Mycobiology

Research Note

Identification and Characterization of New Record of Grape Ripe Rot Disease Caused by *Colletotrichum viniferum* in Korea

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Abstract In 2016, grape fruits showing ripe rot symptom were found in fields of Korea. The fungus was isolated and identified as *Colletotrichum viniferum* based on morphological characteristics and nucleotide sequence data of the internal transcribed spacer, glyceraldehyde-3-phosphate dehydrogenase and β-tubulin. To our knowledge, this is the first report of *C. viniferum* causing grape ripe rot disease of grape fruits in Korea.

Keywords *Colletotrichum viniferum*, Glyceraldehyde-3-phosphate dehydrogenase, Grape, Ripe rot disease

The grape (*Vitis vinifera*) is botanically a berry and belongs to the Vitaceae family, which is one of the most extensively planted fruits worldwide [1]. They are also the most important fruit crops in Korea with regards to crop production in recent years. There are endless ways to consume grapes, as they can be eaten fresh as table grapes or used for producing jam, wine, jelly, juice, raisins, grape seed extract, vinegar, and grape seed oil [1]. The grapes is a non-climacteric fruit that generally occurs in clusters. The potential health benefits of consuming grapes are vast. Grapes contain powerful antioxidants known as polyphenols, which may slow or prevent many health issues such as heart disease, high blood pressure, cancer, constipation, diabetes and allergies [2]. According to the Food and Agriculture Organization (FAO), 75,866 km^2 of the world are dedicated to grapes productions and area dedicated to vineyards is increasing by about 2% per year. Approximately 71% of global grape production is used for wine, 27% as fresh fruit, and 2% as raisins [3]. In 2009, Korea produced 369,000 tons of grapes on 18,900 ha of land with a value of US $785 million [4]. The income from grape production in the Republic of Korea is much higher than that of other fruits. Grape consumption is rising due to the increasing national income and the superior quality of grapes [5]. However, several fungi have been known to infect the fruits and leaves of *V. vinifera* in Korea, such as *Colletotrichum* spp. (ripe rot), *Botrytis cinerea* (gray mold), *Plasmopara viticola* (downy mildew), *Uncinula necator* (powdery mildew), *Elsinoë ampelina* (bird's eye rot or anthracnose), and *Botryosphaeria dothidea* (peduncle rot or black rot) [6]. Among these pathogens, grape ripe rot is especially economically significant in most vineyards and leading to yield loss and deterioration in Korea.

Colletotrichum is an important pathogenic genus worldwide and has been described as both plant pathogens and saprophytes. Generally, it causes anthracnose disease in over 197 plant species [7, 8]. Anthracnose disease is characterized by lesions that appear on the fruits, stalks, and leaves of plants. This disease has economic consequences since fruits and vegetables with lesions are physically disfigured and cannot be sold. *Colletotrichum* thrives under warm, wet conditions and can affect to 80% of a crop. *Colletotrichum* lesions first appear as soft, sunken areas that are light brown in color. Small, pink, concentric rings then cover the lesions. These pink spots are the fruiting bodies (or acervuli) of the fungus that produce spores which further spread the disease.

To date, only 2 species (*Colletotrichum acutatum* and *C. gloeosporioides*) have been reported as causal agents of grape ripe rot in Korea [1]. The objective of this study was to identify the species of grape ripe rot from Korea based on morphology and molecular characteristics and to confirm the pathogenicity of the isolated fungus.

Mycobiology 2017 December, **45**(4): 421-425
https://doi.org/10.5941/MYCO.2017.45.4.421
pISSN 1229-8093 • eISSN 2092-9323

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Received October 18, 2017
Revised November 3, 2017
Accepted November 10, 2017

