Regular Article
pISSN: 2288-9744, eISSN: 2288-9752
Journal of Forest and Environmental Science
Vol. 37, No. 2, pp. 154-162, June, 2021
https://doi.org/10.7747/JFES.2021.37.2.154

JFES
Journal of Forest and Environmental Science

Colletotrichum spp. Agents of Anthracnose on Blueberry Leaves in Gangwon Province, Korea

Byung-Ju Cho[1], Hyo-Won Choi[2], DaeHo Kim[1,*] and JongKyu Lee[1,*]
[1]*Tree Pathology and Mycology Laboratory, College of Forest and Environmental Sciences, Kangwon National University, Chuncheon 24341, Republic of Korea*
[2]*Crop Protection Division, National Institute of Agricultural Sciences, Rural Development Administration, Wanju 55365, Republic of Korea*

Abstract

Blueberry, which produces phenolic compounds, is one of the most popular fruits in Korea. During a survey on blueberry diseases, 16 *Colletotrichum* isolates were obtained from blueberry leaves in Chuncheon and Gosung, Kangwon province, Korea. Using morphological and molecular analyses, the isolates were identified as *Colletotrichum aenigma*, *C. fioriniae*, *C. fructicola*, *C. gloeosporioides*, and *C. nymphaeae*. *C. gloeosporioides* was the most frequently isolated (11 isolates), and only one or two isolates of the other species were found. After inoculation with all isolates, those leaves and fruits with wounds easily developed anthracnose; whereas, fruits without wounds became infected but leaves without wounds were infected by only two of *C. gloeosporioides*. Typically, around seven fungicidal agents are used to control anthracnose on blueberries in Korea. Fluzinam and prochloraz manganese complex strongly (over than 80%) inhibited the growth of all *Colletotrichum* species, while dithianon and mancozeb only weakly (about 80% or less) inhibited their growth.

Key Words: blueberry, blueberry leaf disease, anthracnose, *Colletotrichum*

Introduction

Blueberry is a woody plant of the genus *Vaccinium* sect. *Cyanococcus* that is native to North America and currently cultivated worldwide (Martín-Gómez et al. 2020). In Korea, highbush and rabbiteye blueberry are the most commonly cultivated, along with their various cultivars (Kim et al. 2017). The production and cultivation areas have increased since its introduction into Korea in the 1960s. About 9,222 tons of blueberries were produced from 4,162 ha in 2015 (Kim et al. 2017).

The phenolic compounds, such as phenolic acids, tannins, stilbenes, lignans, and flavonoids (including anthocyanins and flavonols), produced by the blueberry are reported to lower blood pressure and to be effective against cancer, aging, and diabetes (Zhou et al. 2014; Lin et al. 2020), and interest in the properties of blueberries is increasing (Kim et al. 2017; Lin et al. 2020).

There are many plant fungal diseases that affect blueberries, including mummy berry, twig blight, canker, leaf spot, and fruit rot due to *Phomopsis*; stem blight due to *Botryosphaeria*; postharvest pathogen and root rot caused

Received: March 9, 2021. Revised: March 31, 2021. Accepted: April 1, 2021.

Corresponding author: DaeHo Kim
Tree Pathology and Mycology Laboratory, College of Forest and Environmental Sciences, Kangwon National University, Chuncheon 24341, Republic of Korea
Tel: +82-32-250-7225, Fax: +82-32-259-5617, E-mail: aheung1@naver.com

Corresponding author: JongKyu Lee
Tree Pathology and Mycology Laboratory, College of Forest and Environmental Sciences, Kangwon National University, Chuncheon 24341, Republic of Korea
Tel: +82-32-250-8364, Fax: +82-32-259-5617, E-mail: jongklee@kangwon.ac.kr

by *Phytophthora* and *Armillaria*; leaf rust; leaf spot; powdery mildew; anthracnose caused by *Colletotrichum*; and *Alternaria* fruit rot (Caruso and Ramsdell 1995). In Korea, sooty mold due to *Cladosporium sphaerospermum* (Kwon et al. 2019), anthracnose caused by *Colletotrichum acutatum* and *C. gloeosporioides* (Kim et al. 2009), twig dieback due to *Neopestalotiopsis* (Lee et al. 2019), bark dieback due to *Neofusicoccum* and *Botryosphaeria* (Choi 2011; Choi et al. 2012), postharvest pathogen caused by *Botrytis cinerea* (Kwon et al. 2011), etc. have been reported to affect blueberries.

