식물병연구
Note Open Access
Res. Plant Dis. 29(1): 60-63 (2023)
https://doi.org/10.5423/RPD.2023.29.1.60

First Report of Leaf Spot in Fischer's Ragwort Caused by *Didymella ligulariae*

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Received December 6, 2022
Accepted January 3, 2023

During disease surveys from 2019 to 2021, the authors frequently encountered leaf spot symptoms on Fischer's ragwort plants growing at fields at six locations of Gangwon Province, Korea. The symptoms displayed brown to dark brown, circular or irregular spots on the plant leaves. The disease surveys at the six locations revealed 1–90% of diseased leaves of the plants. *Phoma* sp. was dominantly isolated from the diseased leaf lesions. Seven single-spore isolates of the fungus were selected and identified as *Didymella ligulariae* by investigation of their cultural, morphological, and molecular characteristics. Artificial inoculation test to Fischer's ragwort leaves was conducted with three isolates of *D. ligulariae*. The inoculation test revealed that the tested isolates cause leaf spot symptoms in the plants similar to the natural ones. The fungal pathogen has never been reported to cause leaf spot in Fischer's ragwort. Leaf spot of Fischer's ragwort caused by *D. ligulariae* is first reported in this study.

Keywords: *Didymella ligulariae*, Fischer's ragwort, Leaf spot, *Ligularia fischeri*

Fischer's ragwort (*Ligularia fischeri*) is a perennial plant belonging to the family Asteraceae. It is native to China, Japan, Korea, Mongolia, Nepal, Pakistan, etc. (Plants of the World Online, 2022). In Korea, the plant is originally grown in the mountains, but it is also grown as a vegetable by farmers. During disease surveys from 2019 to 2021, the authors frequently encountered leaf spot symptoms on Fischer's ragwort plants growing in fields at six locations of Gangwon Province, Korea. The symptoms initially appeared as brown to dark brown, small circular or irregular spots on the leaves of the plants (Fig. 1A). When the disease progressed, the lesions expanded to 3–5 mm in diameter (Fig. 1B). The disease surveys at the six locations revealed 1–90% of diseased leaves of the plants (Table 1).

Fungal isolates were obtained from leaf lesions of Fischer's ragwort using the methods as previously described (Lee et al., 2021). *Phoma* sp. described by Boerema et al. (2004) was dominantly isolated from the leaf lesions. Seven single-spore isolates were obtained from the *Phoma* sp. isolates using the methods as previously described (Lee et al., 2021). Cultural and morphological characteristics of the isolates were examined following the previous study (Boerema et al., 2004). The isolates were cultured on malt extracted agar (MEA), oatmeal agar (OA), and potato dextrose agar (PDA) at 22°C for 14 days. Diameter of 1-week-old colonies of the isolates was 46–52 mm on MEA, 51–56 mm on OA, and 44–55 mm on PDA, respectively. The colony on MEA showed white wooly aerial mycelia with clear concentric zones in which brown to black pycnidia were formed (Fig. 2A). The colony on OA showed pale brown floccose aerial mycelia with clear concentric zones and black pycnidia around the center (Fig. 2B). The colony on PDA showed white to colorless aerial mycelia with pale concentric zones and salmon conidial mass around the center (Fig. 2C). Morphological characteristics of each isolate were investigated with 30 pycnidia and 30 conidia from 2-week-old cultures on OA. Pycnidia formed in the

Research in Plant Disease
eISSN 2233-9191
www.online-rpd.org

medium were variable in size and shape (Fig. 2D). They were light brown to black, mostly globose to subglobose, solitary or confluent with 1 (–2) or non-papillated ostioles, and measured 65–259 µm (av. 164 µm) in diameter. Conidia were aseptate or 1-septate, hyaline, oblong to ovoid, with bipolar or multiple guttules (Fig. 2E), and measured 5.4–14.2×2.4–4.3 µm (av. 10.2×3.2 µm). Chlamydospores were unicellular in chain, in various form of globose, dark green or black (Fig. 2F), and measured 7.2–23.7 µm. NaOH spot tests (Boerema et al., 2004) on MEA cultures showed negative reactions. The cultural and morphological features of the isolates showed similar to those of *Didymella ligulariae* (Chen et al., 2022).

