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First Report of Leaf Spot of *Datura metel* Caused by *Alternaria tenuissima* in Korea

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In June 2013, we collected leaf spot disease samples of *Datura metel* from Gangneung, Gangwon Province, Korea. The symptoms observed were small circular to oval dark brown spots with irregular in shape or remained circular with concentric rings. We isolated the pathogen from infected leaves and cultured the fungus on potato dextrose agar. We examined the fungus morphologically and confirmed its pathogenicity according to Koch's postulates. The results of morphological examinations, pathogenicity tests, and the rDNA sequences of the internal transcribed spacer regions (ITS1 and ITS4), glycerol-3-phosphate dehydrogenase (G3PDH) and the RNA polymerase II second largest subunit (RPB2) gene sequence revealed that the causal agent was *Alternaria tenuissima*. To the best of our knowledge, this is the first report of leaf spot of *D. metel* caused by *A. tenuissima* in Korea as well as worldwide.

Keywords : *Alternaria tenuissima*, Devil's trumpet, Leaf spot, Pathogenicity

Datura metel L. (devil's trumpet) is a small perennials, usually grown as an annuals herbaceous plant with white flowers, belonging to the family Solanaceae and it is considered as one of the most important medicinal plants throughout the world. The main active constituents of the plant are the medicinally important tropane alkaloids such as hyoscyamine and scopolamine (Cusidó *et al.*, 1999). Due to the presence of bioactive compounds, the plant was widely used in traditional medicine to cure diseases such as asthma, cough, wound treatment, convulsion, headache, insanity, hemorrhoids, and rheumatism (Ali *et al.*, 2004; Dabur *et al.*, 2004). Aqueous and alcoholic extract of the plants possesses good antibacterial (Siva *et al.,* 2011), antifungal (Kagalea *et al.*, 2014), nematicidal (Moosavi, 2012), antitumor (Islam *et al.*, 2008) and anticancer (Nazeema *et al.*, 2014) activities also. Asia as the geographic origin of *D. metel* and recognized its distribution in Asia, Africa, and the tropical and subtropical regions of America (Fuentes, 1980; Satina and Avery, 1959).

In June 2013, serious leaf spots symptoms were observed in *D. metel* plants in Gangneung, Gangwon Province, Korea. The symptoms observed were small circular to oval dark brown spots with an average diameter of 1–5 μm (Fig. 1A). The spots gradually enlarged in size and later became irregular in shape or remained circular with concentric rings (Fig. 1B). To identify the causative agent associated with leaf spot observed on *D. metel*, based on mycological characteristics, molecular phylogenetics, and pathogenicity.

For pathogen isolation, small pieces of infected leaves were sterilized by immersion in 0.1% sodium hypochlorite (NaOCl) for 1 min, rinsed three times with sterile distilled water, and incubated on potato dextrose agar (PDA; Difco, USA) for 5 days at 20±2°C in dark. Afterwards, they were constantly exposed to the fluorescent light for 2 days. The fungus produced gray colonies (65–70 mm diameter) with olive green peripheries and cottony mycelium (Fig. 1D and E). The conidiophores (n=20) were branched, straight measuring 23.7–40.2 μm long and 3.6–5.0 μm thick (Fig. 1H). Short conidia chains consist of 3–7 or more conidia, occasionally (uncommonly) branched (Fig. 1G) were found. The conidia (n=50) were dark brown in color and size of conidia varied from 12.5–48.0 μm in length and 8.1–14.4 μm in width with short taper beak (1.6 to 3.2 μm) or no beak was observed (Fig. 1F). Horizontal and vertical septa of conidia varied from 1–6 and 0–2, respectively. Under light regime, conidia were geminated (Fig. 1I). A representative isolate (A003) was deposited in Gangneung-Wonju National University and used for further studies. Morphological and cultural characters