Anthracnose, one of the most common fungal diseases globally, is caused by fungi of the genus *Colletotrichum* (teleomorph: *Glomerella*) and can occur in many host species (Polashock et al. 2005). Furthermore, it damages the leaves, stems, flowers, and fruits of the host, causing significant economic losses to the fruit growing industry (Freeman et al. 1998). Anthracnose is caused by two main species in the blueberry plant, *C. gloeosporioides* and *C. acutatum*. Kim et al. (2009) isolated 75 *Colletotrichum* strains from blueberry in Korea and identified them as *C. gloeosporioides* and *C. acutatum*. However, this result was reported more than ten years old. The incidence rate of anthracnose has been increasing with climate change (Shin and Yun 2011; Lee 2019). Moreover, the characteristics and composition of the *Colletotrichum* genus have changed (Cannon et al. 2012; Damm et al. 2012; Weir et al. 2012). Therefore, a survey on the current diseases of blueberry plants is needed.

During our study of diseases on blueberry plants in Kangwon province, Korea, we isolated various pathogens from leaves, twigs, and fruit. We aimed to determine the frequency of individual species among the collected pathogens and to isolate the major causative agent of anthracnose on the blueberry. And we also examined fungal growth inhibition by fungicidal agents.

Materials and Methods

Collection of samples and isolation of fungi

To survey diseases of the blueberry, 56 diseased leaf samples from two farms in Chuncheon (37°57'59.9"N 127°44'09.6"E and 37°56'46.0"N 127°41'56.9"E) and 61 samples from two farms in Gosung (38°22'56.5"N 128°27'05.6"E and 38°27'19.7"N 128°27'05.9"E) were collected from June to August, 2017 (Table 1). Samples were sterilized by 1% NaOCl for 60 s after cutting 3.0×5.0 mm samples from lesions. These were placed on water agar and incubated at 25°C for 3 days in the dark. The resulting mycelia were transferred to potato dextrose agar (PDA) and incubated at 25°C for 7 days in the dark. Isolates were preserved at 4°C for further study (Hong et al. 2018).

Morphological and molecular characteristics of isolates

The cultural morphology and growth conditions on PDA were analyzed at six different temperatures (10-35°C at 5°C intervals) after 5 days incubation. The microscopic morphologies of isolates grown on PDA at 25°C for 7 days under 12 h light/12 h dark were also analyzed. To analyze the molecular characteristics, total DNA was extracted using Wizard® Genomic DNA Purification Kit (Promega) and the beta-tubulin (BTU; T1, and bt2b), calmodulin (CAL; CL1C, and CL2C), chitin synthase (CHS; CHS-79F, and CHS-345R), and histone (HIS; CYLH3F, and CYLH3R) genes were amplified following the methods of Damm et al. (2012) and Weir et al. (2012). Sequencing was performed by BIONICS Co. (http://www.bionicsro.co.kr).

Pathogenicity assays

Healthy blueberry (Duke, which is the most widely cultivated cultivar of highbush blueberry in Korea) leaves and fruits with and without wounds were prepared for virulence assay. The conidia were obtained after incubation on PDA at 25°C for 14 days under 12 h light/12 h dark. A conidial suspension containing 1×10^6/mL conidia was inoculated on the prepared blueberry samples and incubated at 25°C in the dark, and virulence was measured after 5 days.

Evaluation of fungicides on mycelial growth in vitro

We selected seven fungicidal agents, typically used to control anthracnose on blueberries in Korea, and we selected suitable concentrations after pre-examination. Various concentrations of each fungicide were dissolved in warm PDA medium after autoclaving. Captan, mancozeb, and dithianon were used at 15.6, 31.6, 62.5, 125, 250, and 500 ppm; benomyl, prochloraz manganese complex, and fluzinam were used at 3.1, 6.3, 12.5, 25, 50, and 100 ppm; and

Table 1. List of fungal isolates and their characteristics and isolation locations