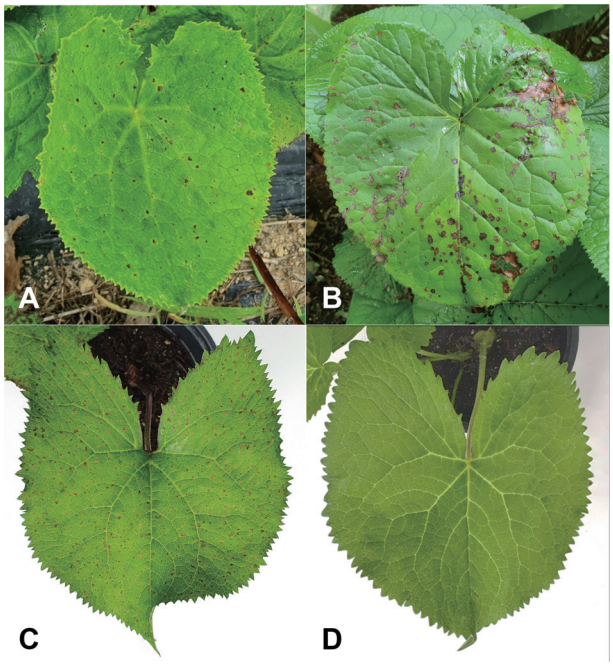

Fig. 1. Leaf spot symptoms of Fischer's ragwort plants. (A, B) Field symptoms of the disease. (C) Symptoms produced 12 days after artificial inoculation with *Didymella ligulariae* isolate. (D) A control plant.

Phylogenetic tree analysis was conducted to verify the identification result. Genomic DNA of the isolates was extracted using a protocol (Park et al. 2021) amended slightly by the authors. Large subunit nrDNA (LSU), internal transcribed spacer (ITS), beta-tubulin (TUB2), and RNA polymerase II (RPB2) genes were investigated with primer sets of LR0R (Rehner and Samuels, 1994) and LR7 (Vilgalys and Hester, 1990), V9G (de Hoog and van den Ende, 1998) and ITS4 (White et al., 1990), Btub2Fd and Btub4Rd (Woudenberg et al., 2009), and RPB2-5f2 (Sung et al., 2007) and fRPB2-7cR (Liu et al., 1999), respectively.

Polymerase chain reaction amplification was done using the conditions in the previous researches (Aveskamp et al., 2010; Chen et al., 2015). Genomic DNA sequencing was done as previously conducted (Chen et al., 2015). MUSCLE (Edgar, 2004) was used to align the sequences. Phylogenetic tree was constructed by MEGA 7 (Kumar et al., 2016) by a maximum likelihood method with general time reversible model performing 1,000 bootstrap replicates. The final alignments showed 2,160 characters containing gaps (860, 404, 297, and 599 for LSU, ITS, TUB2, and RPB2, respectively) of the isolates and 12 ingroup taxa. *Coniothyrium palmarum* (CBS 400.71) was selected for an outgroup taxon. The isolates were clustered with *D. ligulariae* (CGMCC 3.20070) in the tree based on concatenated sequences of four genes (Fig. 3). The accession numbers of the sequences for LSU, ITS, TUB2, and RPB2 deposited in GenBank are OP804095–OP804101, OP804102–OP804108, OP806114–OP806120, and OP806121–OP806127, respectively.

To confirm pathogenicity of the *D. ligulariae* isolates to Fischer's ragwort leaves, three isolates were used for artificial inoculation. The concentration of conidial suspension of each isolate prepared from 3-week-old cultures on MEA

Table 1. Incidence of leaf spot in Fischer's ragwort grown in fields at six locations of Gangwon Province, Korea from 2019 to 2021

Location investigated	Period investigated	No. of fields investigated	No. of fields with leaf spot	Diseased leaves (%)[a]
Cheolwon	Jun 2020	1	1	1–5
Chuncheon	Sep 2019	1	1	50–90
Hongcheon	May 2021	1	1	1–5
Hwacheon	Jun 2020	2	2	2–30
Pyeongchang	Jun 2019	7	5	5–30
	Jun 2021	1	1	1–10
Yeongwol	Sep 2020	1	1	60–90

[a]Three sites were observed in a field, and 100 leaves of the plants at each site were investigated for the disease incidence.

