

First Report of Anthracnose Caused by *Colletotrichum fioriniae* on Chinese Matrimony Vine in Korea

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Abstract A fungus, *Colletotrichum fioriniae*, was isolated for the first time from fruits of Chinese matrimony vine (*Lycium chinense* Mill.) in Korea. It was classified as *C. fioriniae* based on the morphological characteristics and nucleotide sequence of glyceraldehyde-3-phosphate-dehydrogenase and β -tubulin. To the best of our knowledge, this is the first report of *C. fioriniae* causing anthracnose of Chinese matrimony vine in Korea.

Keywords Beta-tubulin, Chinese matrimony vine, *Colletotrichum fioriniae*, Glyceraldehyde-3-phosphate dehydrogenase

Chinese matrimony vine (*Lycium chinense* Mill.), also known as Chinese boxthorn, wolfberry, or Goji berry, belongs to the Solanaceae family. Originally from Asia and southern Europe, the plant is popular in traditional Chinese medicine. In China, Chinese boxthorn is used as an aphrodisiac (seeds) and antifebrile and antirheumatic tonic (roots). It is also applied as a cure for a wide range of ailments, from skin rashes and eyesight problems to diabetes and lack of energy (as a tonic; berries and leaves) [1]. The fruits are well known in Korea and China for their great nutritional value and medicinal applications [1].

In recent years, utilization of Chinese matrimony vine rapidly increased mainly for its high nutrient value (a high proportion of antioxidants, 12% protein, 68% carbohydrates, 10% fat, and 10% fiber) [2]. The use of Chinese matrimony vine in eastern Asia is becoming more and more prevalent but statistics are not available. In Korea, Chinese matrimony vine was cultivated on approximately 259 ha in 2010 [3].

Several fungi that infect the fruits of Chinese matrimony vine have been identified. These include powdery mildew (*Arthrocladiella mougeotii*), leaf spot (*Cercospora lycii*), anthracnose (*Colletotrichum acutatum*, *C. dematium*, *C. gloeosporioides*, and *Gloeosporium* sp.), brown leaf spot (*Phoma* sp.), and boxthorn blight (*Phytophthora drechsleri* and *P. nicotianae*) [4]. Among these, *Colletotrichum* spp. have a tremendous effect on Chinese matrimony vine production because of their wide range of infection capabilities. *Colletotrichum* is an important fungal taxon that causes anthracnose in several economically important plants (crops and grasses) [5].

In October 2015, severe outbreaks of anthracnose on fruits of Chinese matrimony vine occurred, and the pathological signs were observed in the fields of Chungcheongnam-do, South Korea. The infected fruits initially showed small circular brown spots that evolved into sunken zones with concentric rings of orange conidial masses. As the disease progressed, fruits eventually became black, sunken, and blighted (Fig. 1A and 1B). Fresh samples were collected from the infected fruits in the field. The fruits showing the typical disease signs were surface-sterilized with 1% sodium hypochlorite (NaOCl) for 3 min, followed by 3 washes with sterilized distilled water, and were dried on sterilized tissue paper [6]. Then, the fruits were placed in Petri dishes containing blotter paper and incubated at $25 \pm 2^\circ\text{C}$ for 2 days in a 12-hr light and 12-hr dark chamber. After 2 days, spore layers were isolated using autoclaved toothpicks or glass sticks and were mixed with distilled water and streptomycin (300 ppm) in tubes. The mixtures were then spread on a water agar medium containing streptomycin (300 ppm) [7], with incubation for 3 days at $25 \pm 2^\circ\text{C}$. Measurements were taken to ensure that the samples were

