynomics Inc., Daejeon, Korea). A total of 20 μl PCR reaction mixture was prepared, consisting of 2 μl of 10× nPfu-Forte buffer, 0.5 μl of 10 pmol/μl forward primer (5′-ATG CGT GAG ATT GTA TGT ATT TC-3′), 0.5 μl of 10 pmol/μl reverse primer (5′-CTA TTC CTC GCC CTC AAT TG-3′), 2 μl of 2 mM dNTP mixture, 1 μl of 100 ng genomic DNA, 0.2 μl of 2.5 units/μl nPfu DNA

polymerase, and 13.8 µl of UltraPure DNase/RNase-free distilled water (ThermoFisher Scientific, Waltham, MA, USA). The PCR reaction was carried out using the Mini-Amp PCR system by Applied Biosystems (ThermoFisher Scientific) with an initial denaturation at 95°C for 2 min, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 48°C for 30 s, and extension at 72°C for 1 min. A final extension step was performed at 72°C for 5 min. Subsequently, enzymatic purification was performed using the ExoSAP-IT PCR Product Cleanup Reagent (Applied Biosystems, Waltham, MA, USA). The amplicons were sequenced by a commercial sequencing service provider (Macrogen Inc., Seoul, Korea).

Sensitivity assays of *B. cinerea* strains to benzimidazole on strawberry leaves and fruits. Detached strawberry leaves were sterilized with 1% NaOCl for 3 min. The sterilized leaves were then rinsed thrice with sterile ddH_2O for 1 min. After naturally drying to remove the remaining moisture, the leaves were soaked for 3 min in benomyl solutions at concentrations of 250 mg/l (recommended concentration), 125 mg/l (half the recommended concentration), and 500 mg/l (double the recommended concentration). The control group was treated with sterile ddH_2O. After drying, the treated leaves were inoculated with agar plugs (5mm-diameter) taken from the edge of fungal colonies. The inoculated leaves were cultured at 25°C under dark conditions for 5 days, and the resulting lesions were measured using a digital caliper. The relative lesion length was calculated as follow:

$$\text{Relative lesion length rate (\%)} \frac{Lt}{L0} \times 100$$

'Lt' represents the average length of lesions when treated with benomyl, while 'L0' represents the average length of lesions when treated with ddH_2O. For the strawberry fruits, the same surface disinfection with NaOCl was performed, followed by the same method as described above, including benomyl treatment. Fungal spore suspensions obtained after 7 days of cultivation were adjusted to a concentration of 1×10^6 spores/ml using a hemocytometer (Marienfeld-Superior, Lauda-Königshofen, Germany) and a microscope (Olympus BX51, Tokyo, Japan). Sterile pipette tips were used to create consistent wounds on the benomyl-treated strawberry fruits, and 20 µl of the spore suspension was inoculated into the wounds. Subsequently, the inoculated fruits were cultured at 25°C under dark conditions for 4 days, and the length of the lesions were measured using a digital caliper. The relative lesion length was calculated using the aforementioned formula. This experimental procedure was performed in triplicate.

Control efficacy assay of benzimidazole against *B. cinerea* strains in strawberry farm. Field efficacy of benzimidazole against benzimidazole sensitive and resistant *B. cinerea* strains were assayed. For the study, strawberry seedlings were cultivated in a greenhouse, located in Gwangju, South Korea, to promote fruit formation. The strawberry plants, which had never been exposed to any fungicides, were sprayed with 12.5 ml of benomyl at the recommended concentration of 250 mg/l using a sprayer A5-700 (APOLLO INDUSTRIAL Co., Ltd., Siheung, Korea). The control group, on the other hand, was sprayed only with 12.5 ml of sterile ddH_2O. After allowing the sprayed strawberries to dry for a day, 2.5 ml of spore suspensions of each strain, at a concentration of 1×10^5 spores/ml, were sprayed using the sprayer. Two weeks after inoculation, the severity of gray mold disease on each strawberry plant, especially the fruit portion, was visually assessed and scored on a scale of 0 (no disease) to 5 (severe disease), expressed as a percentage. The experiment was conducted twice with five replicates. Using these results, control efficacy was calculated according to the following formula (Paul et al., 2021):

$$\text{Control efficacy (\%)} \frac{A1 - A2}{A1} \times 100$$

Where A1 represents the disease severity in strawberries treated only with sterile ddH_2O, without benomyl treatment, and A2 represents the disease severity in strawberries treated with benomyl.