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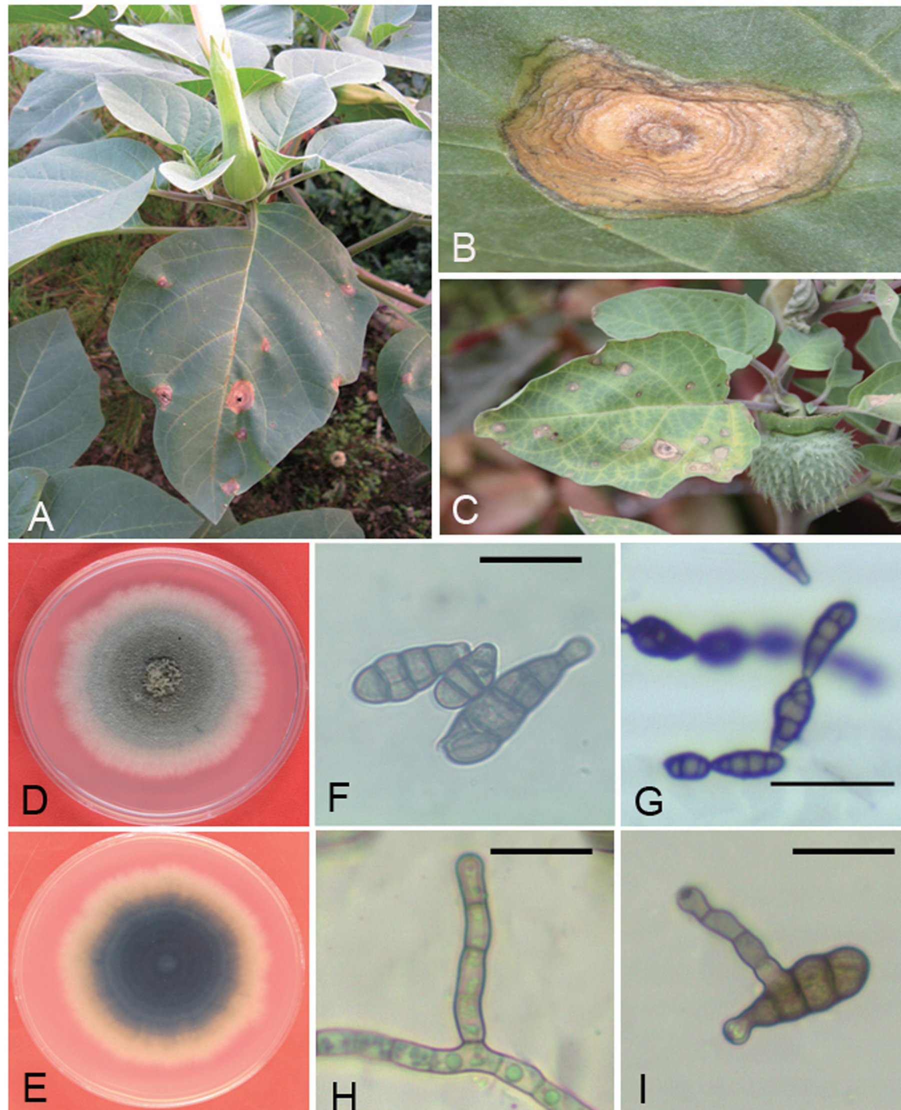

Fig. 1. Leaf spot disease of *Datura metel* caused by *Alternaria tenuissima*. **A**: Symptoms on leaves, **B**: Close-up view of a diseased leaf, **C**: Symptoms on artificially induced leaves 15 days after inoculation, **D** and **E**: One-week-old colony on PDA, **F**: Conidia, **G**: Conidial chain, **H**: Conidiophore, **I**: Germinating conidium. Scale bars: 20 μm (**F**, **H** and **I**), 40 μm (**G**).

were consistent with those of *A. tenuissima* (Simmons, 2007; Yu, 2001).

Molecular characteristics of the studied isolate were determined by DNA extraction from 100 mg mycelia using Plant DNeasy Mini Kit (Qiagen Inc., Valencia, CA, USA). ITS1 and ITS4 primers (White *et al*., 1990) was used to amplify the internal transcribed spacer region (ITS) region containing ITS1-5.8S-ITS2 of nuclear ribosomal DNA (rDNA), and glycerol-3-phosphate dehydrogenase (G3PDH) (Berbee *et al*., 1999), the RNA polymerase II second largest subunit (RPB2) (Liu *et al*., 1999) two nuclear protein-coding genes were sequenced. PCR was performed in a total volume of 25 μl by using 0.5 μl of dNTP, 2.5 units of *Taq* DNA polymerase (0.5 μl of 5 U/μl enzyme; Bioneer, Daejeon, Korea), 2.0 μl of genomic DNA, 2.5 μl of 10× PCR reaction buffer and 5 pmol/l of each primer (0.5 μl each). The reaction was performed in Eppendorf Mastercycler Gradient (Eppendorf, Germany). The PCR amplification conditions were as follows: 94°C for 5 min, followed by 35 cycles of 94°C for 35 sec, 52°C for 55 sec, and 72°C for 1 min, with a final extension step at 72°C for 10 min (for ITS). For RPB2 gene fragments, 94°C for 5 min (1 cycle); 94°C for 30 s, 55°C for 30 s, and 72°C for 90 s (35 cycles), and then 72°C for 10 min (1 cycle). The same program with an annealing temperature of 64°C was used for G3PDH gene fragments. DNA was purified using QIAquick PCR Purification Kit (Qiagen) following the instructions of the producer. Sequence was performed by Bioneer Sequencing Service (Bioneer, Daejeon, Korea) with the same primers used for the PCR amplifications. The resulting 540 bp ITS, 602 bp G3PDH and 733 bp RPB2 gene nucleotide sequence were deposited in GeneBank with accession number KP731980, KT955743 and KT955744, respectively. The obtained nucleotide sequences were searched by using BLASTn available from the