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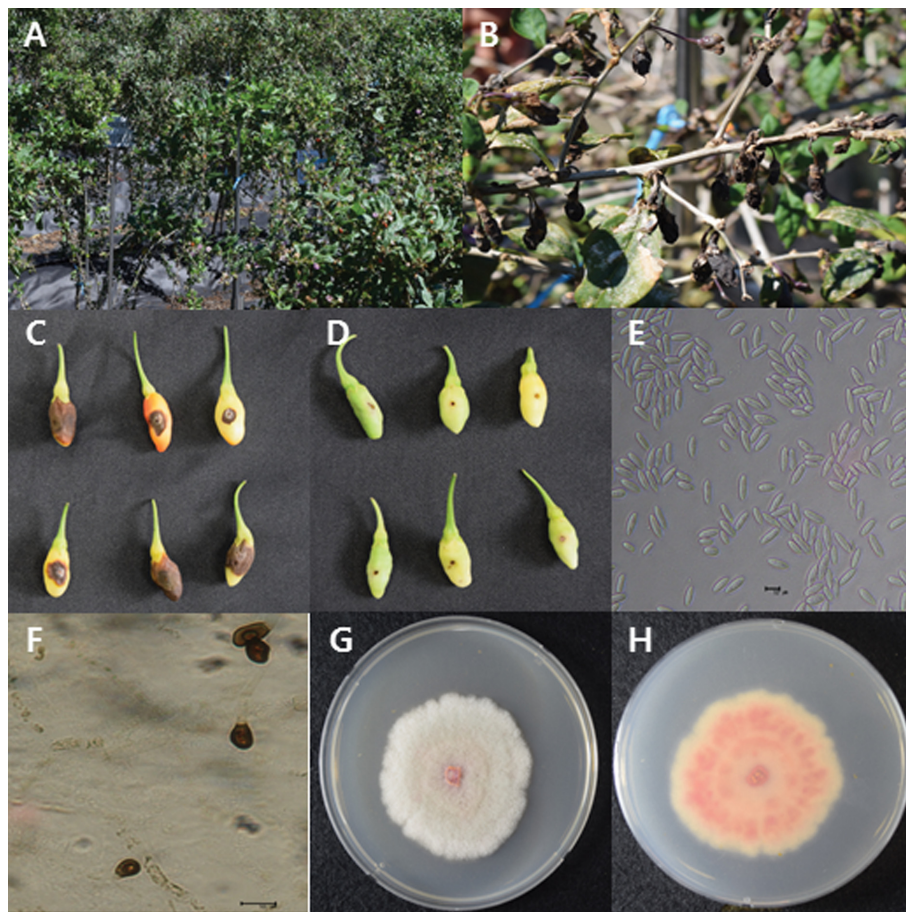


Fig. 1. Anthracnose caused by *Colletotrichum fioriniae* on Chinese matrimony vine fruits. A, An anthracnose-infected field of matrimony vine; B, Signs of the causal fungus of anthracnose on matrimony vine; C, Signs appearing on inoculated fruits; D, No pathological signs on control fruits; E, Morphological characteristics of conidia (scale bar = 10 µm); F, Appressoria (scale bar = 10 µm); G, H, Fungal colonies on V8 juice agar plates (G, front view; H, back view).

free of fungal or bacterial contaminants. After 3 days, single-spore isolates of the emerging fungus were transferred to a new potato dextrose agar (PDA; Difco, Sparks, MD, USA) plate to obtain pure culture.

To prove the pathogenicity of the isolated fungus, isolate CNU150701 was inoculated into healthy fruits by wounding and nonwounding methods. Three cultivars (cv. Cheongun, cv. Cheongmyeong, cv. Hwasuu) of matrimony vine were used in the pathogenicity test. A conidial suspension of fungus CNU150701 was prepared from 7-day-old cultures grown on the V8 juice medium at 25°C in the dark. Uninfected fruits were surface-sterilized with 1% NaOCl for 3 min followed by 2 washes with sterilized distilled water. The fruits were dried with sterilized tissue paper and inoculated using the wounding or nonwounding method [8, 9]. In the wounding method, the fruits were wounded with a sterile nail to 1-mm depth and inoculated with 10 µL of a conidial suspension (10^6 conidia/mL) of the pathogen. The nonwounding method involved spraying the conidial suspension (10^6 conidia/mL) of the pathogen onto the fruits. The fruits sprayed with sterilized distilled