Sampling for diagnosis of benzimidazole resistance using TaqMan qPCR analysis. Diseased samples of strawberry leaves and tomato fruits were collected from two greenhouses at two different locations, respectively, for diagnosis of benzimidazole resistance. One of the greenhouses was situated in Gwangju, where strawberries had been previously grown and tomato cultivation was in progress (35°10′05.7″N, 126°.49′22.1″E), while the other was located in Damyang, where strawberry cultivation was still in progress (35°15′32.2″N, 126°55′46.1″E). The greenhouse in Gwangju was visited to gather tomato fruits for sampling in May 2023, and in Damyang, strawberry leaves were collected for sampling in July 2023.

Table 1. TaqMan probes and primers' designs for benzimidazole resistance assessment in *Botrytis cinerea* strains

Type	Sequence (5′-3′)	Label	Target
Probe	GAACTCTGACGAGACCTTCTGTATCG	Cy5	Wild type
Probe	GAACTCTGACGCGACCTTCTGTATCG	FAM	E198A
Probe	GAACTCTGACGTGACCTTCTGTATCG	Texas Red	E198V
Probe	GAACTCTGACGGGACCTTCTGTATCG	HEX	E198G
Primer (forward)	TACCGTTGTCGAGCCATATA	-	-
Primer (reverse)	CTGAGCTTCAAGGTTCTCAT	-	-

TaqMan qPCR analysis. TaqMan qPCR analysis was performed using a CFX96 Real-Time System (Bio-Rad, Carlsbad, CA, USA). Each reaction consisted of a mixture of 10 µl of SsoAdvanced Universal Probe Supermix (Bio-Rad), 400 nM of each primer, 200 nM of the probe, 1 µl of genomic DNA, and 6 µl of DNase-free water. The primer and probe information are described in Table 1. The probes were designed with different single base modifications to detect point mutations conferring resistance to benzimidazole. The probe for detecting the wild type without mutations was labeled with Cy5 fluorescence; the probe for detecting E198V mutation, with Texas Red; the probe for detecting E198A mutation, with FAM; and the probe for detecting E198G mutation, with HEX. The initial denaturation was carried out at 95°C for 3 min, followed by 45 cycles of denaturation at 95°C for 15 s, and annealing at 63°C for 30 s. For the qPCR sensitivity assay, DNA of CMML22-BC67 was tested with concentrations ranging from 100,000,000 fg/µl (100 ng) to 10 fg/µl (10-fold serial dilutions) (Supplementary Fig. 1).

Statistical analysis. Statistical analysis of this study was conducted using R software (v4.2.3, R Development Core Team 2023). After calculating parameters of relative growth and disease severity, Tukey's honestly significant difference test was performed to evaluate the significance. The significance level was set at $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

Results

***In vitro* benzimidazole sensitivity assay.** *In vitro* benzimidazole sensitivity assays of 78 strains collected from 78 farms resulted in the differentiation of 14 sensitive strains and 64 resistant strains. The strains with an EC_{50} value of 300 mg/l or higher were classified as the resistant group, while those with an EC_{50} value of less than 1 mg/l were classified as sensitive. The EC_{50} values of the resistant group ranged from 377.62 mg/l (CMML22-BC04) to 5,955.72 mg/l (CMML22-BC29). For the sensitive group, EC_{50} values ranged from 0.04 mg/l (CMML22-BC25) to 0.15 mg/l (CMML22-BC94) (Fig. 1). The investigation covering a wide geographic area with five different regions revealed that Gyeongsang Province had 93.75% resistant isolates; Jeolla Province, 87.50%, Chungcheong Province, 85.71%; Gangwon Province, 68.75%; and Gyeonggi Province, 75.00%.

Benzimidazole sensitivity tests on strawberry leaves and fruits. The effectiveness of benzimidazole fungicides in controlling *B. cinerea* infection in strawberry leaves and fruits and the phenotypic differences between sensitive and resistant strains were examined. It was observed that even when treated with half of the recommended concentration of benomyl (125 mg/l) typically used in farms, disease control was effectively achieved in the sensitive strain when inoculated on strawberry leaves. However, no control of the disease was observed in the resistant strains even when exposed to double the recommended concentration of benomyl (500 mg/l) (Fig. 2). Furthermore, in strawberry fruits, it was evident that the sensitive strains responded positively to the treatment with the recommended concentration of benomyl (250 mg/l), whereas no disease control was observed in the resistant strains (Fig. 3).