Colletotrichum species	Isolation no.	Isolated location*	Conidia (um)	Colony (mm)						GeneBank no.			
				10°C	15°C	20°C	25°C	30°C	35°C	BTU	CAL	CHS	HIS
C. aenigma	GO084	Gosung A	12.95-18.47×4-6.72	3.0	14.5	40.7	56.2	44.5	3.3	MT321668	MT321654	MT321684	-
C. fioriniae	GO098	Gosung A	10.82-14.87×4.04-5.65	10.7	23.7	38.5	42.8	29.7	1.2	MT321669	-	MT321685	MT321700
C. fructicola	CH062	Chuncheon A	10.81-17.08×4.11-6.98	9.5	25.2	51.3	57.8	41.7	3.8	MT321670	MT321655	MT321686	-
	GO113	Gosung A	10.78-18.73×4.63-7.06	4.0	19.0	44.2	58.2	49.8	3.8	MT321671	MT321656	MT321687	-
C. gloeosporioides	CH052	Chuncheon A	10.8-15.36×3.53-6.01	7.0	19.8	46.5	57.7	53.8	10.2	MT321672	MT321657	MT321688	-
	GO073	Gosung A	11.62-19.02×4.63-6.98	14.8	31.2	53.7	63.8	64.2	12.2	MT321673	MT321658	MT321689	-
	GO074	Gosung A	9.4-15.5×4.94-9.35	8.3	23.3	38.5	47.5	53.2	14.2	MT321674	MT321659	MT321690	-
	GO079	Gosung A	14.59-20.54×4.46-6.72	5.8	25.5	50.5	61.5	70.0	13.5	MT321675	MT321660	MT321691	-
	GO089	Gosung A	13.55-19.95×4.33-6.68	11.8	29.0	57.0	66.2	60.0	11.7	MT321676	MT321661	MT321692	-
	GO090	Gosung A	13.22-19.72×5.03-6.61	10.7	29.7	56.5	64.8	60.2	11.8	MT321677	MT321662	MT321693	-
	GO092	Gosung A	12.47-19.53×4.25-6.39	10.8	28.5	54.5	69.0	61.2	10.8	MT321678	MT321663	MT321694	-
	GO103	Gosung A	10.75-18.84×4.52-6.98	10.0	23.8	28.3	57.8	62.8	13.7	MT321679	MT321664	MT321695	-
	GO110	Gosung A	12.33-17.46×4.63-7.06	9.0	29.5	56.7	64.2	65.0	15.0	MT321680	MT321665	MT321696	-
	GO116	Gosung A	12.03-18.7×4.66-6.11	6.7	24.8	55.5	67.8	58.2	8.8	MT321681	MT321666	MT321697	-
	GO122	Gosung A	12.71-17.96×4.02-6.01	10.2	28.7	56.0	67.7	60.5	11.7	MT321682	MT321667	MT321698	-
C. nymphaeae	GO065	Gosung B	7.11-15.64×3.13-5.65	7.5	18.3	34.7	37.8	20.5	1.3	MT321683	-	MT321699	MT321701

*, The listed location indicated that Gosung A is 38°27'19.7"N 128°27'05.9"E, Gosung B is 37°57'59.9"N 127°44'09.6"E, Chuncheon A is 37°57'59.9"N 127°44'09.6"E and Chuncheon B is 37°56'46.0"N 127°41'56.9"E.

oxine-copper was used at 7.8, 15.6, 31.3, 62.5, 125, and 250 ppm. The mycelial growth was measured after incubation on each prepared medium at 25°C in the dark for 5 days.

Results and Discussion

Isolation and identification

A total of 61 isolates were isolated from blueberry leaves. These included 16 strains of *Colletotrichum*, which was the most frequently isolated genus, isolated from 16 samples