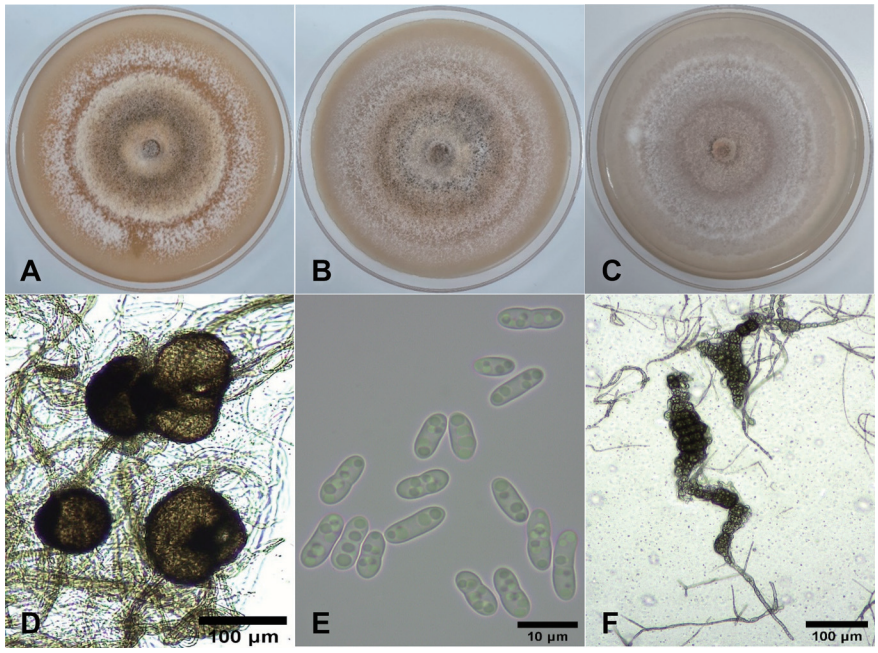

Fig. 2. Appearance in culture and morphology of *Didymella ligulariae* from Fischer's ragwort. Colonies of the fungus grown on malt extract agar (A), oatmeal agar (B), and potato dextrose agar (C) 14 days after incubation at 22°C. Pycnidia (D), conidia (E), and chlamydospores (F) of the fungus produced in oatmeal agar.

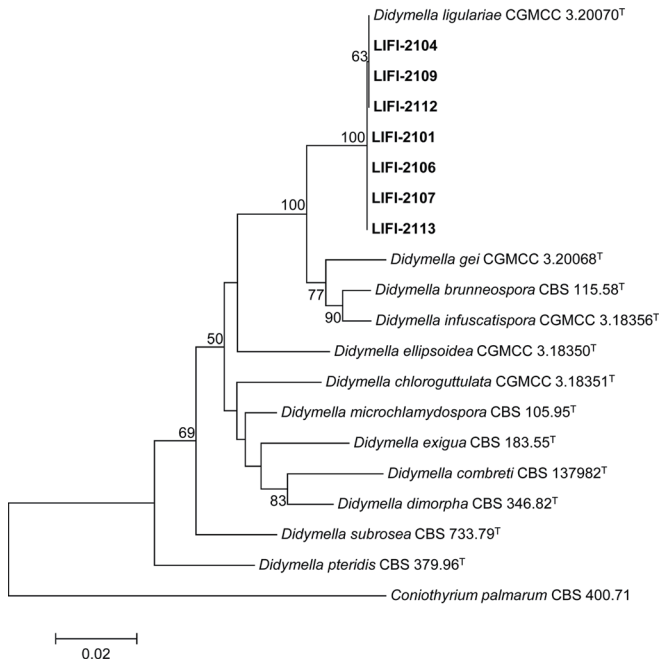

Fig. 3. The maximum likelihood phylogenetic tree using concatenated sequences of LSU, ITS, TUB2, and RPB2 of seven isolates (LIFI-numbers) from Fischer's ragwort and reference species. Bootstrap support values are indicated at nodes. The scale bar shows the number of nucleotide substitutions per site. *Coniothyrium palmarum* (CBS 400.71) was served as an outgroup. ^{T} indicates the type strain. LSU, large subunit nrDNA; ITS, internal transcribed spacer; TUB2, beta-tubulin; RPB2, RNA polymerase II.

was adjusted to 1–2×10^{7} conidia/ml). A 30 ml of conidial suspension was sprayed onto each 13-month-old Fischer's ragwort plant grown in a plastic pot (height, 16 cm; upper diameter, 17 cm; lower diameter, 10 cm) in a vinyl greenhouse. Control plants were treated with the same quantity of sterile distilled water. The inoculated plant pots were placed in plastic boxes (71.0×53.5×40.5 cm) under the same conditions for 3 days as previously described (Lee et al., 2021). After that, the pots were removed from the boxes and placed in the greenhouse. Pathogenicity of the isolates was checked depending on the lesion formation 12 days after inoculation. The inoculation test was conducted in triplicate. The inoculation test revealed that the tested isolates caused leaf spot symptoms in the plants similar to the natural ones (Fig. 1C). No symptom was formed in the control plant (Fig. 1D). Re-isolation of the inoculated isolates was confirmed.

The present study revealed that *D. ligulariae* caused leaf spot in Fischer's ragwort. *D. ligulariae* isolated from diseased leaves of *Ligularia sibirica* was recently named (Chen et al., 2022). However, leaf spot caused by the fungus has not been reported in other plants except *L. sibirica*. Leaf spot of Fischer's ragwort caused by *D. ligulariae* is first reported in this study.

Conflicts of Interest

No potential conflict of interest relevant to this article was reported.

Acknowledgments

This study was supported by a research grant (PJ014507012021) from the Rural Development Administration, Korea.

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