Control efficacy of benzimidazole against *B. cinerea* strains in strawberry farm. The disease severity of strawberry plants caused by sensitive and resistant strains in response to benzimidazole were observed in strawberries grown in a greenhouse setting. Disease severity caused by the sensitive strain CMML22-BC67 was significantly reduced from 66.66% to 13.33% by application of 250 mg/l of benomyl ($**P < 0.01$). However, the severity caused by resistant strains (CMML22-BC03, -BC27, -BC30, and -BC42) was not significantly reduced even after benomyl treatment. For the disease severity of resistant

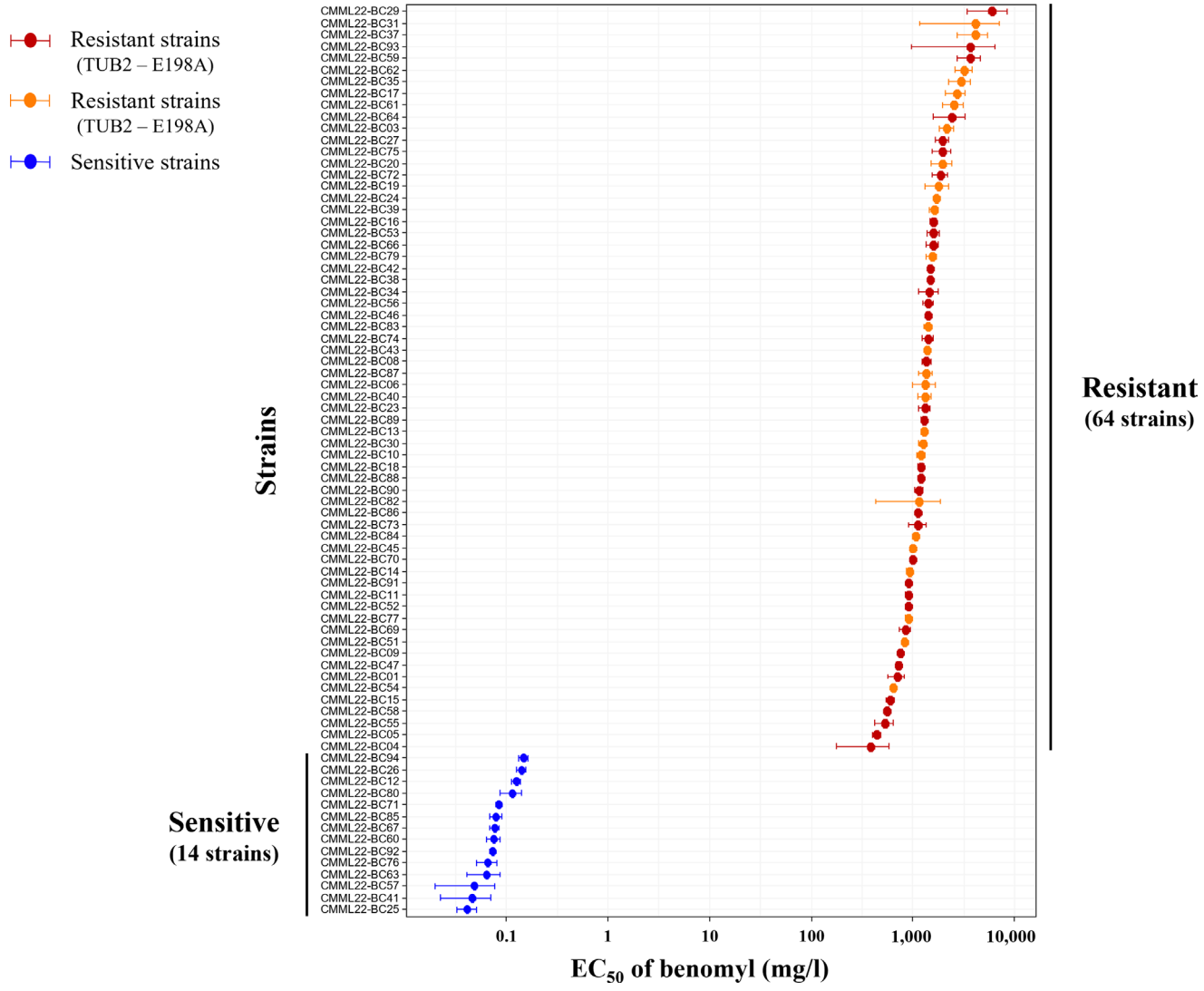

Fig. 1. Benzimidazole sensitivity of *Botrytis cinerea* strains isolated from strawberries grown in 78 different farms in Korea. The EC_{50} values of *B. cinerea* strains to a benzimidazole fungicide, benomyl, were divided into 14 benzimidazole sensitive strains and 64 resistant strains. Strains with EC_{50} values equal to or below 0.147 mg/l, including CMML22-BC94, were classified as sensitive and marked in blue. On the other hand, strains with EC_{50} values equal to or above 377 mg/l, including CMML22-BC04, were classified as resistant strains. Among the resistant strains, those with the E198A mutation in the *TUB2* gene were marked in red, while those with the E198V mutation were marked in yellow.

strains, CMML22-BC03 slightly decreased after treatment of benomyl from 86.66% to 80.00%; CMML22-BC27 remained 100.00%; CMML22-BC30 increased from 45.00% to 71.42%; and CMML22-BC42 increased from 80.00% to 88.88% (Fig. 4). In the case of the sensitive strain, CMML22-BC67, the control efficacy was 80.00%, indicating a high effectiveness of benomyl. However, for the resistant strain, CMML22-BC03, the control efficacy was only 7.69%, indicating a very low effectiveness of benomyl. Additionally, other resistant strains, CMML22-BC27, -BC30, and -BC42, showed a control efficacy of 0%, indicating that they were not controlled at all by benomyl.