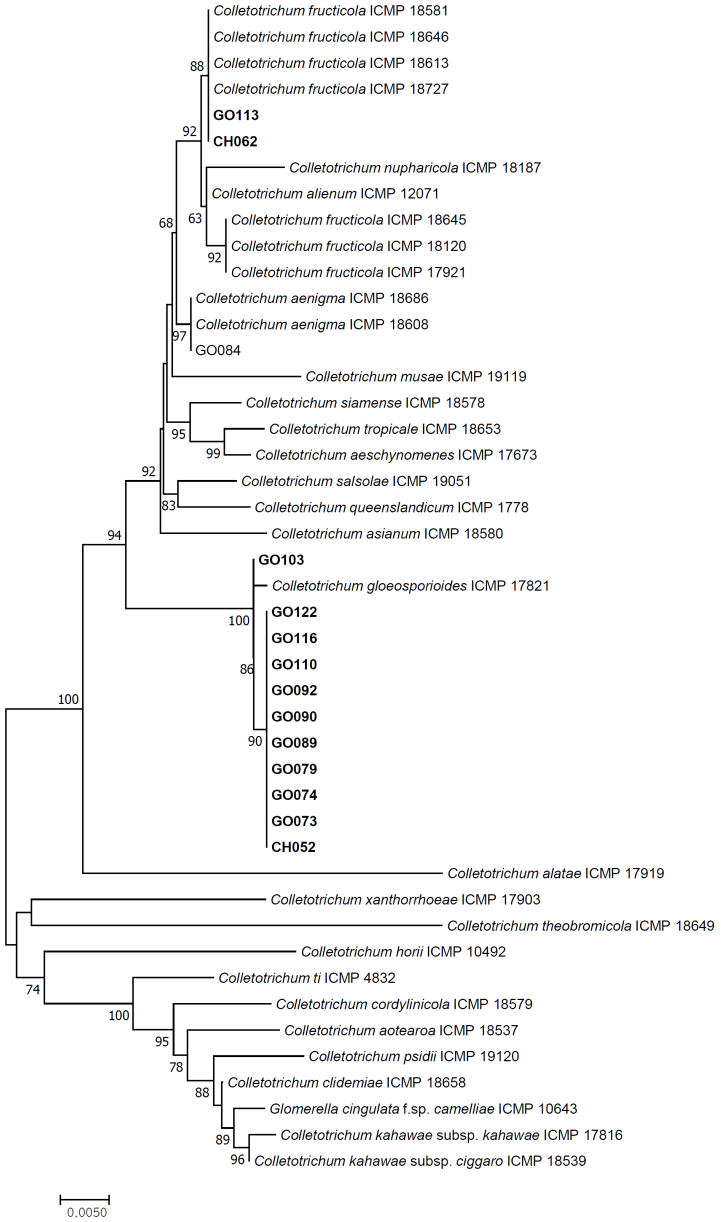

Fig. 1. Phylogenetic tree of members of *C. gloeosporioides* complex based on the combined three loci BTU, CAL, and CHS. Combined data for the isolates were compared with those of other species of the *C. gloeosporioides* complex from Weir et al. (2012). The combined data were analyzed using Tamura-Nei parameter distance calculation model, and then used to construct the Neighbor-Joining (NJ) tree with MEGA ver. 7.0. Bootstrap analysis was performed with 1,000 replications.

from all collecting sites, except one in Chuncheon. *Colletotrichum* spp. were only isolated in August, probably because anthracnose occurrence is more closely related to rainfall than temperature (Shin and Yun 2011). For the same reason, diseased tissue was only found on leaves. Furthermore, blueberries are usually harvested in June and July, before heavy rain and before anthracnose on the fruit has become well developed and begun to spread.

Using morphological and molecular characteristics, 16 isolates were identified from the genus *Colletotrichum*, including the five species *C. aenigma*, *C. fioriniae*, *C. fructicola*, *C. gloeosporioides*, and *C. nymphaeae* (Table 1). This is the first report of these species, except *C. gloeosporioides*, as pathogens of blueberry plants in Korea.