water only served as a control. The inoculated fruits were incubated at room temperature in boxes on paper trays. Six fruits were inoculated per cultivar for each method. With the wounding method, all the inoculated fruits showed typical signs (sunken zones with concentric rings of orange conidial masses similar to those observed in the field) after 4 days, whereas no pathological signs were observed in control fruits (Fig. 1C and 1D). With the nonwounding method, the inoculated fruits also showed signs after 7 days, while the control fruits were still symptomless. All 3 cultivars of matrimony vine showed a pathogenicity effect with the wounding method and nonwounding method. The experiment was repeated thrice (each time in triplicate), and the results were similar. Fungus CNU150701 was reisolated from the inoculated fruits and compared with the original pathogen to accomplish Koch's postulates in each test.

For morphological analyses, a small portion of a mycelium from the fungal culture was removed and mounted in a drop of lacto phenol [10]. Colonies on the PDA medium were pale wine in color with an aerial mycelium containing

Table 1. Comparison of morphological characteristics of the isolate under study with those of previously reported *Colletotrichum fioriniae*

Characteristic	Study isolate <i>C. fioriniae</i> CNU 150701	<i>Colletotrichum fioriniae</i> ^a
Conidia		
Shape	Straight, fusiform, cylindrical with both ends pointed or slightly acute	Smooth-walled, aseptate, straight, fusiform to cylindrical with both ends acute
Color	Hyaline	Hyaline
Size (µm)	9.5~17.5 × 4~5.5	(15.0 ± 1.6) × (4.5 ± 0.3)
Appressoria		
Shape	Ellipsoidal, clavate to irregular outline	Solitary or in loose group, clavate to irregular outline
Color	Brown	Pale to medium brown
Size (µm)	9~12 × 5~7	(9.2 ± 2.2) × (5.6 ± 1.2)

^aSource of description and illustration [11].

an orange cluster of conidia. The growth rate was 35~40 mm/wk. Colonies on V8 juice were pale smoke-grey to olivaceous on one side and purplish grey on the reverse side, and the growth rate was 40~45 mm/wk (Fig. 1G and 1H). Setae were not observed. Acervuli were salmon to orange in color. Conidia were hyaline, straight, aseptate, smooth-walled, fusiform to cylindrical with both ends pointed or slightly acute, 9.5~17.5 × 4~5.5 µm (Fig. 1E) in size. Appressoria were solitary or in a loose group having clavate to irregular outline, smooth walled, brown colored, with the entire edge undulating, 9~12 × 5~7 (Fig. 1F). Actually, *C. fioriniae* is similar to *C. acutatum* in morphological characteristics. However, appressoria shapes are a little different between these 2 species. Appressoria of *C. acutatum* were solitary, medium brown, smooth-walled, ellipsoidal to obovate, (7.3 ± 2.0) × (5.4 ± 1.2) µm in size [11]. Morphological

characteristics are typical of *C. fioriniae* [11] as described in Table 1.

Sequence analysis of the type strain is an important method in the research on identification of *Colletotrichum* [12, 13]. Morphological analyses were validated by molecular analysis. For this purpose, genomic DNA was extracted from a representative isolate using the method described by Cenis [14]. For amplification of the internal transcribed spacer (ITS) rDNA regions, glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), and β-tubulin-2 (*BT2*) genes, we used primers ITS1 and ITS4 [15]; GDF and GDR [16], and T1 [17] and BT-2b [18], respectively. Conditions for PCR amplification were described by Prihastuti *et al.* [7]. The PCR products were purified and directly sequenced with the same primers. The obtained sequence data were compared with all fungal sequence data available in the NCBI-GenBank