Mutation in *TUB2* associated with benzimidazole resistance. Mutations were investigated in the amino acid sequence of β-tubulin from the 1st to the 238th position. The results revealed no mutations in the sensitive strains, whereas a mutation, E198A or E198V, was identified in the resistant strains. Among the resistant strains, 37 strains contained the E198A mutation, while 27 strains contained

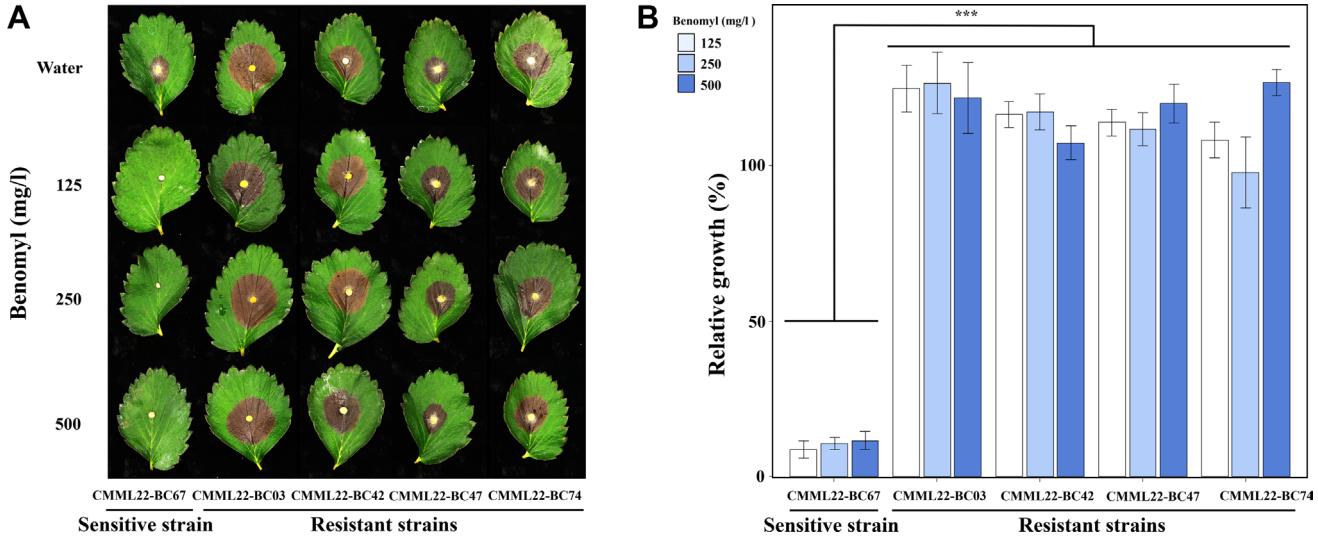

Fig. 2. Sensitivity of benzimidazole sensitive and resistant *Botrytis cinerea* strains on strawberry leaves against benomyl. (A) Pictures of lesions on strawberry leaves caused by *B. cinerea* strains in response to benomyl at different concentrations. (B) Relative growth of *B. cinerea* strains on strawberry leaves treated with benomyl. In all three concentrations of benomyl treatment, the sensitive strain CMML22-BC67 exhibited significantly lower disease severity compared to the resistant strains. Error bars represent the standard error of the mean (***$P < 0.001$).

Table 2. Mutation within β-tubulin for each *Botrytis cinerea* strain and the average EC_{50} value for each genotype

Genotype	Benzimidazole sensitivity	Strain no. (CMML22-)	No. of strains	Mean EC_{50} (mg/l)[a]
Wild type	Sensitive	BC12, BC25, BC26, BC41, BC57, BC60, BC63, BC67, BC71, BC76, BC80, BC85, BC92, BC94	14	0.08 ± 0.01 a
E198A	Resistant	BC01, BC04, BC05, BC08, BC09, BC11, BC15, BC16, BC18, BC23, BC27, BC29, BC34, BC38, BC42, BC46, BC47, BC52, BC53, BC55, BC56, BC58, BC59, BC64, BC66, BC69, BC70, BC72, BC73, BC74, BC75, BC86, BC88, BC89, BC90, BC91, BC93	37	1,453.55 ± 173.52 b
E198V	Resistant	BC03, BC06, BC10, BC13, BC14, BC17, BC19, BC20, BC24, BC30, BC31, BC35, BC37, BC39, BC40, BC43, BC45, BC51, BC54, BC61, BC62, BC77, BC79, BC82, BC83, BC84, BC87	27	1,750.32 ± 180.68 b

[a]Statistical differences of means ± standard errors were tested by Tukey's honestly different test ($P < 0.001$).

the E198V mutation (Table 2).