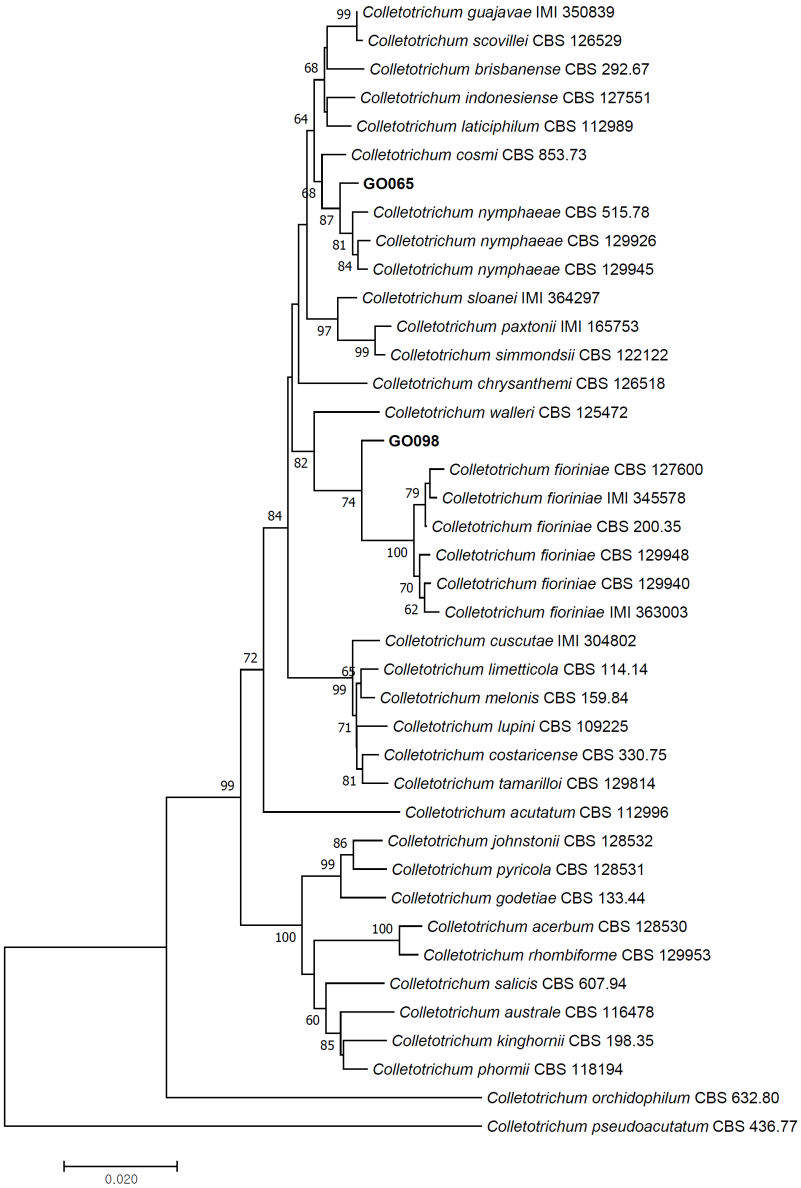

Fig. 2. Phylogenetic tree of members of *C. acutatum* complex inferred from three loci BTU, CHS, and HIS. Combined data for the isolates were compared with those of other species of *C. acutatum* complex from Damm et al. (2012). The combined data were analyzed using Tamura-Nei parameter distance calculation model, and then used to construct the Neighbor-Joining (NJ) tree with MEGA ver. 7.0. Bootstrap analysis was performed with 1,000 replications.

The microscopic morphologies of all strains were similar to that of *Colletotrichum*, but we were unable to distinguish between species. However, the species were clearly defined using molecular characteristics. In total, 11 strains were clustered within the *C. gloeosporioides* clade and two strains were of the *C. fructicola* clade (Fig. 1). Another three strains were clustered in the same clades as *C. aenigma*, *C. fioriniae*, and *C. nymphaeae*, respectively (Fig. 2). *Colletotrichum fioriniae* and *C. nymphaeae*, members of *C. acutatum* complex, were clearly defined by all genes studied (ACT, TUB, and HIS). With regards to the *C. gloeosporioides* complex, *C. aenigma* and *C. gloeosporioides* were clearly divided using TUB and CHS, and *C. fructicola* was clearly defined using ACT and CHS (data not shown). Therefore, multilocus sequence typing analysis is necessary for the correct identification of *Colletotrichum* species.

C. gloeosporioides was the most frequently isolated species in this study, while the four other species were isolated from only one or two strains. Kim et al. (2009) isolated 82 strains, which were identified as *C. gloeosporioides* (75 strains) and *C. acutatum* (14 strains); the most frequently isolated species was the same as in this study. However, we isolated *C. aenigma*, *C. fructicola*, *C. fioriniae*, and *C. nymphaeae* but did not find *C. acutatum*. These differences are likely to be due to the identification methods employed. Kim et al. (2009) used only morphological identification, but *Colletotrichum* cannot be defined using morphology alone. Furthermore, the classification of *Colletotrichum* species has recently changed. The isolation frequencies of *C. gloeosporioides* and *C. acutatum* complexes in this study were similar to that of *C. gloeosporioides* and *C. acutatum* in the study by Kim et al. (2009). Therefore, the stains identified by Kim et al. (2009) could potentially be reclassified as other species.