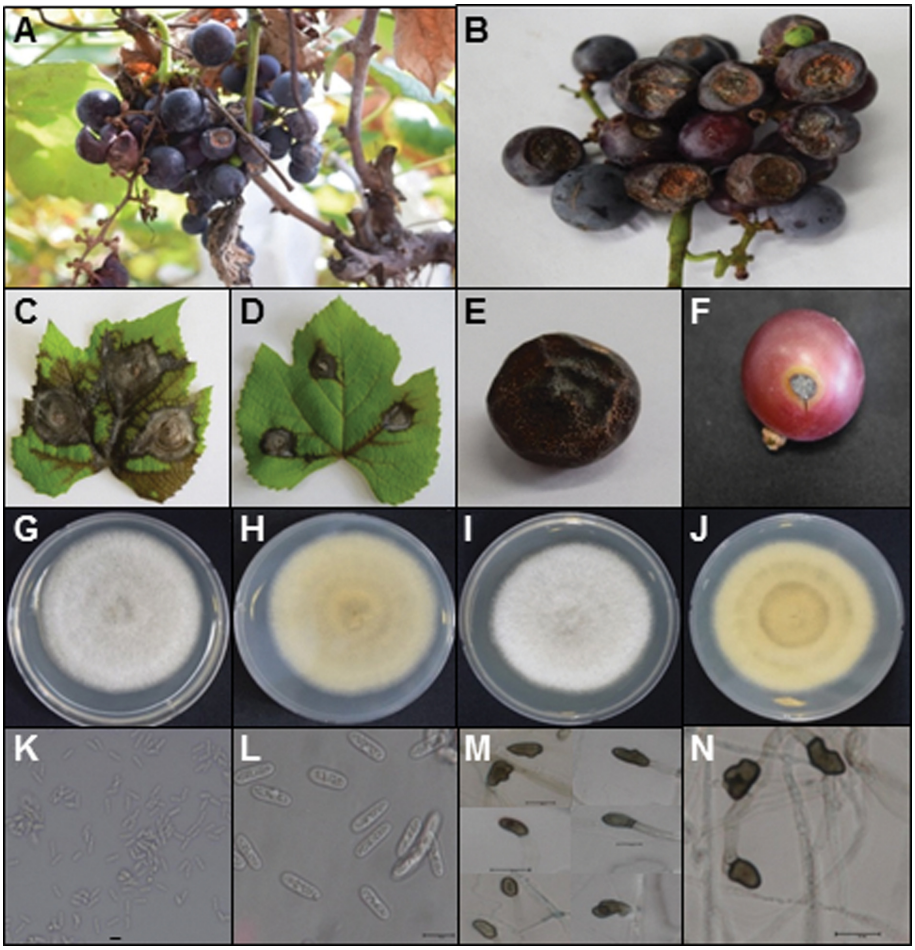

Fig. 1. Ripe rot disease caused by *Colletotrichum viniferum* on grape fruits and morphological characteristics of ripe rot. A, Ripe rot-infected field of grape; B, Symptoms of grape ripe rot; C, Symptoms on wound inoculated leaves after 14 days (1×10^6 conidia/mL); D, Symptoms on wound inoculated leaves after 7 days (agar discs); E, Symptoms on wound inoculated fruits after 14 days (1×10^6 conidia/mL); F, Symptoms on wound inoculated fruits after 7 days (agar discs); G, H, Fungal colonies on V8 juice agar plates (G, upper view; H, reverse view); I, J, Fungal colonies on potato dextrose agar plates (I, upper view; J, reverse view); K, L, Morphological characteristics of conidia; M, N, Various shape of appressoria (scale bars: K–N = 10 μm).

In the summer of 2016, suspected grape ripe rot fruits were observed in grape fields of Eoeun-dong, Yuseong-gu, Daejeon, Korea. Symptoms were characterized by circular area of decay on individual fruits. The decaying and sunken areas enlarge, causing the berry to rot and collapse. As the berries rot, fungal fruiting bodies develop under the surface of the skin. These fruiting bodies rupture through the skin when mature, and masses of pink spores may be evident on the berry surface (Fig. 1A and 1B).

Fresh fruits specimens were collected from infected plants in the field. Fruits exhibiting typical disease symptoms were cut into 5-mm fragments and small pieces of diseased portion were surface-sterilized by dipping into 1% NaOCl (sodium hypochlorite) for 3 min and rinsed 3 times with sterilized distilled water, and were dried on sterilized tissue paper [9]. Then the samples were placed onto 90-mm Petri dishes containing blotter paper and were incubated 25 ± 2°C in a light/dark chamber with 12-hr light and 12-hr dark respectively. Petri dishes were monitored daily to check spore layers or clusters. After 2 days, spore layers were isolated using autoclaved toothpicks or glass sticks and mixed with distilled water and streptomycin (300 ppm for 1 L) in tubes. The mixture was then spread onto a water agar medium containing streptomycin (300 ppm for 1 L) [10]. Three days after incubating at room temperature, single hyphal tips of the emerging fungus were transferred to a potato-dextrose agar medium (PDA; Difco, Sparks, MD, USA) plate to obtain pure culture.