Detection of benzimidazole resistant *B. cinerea* using TaqMan qPCR analysis. To confirm the presence of mutations in β-tubulin associated with resistance to benzimidazole, TaqMan qPCR analysis was performed on the sensitive strain CMML22-BC67 and the resistant strains CMML22-BC03 and CMML22-BC42, which harbor E198V and E198A mutations in TUB2, respectively. The results showed that only the Cy5 fluorescence of the probe targeting the TUB2-198E was detected in CMML22-BC67 (Fig. 5A), while the Texas Red fluorescence of the probe targeting the TUB2-198V was detected in CMML22-BC03 (Fig. 5B), which acquired resistance due to the E198V mutation. Additionally, the FAM fluorescence of the probe targeting the TUB2-198A was detected in CMML22-BC42, where the E198A mutation had occurred (Fig. 5C).

Diagnosis of benzimidazole resistance in strawberry and tomato growing farms using TaqMan qPCR analysis. DNA was extracted from strawberry leaves sampled in Damyang and subjected to TaqMan qPCR analysis us-

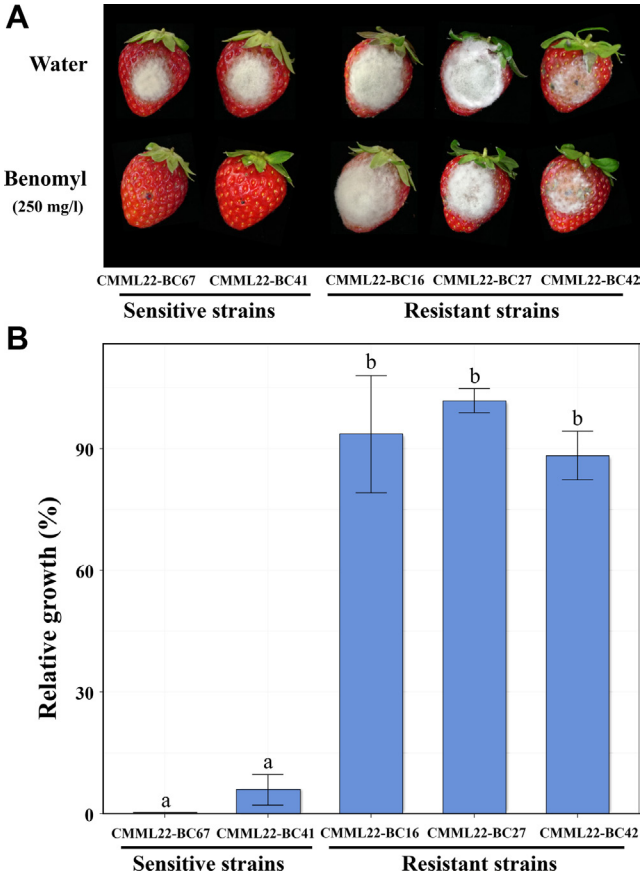

Fig. 3. Sensitivity of benzimidazole sensitive and resistant *Botrytis cinerea* strains on strawberry fruits against benomyl (250 mg/l). (A) Pictures of strawberry fruits caused by *B. cinerea* strains in response to benomyl. (B) Relative growth of *B. cinerea* strains on strawberry fruits treated with benomyl. Significant differences were observed only between the sensitive strains and the resistant strains. Statistical differences of means ± standard errors were tested by Tukey's honestly different test ($P < 0.001$).