Growth rate

Fungal growth at various temperatures showed that *C. aenigma*, *C. fioriniae*, *C. fructicola*, and *C. nymphaeae* had the highest growth rates at 25°C. Five strains of *C. gloeosporioides* had the highest growth rates at 25°C, while the other five strains grew better at 30°C. Except *C. fioriniae*,

Table 2. Pathogenicity assay on leaves and fruit with/without wounds

Scientific name	Strain no.	Leaf*[1]		Fruit*[2]	
		With wound	Without wound	With wound	Without wound
C. aenigma	GO084	+	-	+++	+
C. fioriniae	GO098	++	-	+++	+
C. fructicola	CH062	+++	-	+++	+
	GO113	+++	-	+++	+
C. gloeosporioides	CH052	+	+	+++	+
	GO073	++	-	+++	+
	GO074	+	-	+++	+
	GO079	+	-	+++	+
	GO089	+	-	+++	+
	GO090	++	-	+++	+
	GO092	++	+	+++	+
	GO103	+	-	+++	+
	GO110	++	-	+++	+
	GO116	++	-	+++	+
	GO122	+	-	+++	+
C. nymphaeae	GO065	+	-	+++	+

Lesions and symptoms were observed after 7 days incubation.

*[1]: Pathogenicity on leaves was indicated by the size of lesion (-, no lesion; +, < 5.0 mm; ++, 5.0-10.0 mm; +++, > 10.0 mm).

*[2]: The development of disease on fruit was indicated by -, no symptoms; +, weak symptoms; ++, developed only on surface; +++, developed into fruit.

C. nymphaeae, and one of the two *C. fructicola* strains, which had relatively higher growth rates at 20°C, almost all strains showed higher growth rates at 30°C or 20°C and substantially lower growth rates at 10°C and 35°C (Table 1).

Pathogenicity assays

All strains resulted in anthracnose symptoms when inoculated onto leaves with wounds, and only two of those *C. gloeosporioides* strains (CH052 and GO092) infected