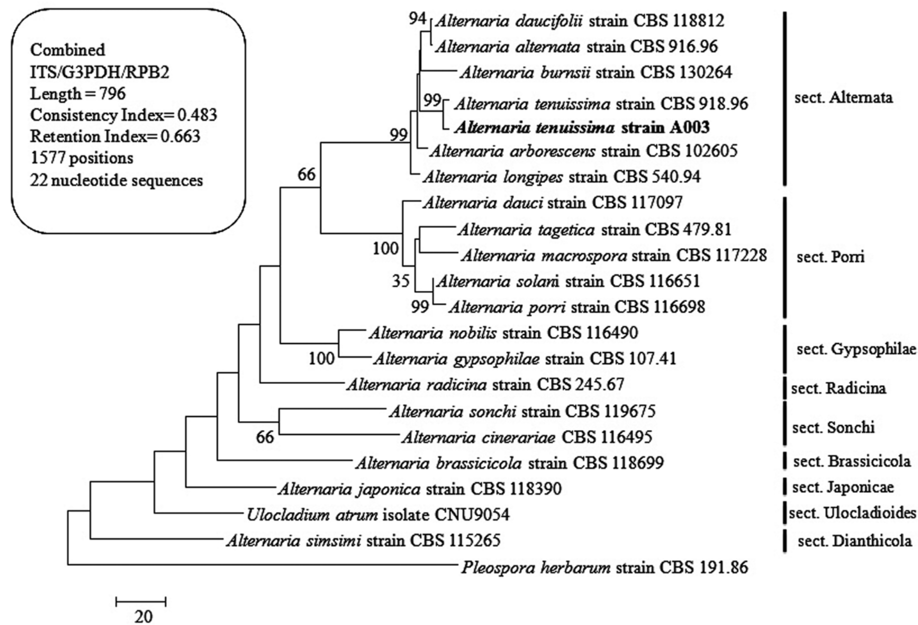

Fig. 2. Phylogenetic tree showing the placement of the *Alternaria* isolates from the present study and their related species generated using the maximum parsimony analysis of combined dataset of ITS, G3PDH and RPB2 gene sequences. Numbers at the nodes indicate bootstrap values (>50%) from 1,000 replications. The bar indicates the number of substitutions per position. The tree is rooted with *Pleospora herbarum* strain CBS 191.86.

GenBank database (http://www.ncbi.nlm.nih.gov/BLAST/). The sequences identified based on ITS, G3PDH and RPB2 gene alignment were 100% similar to *A. tenuissima* species (AF347032, AY278809, KC584435). For phylogenetic analysis, combined ITS, G3PDH and RPB2 gene sequence of some species belongs to the *Alternaria* were retrieved from GeneBank. *Pleospora herbarum* was used as an outgroup taxon. Maximum parsimony trees were constructed for the combined datasets of ITS, G3PDH and RPB2 gene sequences using MEGA6 (Tamura *et al*., 2013) program. Bootstrap analysis using 1000 replications was performed to assess the relative stability of the branches. The phylogenetic relationship using combined ITS, G3PDH and RPB2 gene sequence showed that one isolate clustered with *A. tenuissima*, distinct from other species in the *Alternaria* in a maximum parsimony trees (Fig. 2).

To determine the fungal pathogenicity, inoculum was prepared by harvesting conidia from 2-week-old cultures on PDA. A conidial suspension (5×10^5 conidia/ml) was sprayed onto healthy leaves of three potted *D. metel* plants. Another three potted plants were sprayed with sterilized water, serving as controls. After inoculation, plants were transfer into humid chamber for 2 days (25°C and 80–100% RH) and placed in the greenhouse. The first foliar lesions developed on leaves 15 days after inoculation (Fig. 1C), whereas control plants remained symptomless. The pathogenicity test was carried out twice with similar results. The pathogen was successfully re-isolated from inoculated leaves, fulfilling Koch's postulates.

Alternaria species are important plant pathogens. The leaf spot disease has been reported on *D. metel* in the USA by *A. crassa* and *A. solani* (Farr and Rossman, 2015). A variety of crops other than the *D. metel* are infected with *A. tenuissima*, including eggplant leaf spot in Malaysia (Nasehi *et al*., 2012), and potato leaf blight in China (Zheng and Wu, 2013), pepper leaf spot and fruit rot in China (Li *et al*., 2011), and blueberry leaf spot in Australia (You *et al*., 2014). However, this is the first report that *A. tenuissima* is a causal pathogen of the leaf spot disease of *D. metel* in Korea as well as worldwide.

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