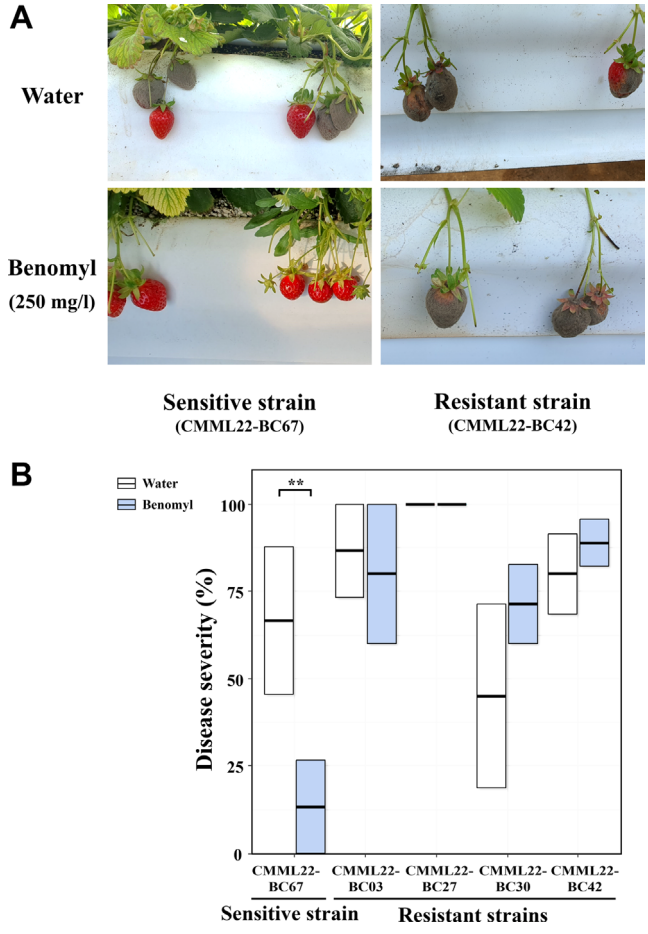

Fig. 4. Assessment of gray mold disease severity in strawberry farm. (A) Pictures of strawberry gray mold caused by benzimidazole sensitive and resistant *Botrytis cinerea* strains after treatment of sterile ddH_2O or benomyl (250 mg/l). The sensitive strain CMML22-BC67 demonstrated effective disease control, while CMML22-BC42 showed no response to the treatment. (B) Disease severity of the strawberry plants caused by the sensitive strain CMML22-BC67 and resistant strains, CMML22-BC03, CMML22-BC27, CMML22-BC30, CMML22-BC42, before and after the treatment of 250 mg/l of benomyl. This graph is represented as a crossbar plot, where the line inside the box indicates the mean value, and the range of the box represents the standard error. The severity caused by the sensitive strain was significantly reduced after the treatment of benomyl (**$P < 0.01$).

ing the same method, resulting in the appearance of Texas Red fluorescence after 27 cycles in 4 individual samples (Fig. 6A). Similarly, DNA extraction was performed from four sampled tomatoes in a Gwangju farm, followed by TaqMan qPCR analysis. The results revealed the detection of Texas Red fluorescence corresponding to E198V in all samples after 25 cycles (Fig. 6B). Diagnosis of *B. cinerea* populations in strawberry and tomato farms by TaqMan qPCR analysis revealed both harbored resistance to benzimidazole.

Discussion

In this study, the sensitivity of 78 strains isolated from geographically diverse strawberry farms in Korea to benzimidazole was assayed. Results showed that 82.05% of strains were resistant to benomyl and contained two types of mutation in the β-tubulin target site of benzimidazole. Reduced sensitivity of the strains to benomyl was confirmed in detached leaf and fruit assays and field trials. Furthermore, a TaqMan qPCR assay was applied to detect the target gene