Pathogenicity of the isolated fungus (isolate CNU171001) was confirmed on healthy leaves and fruits by wound/drop and wound/plug inoculation method [11, 12]. Cultures were grown in a V8 juice medium at 25°C for 7 days under a black light. These cultures were used to create a conidial spore suspension in sterilized distilled water. For the wound/

drop method, pinpricked leaves and fruits were inoculated with 10 μL of the spore suspension (1×10^6 conidia/mL) of the pathogen. For the wound/plug method, 6-mm fungal plug were placed on pinpricked healthy leaves and fruits. Fruits and leaves sprayed with sterilized distilled water served as the control. After 7 days of inoculation, circular spots of decay with sunken area symptoms similar to those observed in the field had developed on the inoculated leaves and fruits from both wound/drop and wound/plug experiments (Fig. 1C–1F), and no symptoms were observed in the control. Three replications were performed to test pathogenicity, and the results were similar to each other. The causal fungus was re-isolated from the inoculated fruits and compared to the original pathogen to satisfy the Koch's postulates in each test.

For morphological observation, a small portion of mycelium from the fungal culture was removed and mounted in a drop of lacto-phenol [13]. Colonies grown on the V8 juices mediums were round with entire margins; the mycelia appeared light gray, while the bottom was light orange with no black generation. Aerial mycelia were white and feathery with no obvious stripe (Fig. 1G and 1H). Colonies of the pathogen on PDA were nearly round and were initially white but turned gray before reversing to a pale yellow color (Fig. 1I and 1J). Setae were not observed. Conidia were broad-cylindrical with ends broadly rounded and longer conidia sometimes tapering slightly toward the base (12–18 × 4.5–5.5 μm) (Fig. 1K and 1L). Appressoria were dark brown in color and had various shapes (e.g., ovoid, simple, sub-globose, and clavate). They typically had a small number of broad, sometimes irregular lobes which usually contained guttules (6.5–10.5 × 4.5–6.5 μm) (Fig. 1M and 1N). Regarding morphological characteristics, the fungus matched well with the description of *C. viniferum* (Table 1) [14].

To validate the molecular identification of the isolated fungus, Genomic DNA was extracted from fungal mycelia using the Cenis's method [15]. The internal transcribed spacer (ITS) rDNA regions, glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene, and β-tubulin (*TUB2*) genes were amplified with the primers ITS1&4 [16], GDF&GDR [17], and T1&BT-2b [18], respectively. The protocol for PCR amplification was performed as described by Prihastuti *et al.* [10]. The PCR products were purified and directly sequenced using the same primers. The obtained sequence data were compared to all fungal sequences available in the NCBI-GenBank database using the BLAST search program [19]. Compared to the *C. viniferum* strain in GenBank, the amplification products demonstrate 100% homology with the ITS sequences (GenBank accession No. JN412807), 100% with the *GAPDH* sequences (GenBank accession No. KF377484) and 100% with the TUB2 sequences (GenBank accession No. KX621365), respectively. The ITS, *GAPDH*, and *TUB2* sequences from a representative isolate (CNU171001) were deposited in GenBank under the accession No. MG182340, No. MG182341 and No. MG182342. Ten reference sequences of *Colletotrichum* spp. were taken from GenBank to determine the phylogenetic relationship among the species. *C. acutatum* (CBS 126521) was used as the outgroup in the phylogenetic analysis. The sequences were initially aligned with closely-related strains using the ClustalW2 program (http://www.ebi.ac.uk/Tools/phylogeny/clustalw2_phylogeny/) and edited manually [20]. The reliability of the tree was evaluated using 1,000 bootstrap replications for branch stability. The phylogenetic tree (Fig. 2) was constructed using the distance-based neighbor-joining method in MEGA7 software using ver. 7.0 [21]. The molecular analysis confirmed the morphological identification for the fungal pathogen. Therefore, on the basis of observed pathological signs, symptoms, pathogenicity, morphology and molecular characterization, the fungus was identified as *C. viniferum* [14].