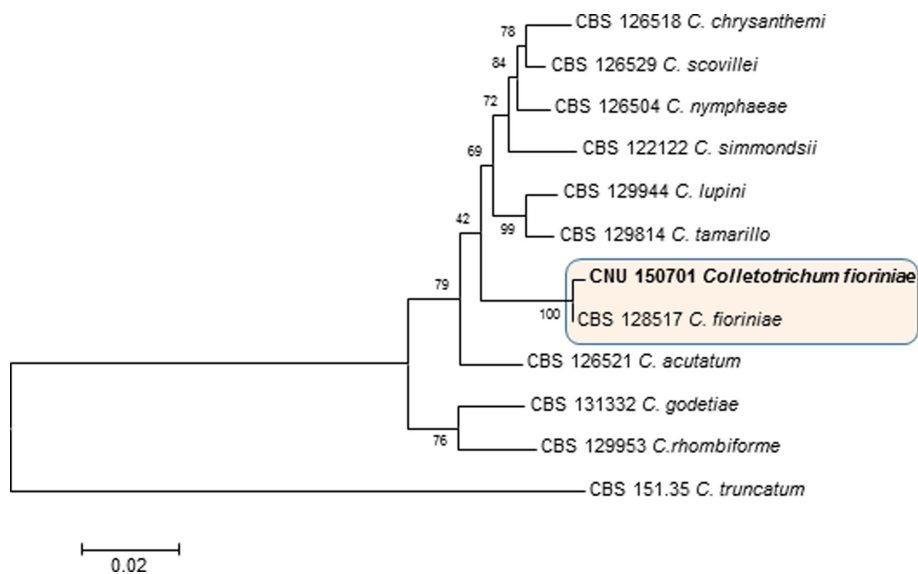


Fig. 2. The phylogenetic tree generated by a maximum parsimony analysis of a combined dataset of ITS, *GAPDH*, and *BT2* gene sequences of *Colletotrichum fioriniae* and those of other *Colletotrichum* spp. obtained from GenBank. The numbers above the branches represent bootstrap values. *Colletotrichum truncatum* (CBS 151.35) served as the outgroup. The fungal strain identified in this study is shown in boldface. ITS, internal transcribed spacer; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase; *BT2*, β-tubulin-2.

database using the BLAST search tool [19]. Compared with the sequence of *C. fioriniae* in GenBank, the amplicons showed 100% homology with the *GAPDH* sequences (GenBank accession No. KM252196), 99% with the ITS sequence (GenBank accession No. JI121192), and 99% with the *BT* sequence (GenBank accession No. KT728899). The *GAPDH* and β -tubulin sequences from a representative isolate (CNU150701) were deposited in GenBank under accession No. KX619446 and No. KX685944. The sequence was aligned manually with those of closely related strains in the ClustalW2 software (http://www.ebi.ac.uk/Tools/phylogeny/clustalw2_phylogeny/). The evolutionary tree was constructed using the distance-based neighbor-joining method of the MEGA 5 software [20]. The reliability of the evolutionary relations in this tree was evaluated by 1,000 bootstrap replications to determine the percentage for each clade (Fig. 2). The sequence of *C. truncatum* served as the outgroup for both *GAPDH* and β -tubulin data. Accordingly, the molecular analysis confirmed the morphological identification of the fungal pathogen. Based on the observed pathological signs, pathogenicity, morphology, and molecular characterization, this fungus was identified as *C. fioriniae* [11].

C. fioriniae is the only representative of Clade 3 and a phylogenetically distinct species of the *C. acutatum* complex [11]. The clade has been recognized as a distinct species complex for some years now [21] and was accepted as a separate species [22]. This species commonly causes anthracnose in many plants worldwide and has been reported to be entomopathogenic [11-23]. Anthracnose on Barbary wolfberry caused by *C. fioriniae* was reported in China [24]. However, there are no reports of the occurrence of anthracnose caused by *C. fioriniae* on Chinese matrimony vine in Korea, and to the best of our knowledge, this is the first such study.

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