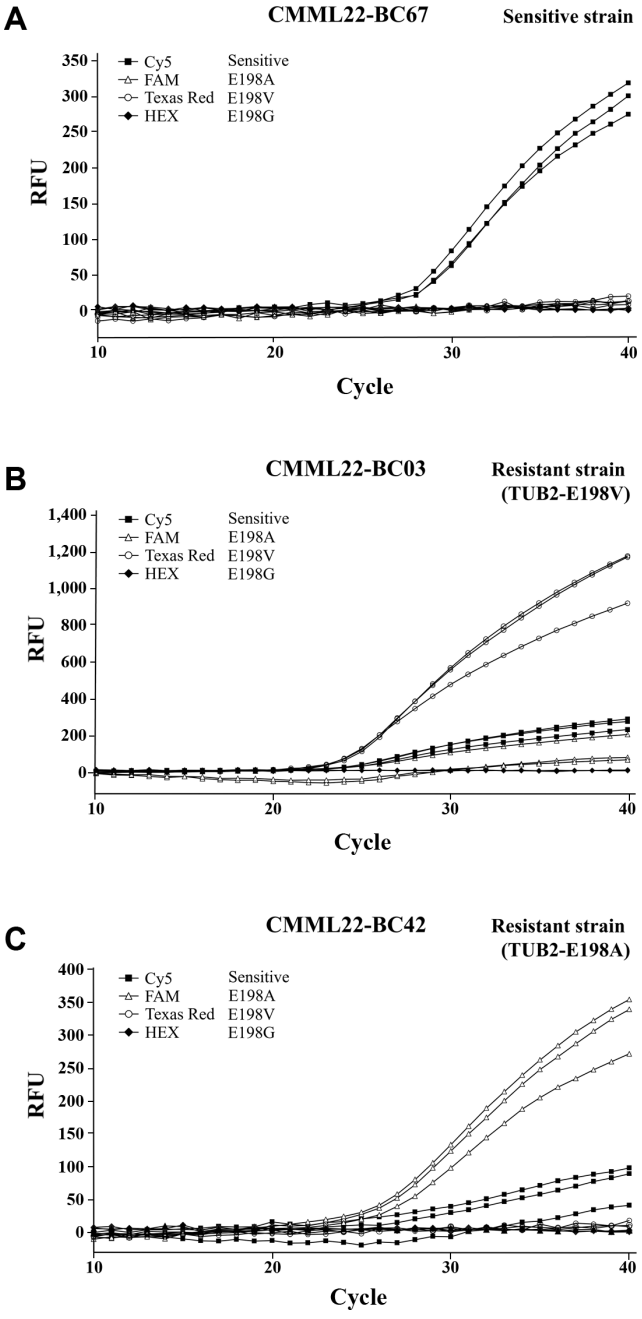

Fig. 5. Detection of benzimidazole resistant *Botrytis cinerea* strains using TaqMan qPCR analysis. Genomic DNA extracted from each strain was mixed individually with primer pairs and supermix. Subsequently, each of these mixtures was mixed with four distinct fluorescent detection probes, including Cy5, FAM, Texas Red, and HEX. Each of these four mixtures was subjected to three technical replicates as part of the experimental procedure. (A) In the benzimidazole sensitive strain CMML22-BC67, only Cy5 fluorescence was detected. (B) In the resistant strain CMML22-BC03 harboring the E198V mutation, Texas Red was detected. (C) In the resistant strain CMML22-BC42 carrying the E198A mutation, FAM was detected.

mutation to diagnose benzimidazole resistance.

Benzimidazole resistance in *B. cinerea* has previously been reported in Korea, and it has been observed that the proportion of resistant strains is higher in farms with a longer strawberry cultivation history (Park et al., 1992). Due to the high proportion of resistant strains, caution should be exercised in the use of benzimidazoles. However, among the fungicides used in strawberry farms in 2013, benomyl was the most frequently used, at a rate of 0.3 kg a.i./ha (Ha et al., 2016). A study of benzimidazole resistance in 1993 revealed that the resistance proportion among *B. cinerea* isolates from different crops in Korea was 60.70%, and that six among eight isolates from strawberry showed resistance to benzimidazole (Kim et al., 1993). The increased proportion of benzimidazole resistance in the current study indicates continuous use of benzimidazole fungicides.

The benzimidazole resistant *B. cinerea* strains investigated in this study possessed the mutation (E198V or E198A) in the β-tubulin target site of benzimidazole. The strains containing the mutations exhibited EC_{50} values exceeding 300 mg/l for benzimidazole (Fig. 1). The average EC_{50} values for strains with the E198V mutation and those with the E198A mutation were both higher than 1,400 mg/l, with no significant difference in EC_{50} values between the two mutation groups (Table 2). Since benomyl is typically applied at a concentration of 250 mg/l in actual farms, it was confirmed that strains with E198V or E198A mutation were not effectively controlled in strawberry plants at this concentration (Figs. 2-4). In addition, the growth rate of resistant strains on the culture plates and pathogenicity of the strains in strawberry plants were not different from the sensitive strains, indicating that the mutation might not affect fitness of the fungus. It was reported that benzimidazole resistant *B. cinerea* strains displayed no significant reduction in fitness compared to sensitive strains and maintained similar fitness levels in an environment devoid of fungicide exposure (LaMondia and Douglas, 1997; Schüepp and Küng, 1981).

Our investigations underscore the importance of prudently managing benzimidazole use. To achieve this, TaqMan qPCR analysis were adopted to rapidly detect benzimidazole resistant *B. cinerea* strains on working farms. A single round of PCR yields fluorescence detection of probes targeting each mutation. This allows intuitive determination of resistance in multiple samples. Compared to methods such as loop-mediated isothermal amplification and random amplified polymorphic DNA analyses, which detect single base mutations, this approach requires fewer primers and eliminates the need for gel electrophoresis,