Grape ripe rot is a serious disease that occurs on mature fruits and damages the quality of grape production. In a previous report, *C. gloeosporioides* and *C. acutatum* were identified as the causal agents of grape ripe rot in Korea [1]. However, the result of this present work has identified *C. viniferum* as a causal agent of grape ripe rot pathogen in Korea. Nowadays, *C. gloeosporioides* is a species complex that was formerly regarded as a cosmopolitan species that

Table 1. Comparison of morphological characteristics of the isolate (CNU171001) under study with those of previously reported *Colletotrichum viniferum*

Characteristic	Study isolate *C. viniferum* CNU 171001	*Colletotrichum viniferum*[a]
Conidia		
Shape	Broad-cylindrical with the ends broadly rounded longer conidia sometimes tapering slightly toward the base	Cylindrical
Color	Hyaline	Hyaline
Size (μm)	12–18 × 4.5–5.5	12–16 × 4.6–6.0
Appressoria		
Shape	Various shapes (ovoid, simple, sub-globose, clavate), irregular lobes	Ovoid, clavate, long clavate and sometimes irregularly weakly lobed
Color	Dark brown	Brown
Size (μm)	6.5–10.5 × 4.5–6.5	6.3–10.3 × 4.8–6.3

[a]Sources of description and illustration [14].

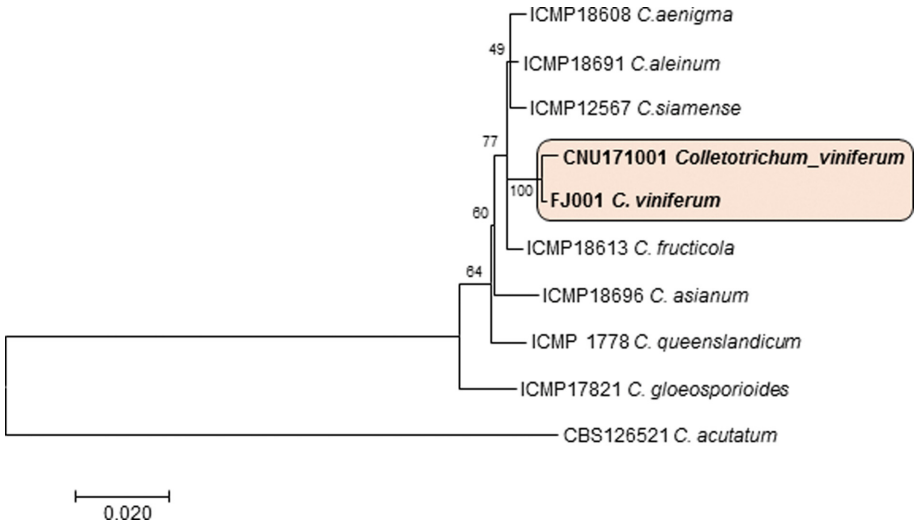

Fig. 2. Phylogenetic tree generated from a maximum parsimony analysis of a combined dataset of internal transcribed spacer, glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), and β-tubulin (*TUB2*) gene sequences of *Colletotrichum viniferum* and those of other related *Colletotrichum* spp. obtained from the GenBank database. The numbers above the branches represent the bootstrap values obtained from a bootstrap with 1,000 replications. *C. acutatum* (CBS 126521) was used as the out-group. The fungal strain identified in this study is shown in boldface.

infects various hosts including grape and other tropical fruits [22]. Yan *et al.* [22] mentioned that *C. viniferum* is a cryptic species, exhibits a phylogenetic divergence and its taxonomy needs to be resolved in the future. There is one report on ripe rot disease caused by *C. viniferum* on grape in China [14]. However, there are currently no reports of the occurrence of the anthracnose disease caused by *C. viniferum* on grape in Korea. To the best of our knowledge, this is the first of such study of ripe rot on grape caused by *C. viniferum* in Korea.

ACKNOWLEDGEMENTS

This work was supported by the Next-Generation BioGreen21 Program (Project No. PJ01118702), of Rural Development Administration, Republic of Korea.

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