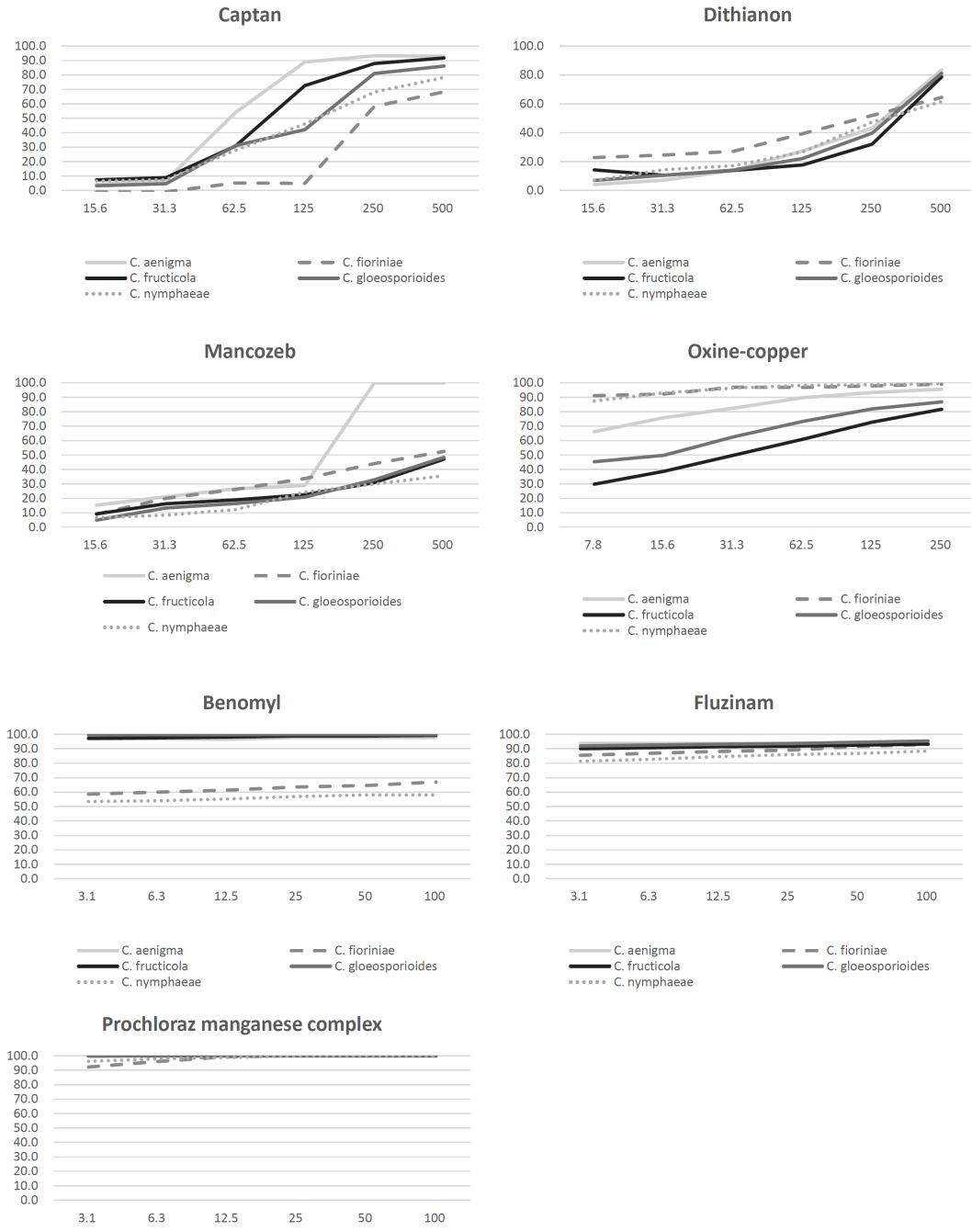

Fig. 3. Growth inhibition by seven anti-fungal agents typically used to control anthracnose on blueberry plants in Korea.

leaves without wounds. Both *C. fructicola* strains (CH062 and GO113) resulted in the largest lesions on leaves with wounds. With regards to the fruit, samples both with and without wounds were infected by all strains (Table 2). Therefore, all the strains had the potential to infect the leaves as well as the fruit of the blueberry plant, although they were only isolated from the leaves.

Fungal growth inhibition

Among the seven fungicidal agents tested, fluzinam and prochloraz manganese complex showed higher growth inhibition on all fungal species, at over 80% and 90%, respectively. Benomyl almost completely inhibited the growth of *C. aenigma*, *C. fructicola*, and *C. gloeosporioides* but resulted in 50-70% inhibition of *C. fioriniae* and *C. nymphaeae* growth. Captan inhibited fungal growth only at higher concentrations, i.e., 250 and 500 ppm. Dithianon showed lower (below 52%) growth inhibitory effects on all species at 250 ppm or lower concentrations. At 500 ppm, dithianon inhibited the growth of *C. aenigma*, *C. fructicola*, and *C. gloeosporioides* by approximately 80% and the growth of *C. fioriniae* and *C. nymphaeae* by about 60%. Mancozeb resulted in 50% or lower growth inhibition, except on *C. aenigma*, which was completely inhibited by 250 and 500 ppm. Oxine-copper inhibited *C. fioriniae* and *C. nymphaeae* growth by over 90%, while it inhibited the growth of other species to various degrees. The members of the *C. gloeosporioides* complex, such as *C. aenigma*, *C. fructicola*, and *C. gloeosporioides*, showed nearly 100% inhibited growth in all concentrations of benomyl, fluzinam, and prochloraz manganese complex. However, *C. acutatum* complex species, such as *C. fioriniae* and *C. nymphaeae*, showed 80% or higher inhibited growth in all concentrations of oxine-copper, fluzinam, and prochloraz manganese complex (Fig. 3). In a recent report describing the current characteristics of *Colletotrichum*, resistance to benomyl is one of features by which *C. gloeosporioides* and *C. acutatum* can be distinguished (Talhinhas et al. 2005). The same phenomenon was seen for the growth inhibition of the two species by benomyl in this study. The correct identification of pathogens is necessary for the effective control of diseases, as the fungicide-induced growth inhibition rates differ depending on the species. There are many fungicidal agents used to control anthracnose, thus, further research into the effect of other fungicidal agents is needed. Furthermore, a study of the effects of the agents on other pathogens is also required.

Conclusion

The genus *Colletotrichum*, which causes anthracnose in the blueberry plant, was the most frequently found pathogen in Kangwon province, Korea. We identified five distinct species, *C. aenigma*, *C. fioriniae*, *C. fructicola*, *C. gloeosporioides*, and *C. nymphaeae*, using molecular characterization, of which *C. gloeosporioides* was the most frequently isolated. All isolations, except *C. fioriniae*, one strain of *C. fructicola*, and *C. nymphaeae*, showed the strongest growth at 25-30°C. All isolations showed pathogenicity on leaves with wounds, and *C. fructicola* was a particularly pathogenic species. We observed symptoms on fruit both with and without wounds after inoculation by all strains. Among the seven antifungal agents tested, which are those typically used to control anthracnose on blueberry plants in Korea, fluzinam and prochloraz manganese complex resulted in the greatest growth inhibition of all fungal species. On the other hand, only weak growth inhibition was seen with dithianon and mancozeb, the growth inhibition properties of the other chemicals varied with fungal species.