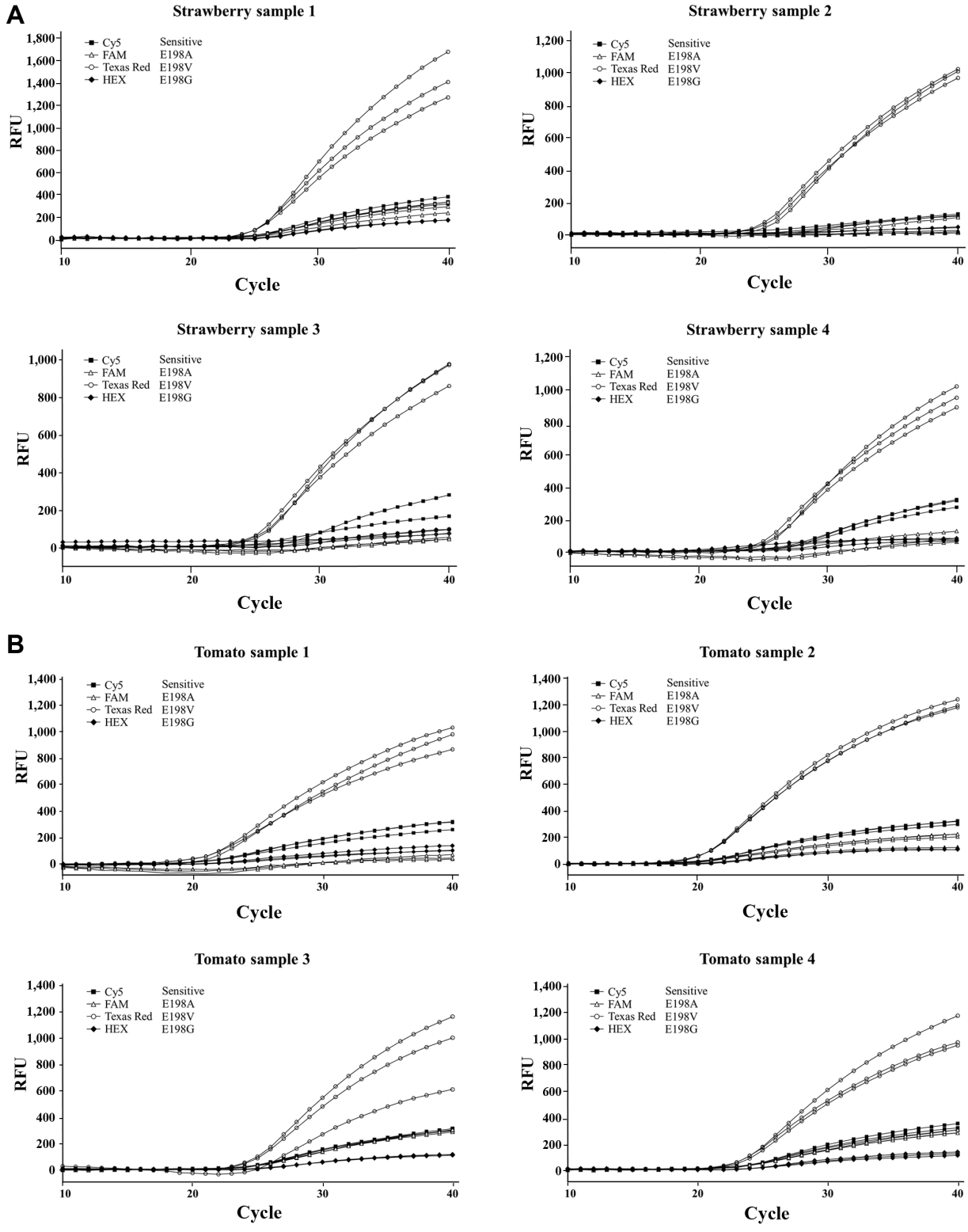

Fig. 6. Benzimidazole resistance diagnosis of *Botrytis cinerea* within greenhouses located in Gwangju and Damyang, Korea. (A) Results of TaqMan qPCR analysis using genomic DNA extracted from strawberry leaves collected in Damyang showed that among four fluorescent probes, TexasRed fluorescence, which detects E198V, was detected in all samples. (B) Results of diagnosis using genomic DNA extracted from tomato fruits collected in Gwangju showed that TexasRed was detected in all samples.

promising swifter detection (Liu et al., 2019; Paplomatas et al., 2004).

The present study demonstrates the recent status of benzimidazole resistance in *B. cinerea* in Korea and highlights the utility of a rapid diagnostic method detecting resistance by using a TaqMan qPCR analysis to propose appropriate strategies for benzimidazole use. In the future, utilizing TaqMan qPCR based risk assessment will reduce unnecessary benzimidazole use and potentially decrease the prevalence of benzimidazole resistant strains, leading to efficient management of gray mold and economic benefits in strawberry and tomato farms.

Conflicts of Interest

No potential conflicts of interest relevant to this article was reported.

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Electronic Supplementary Material

Supplementary materials are available at The Plant Pathology Journal website (http://www.ppjonline.org/).

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