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 km² 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.
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 reverting 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 μm × 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 μm × 4.5–6.5 μm) (Fig. 1M and 1N). Regarding morphological characteristics, the fungus matched well with the description of C. viniferum [1]. However, the result of this present work has identified as the causal agents of grape ripe rot in Korea [1].

Pathological signs, symptoms, pathogenicity, morphology and molecular characterization, the fungus was identified as 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* |
|----------------------|---------------------------------------|---------------------------|
| Conidia              | Broad-cylindrical with the ends broadly rounded longer conidia sometimes tapering slightly toward the base | Cylindrical               |
| Shape                | Hyaline                               |                            |
| Color                | 12–18 × 4.5–5.5                       | Hyaline                   |
| Size (μm)            |                                        | 12–16 × 4.6–6.0           |
| Appressoria          | Various shapes (ovoid, simple, sub-globose, clavate), irregular lobes |                            |
| Shape                | Dark brown                            | Ovoid, clavate, long clavate and sometimes irregularly weakly lobed |
| Color                |                                        | Brown                     |
| Size (μm)            | 6.5–10.5 × 4.5–6.5                   | 6.3–10.3 × 4.8–6.3        |

Sources of description and illustration [14].
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.

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