References

Cannon PF, Damm U, Johnston PR, Weir BS. 2012. *Colletotrichum*-current status and future directions. Stud Mycol 73: 181-213.

Caruso FL, Ramsdell DC. 1995. Compendium of Blueberry and Cranberry Diseases. APS Press, St. Paul, MN, pp 9-134.

Choi IY, Sharma PK, Cheong SS. 2012. First Report of *Neofusicoccum parvum* associated with Bark Dieback of Blueberry in Korea. Plant Pathol J 28: 217.

Choi IY. 2011. First report of bark dieback on blueberry caused by *Botryosphaeria dothidea* in Korea. Plant Dis 95: 227.

Damm U, Cannon PF, Woudenberg JH, Crous PW. 2012. The *Colletotrichum acutatum* species complex. Stud Mycol 73: 37-113.

Freeman S, Katan T, Shabi E. 1998. Characterization of *Colletotrichum* Species Responsible for Anthracnose Diseases of Various Fruits. Plant Dis 82: 596-605.

Hong AR, Yun JH, Yi SH, Lee JH, Seo ST, Lee JK. 2018. Screening of Antifungal Microorganisms with Strong Biological Activity against Oak Wilt Fungus, *Raffaelea quercus-mongolicae*.

J For Environ Sci 34: 395-404.

Kim SJ, Jung SM, Hur YY, Nam JC, Kim SH, Cho KH, Park JG, Park SJ. 2017. Analysis of Relationship Based on Growth Period and Morphological Characteristics in Blueberry (*Vaccinium* spp.). Korean J Plant Res 30: 101-109.

Kim WG, Hong SK, Choi HW, Lee YK. 2009. Occurrence of anthracnose on highbush blueberry caused by *Colletotrichum* species in Korea. Mycobiology 37: 310-312.

Kwon JH, Cheon MG, Choi O, Kim J. 2011. First Report of *Botrytis cinerea* as a Postharvest Pathogen of Blueberry in Korea. Mycobiology 39: 52-53.

Kwon JH, Park K, Lee Y, Kang B, Choi O, Kim J. 2019. The Occurrence of Sooty Mold of Blueberry Caused by *Cladosporium sphaerospermum* in Korea. J Agric Life Sci 53: 151-156.

Lee S. 2019. Climate Change Adaptive Implementation Assessment Proposal for Local Governments Utilizing Vulnerability Index. J For Environ Sci 35: 47-53.

Lee Y, Kim GH, Kim Y, Park SY, Koh YJ. 2019. First Report of Twig Dieback Caused by *Neopestalotiopsis clavispora* on Blueberry in Korea. Plant Dis 103: 1022.

Lin Y, Huang G, Zhang Q, Wang Y, Dia VP, Meng X. 2020. Ripening affects the physicochemical properties, phytochemicals and antioxidant capacities of two blueberry cultivars. Postharvest Biol Technol 162: 111097.

Martín-Gómez J, Ángeles Varo M, Mérida J, Serratosa MP. 2020. Influence of drying processes on anthocyanin profiles, total phenolic compounds and antioxidant activities of blueberry (*Vaccinium corymbosum*). LWT 120: 108931.

Polashock JJ, Ehlenfeldt MK, Stretch AW, Kramer M. 2005. Anthracnose Fruit Rot Resistance in Blueberry Cultivars. Plant Dis 89: 33-38.

Shin JW, Yun SC. 2011. Impact of Climate Change on Fungicide Spraying for Anthracnose on Hot Pepper in Korea during 2011-2100. Korean J Agric For Meteorol 13: 10-19.

Talhinhas P, Sreenivasaprasad S, Neves-Martins J, Oliveira H. 2005. Molecular and phenotypic analyses reveal association of diverse *Colletotrichum acutatum* groups and a low level of *C. gloeosporioides* with olive anthracnose. Appl Environ Microbiol 71: 2987-2998.

Weir BS, Johnston PR, Damm U. 2012. The *Colletotrichum gloeosporioides* species complex. Stud Mycol 73: 115-180.

Zhou Q, Ma C, Cheng S, Wei B, Liu X, Ji S. 2014. Changes in antioxidative metabolism accompanying pitting development in stored blueberry fruit. Postharvest Biol Technol 88: 88-95.