RESEARCH ARTICLE

Leaf Spot of Coastal Hogfennel (*Peucedanum japonicum*) Caused by *Didymella acutilobae*

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ABSTRACT

During crop disease surveys in October 2022, we encountered leaf spot symptoms in coastal hogfennel (*Peucedanum japonicum*) grown in a field in Buyeo, Korea. Disease symptom outbreaks in the field ranged from 5 to 10%. The three fungal isolates obtained from leaf lesions were morphologically identified as *Didymella* sp. Phylogenetic analyses confirmed a close relationship between the isolates and *Didymella acutilobae*, and the mycological characteristics were generally consistent with those of *D. acutilobae.* Pathogenicity of the isolates on leaves of coastal hogfennel was confirmed via artificial inoculation test. To date, this is the first report on the onset of *D. acutilobae*-induced leaf spot in coastal hogfennel.

Keywords: Coastal hogfennel, *Didymella acutilobae*, Leaf spot, *Peucedanum japonicum*

INTRODUCTION

Coastal hogfennel (*Peucedanum japonicum* Thunb.) is perennial and a member of the family Apiaceae. It is native to countries in East Asia, including China, Korea, Japan, the Philippines, and Taiwan, and typically thrives in temperate biomes [1]. Coastal hogfennel is usually used as an edible wild vegetable in cuisine [2], with its phytochemical constituents having been studied for their potential anti-inflammatory and oxidant activities [3,4].

Phoma is one of the largest fungal genera, encompassing approximately 3,000 taxa that exhibit diverse lifestyles, ranging from saprobic and opportunistic to endophytic and pathogenic across various substrates, including animals, plants, and even inorganic matter [5–8]. The identification of *Phoma* spp. has undergone significant changes with the development and application of new molecular tools and techniques. Many species in the genus *Phoma* (anamorphs), identified using traditional methods, have been reclassified within the genus *Didymella* (teleomorphs) of the family Didymellaceae. As a teleomorph, the genus *Didymella* produces eight didymospores in cylindrical bitunicate asci in a pseudothecium. Didymellaceae is the largest family, encompassing more than 30 genera, including *Ascochyta* and *Phoma* with over 5,400 taxa [9]. Nowadays, integrated identification methods combining traditional approaches with molecular phylogenetic analysis are mandatory and have become routine for the identification of ambiguous species in families.

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Leaf spot symptoms were observed in coastal hogfennel plants grown in a field located in Buyeo, Korea, during a survey of crop diseases in October 2022. We investigated fungi isolated from the symptoms and found that they produced conidia within the pycnidia. The morphological features of the pycnidial fungi were generally consistent with those of the genus *Phoma* [5].

Leaf spot in coastal hogfennel caused by other fungi (*Alternaria peucedani* and *Ascochyta* sp.) has been reported in Korea [10]. However, the disease caused by *Phoma* sp. has not yet been reported. In this study, to identify *Phoma* sp. isolates from diseased leaves of coastal hogfennel, the multilocus phylogenetic characteristics, morphological, and cultural characteristics of the isolates were analyzed. In addition, pathogenicity of the isolates on coastal hogfennel was assessed to determine the disease-inducing pathogen.

MATERIALS AND METHODS

Disease survey and isolation of fungi

In October 2022, disease surveys of in-field crops were conducted in Buyeo, Korea. During which, we encountered leaf spot symptoms in coastal hogfennel plants grown in a field and collected these diseased leaves. Leaf lesions were cut into small pieces (3 × 3 mm) and immersed in 1% sodium hypochlorite for 1 min for surface sterilization. Surface-sterilized lesion pieces were plated on 2% water agar (WA; FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan). The WA plates were incubated at 25°C for 3–4 days. Three single-conidium isolates were obtained from pycnidia produced in lesion pieces on the WA and used for identification and pathogenicity tests.

Investigation of mycological characteristics

The mycological characteristics of the isolates were investigated in accordance with previously described protocols [5,11]. Malt extracted agar (MEA; Sigma-Aldrich, St. Louis, MO, USA), oatmeal agar (OA; Sigma-Aldrich), and potato dextrose agar (PDA; BD Difco, Sparks, USA) were used to investigate cultural features of the isolates, which were described according to the Rayner color chart [12]. To determine growth rates, the colony diameters of the isolates were measured after 7 days of incubation in quadruplicate. Morphological characteristics were assessed utilizing 14-day-old cultures of the isolates on OA through collecting 30 pycnidia and 30 conidia and using a light microscope (Nikon Eclipse Ci-L, Tokyo, Japan). Additionally, 15 pycnidial sections per isolate were prepared and examined using a previously described method [13]. One-week-old cultures of the isolates on MEA were used for the NaOH spot reactions. All reaction tests were triplicated.

DNA extraction and PCR

The protocol described by Dong et al. [14] was used to extract genomic DNA from the isolates, with a slight modification: boiling time was shortened. The 28S large subunit of the nrDNA gene (LSU), internal

transcribed spacer regions 1 and 2 including 5.8S nrDNA gene (ITS), β-tubulin (*TUB2*), and RNA polymerase II second largest subunit (*RPB2*) gene regions of the isolates were amplified using specific primer sets [15–21] (Table 1) and cycling conditions [8,22]. PCR amplification was conducted using the DNA Free-Taq Master Mix (CellSafe, Yongin, Korea), while DNA purification was performed using the Universal DNA Purification Kit (Tiangen, Beijing, China); both procedures were performed according to the manufacturers' protocols. The same primers were used for sequencing by Bionics Co., Ltd. (Seoul, Korea). Sequencing data were deposited in the National Center for Biotechnology Information (NCBI) GenBank database.

Table 1. Information of the primer sets used for molecular identification of *Didymella* sp. isolates from coastal hogfennel

Gene region	Primer	Sequence (5'–3')	Reference
LSU	LR0R	ACCCGCTGAACTTAAGC	16
	LR7	TACTACCACCAAGATCT	17
ITS	V9G	TTACGTCCCTGCCCTTTGTA	18
	ITS4	TCCTCCGCTTATTGATATGC	19
TUB2	Btub2Fd	GTBCACCTYCARACCGGYCARTG	20
	Btub4Rd	CCRGAYTGRCCRAARACRAAGTTGTC	20
RPB2	RPB2-5f2	GGGGWGAYCAGAAGAAGGC	21
	fRPB2-7cR	CCCATRGCTTGYTTRCCCAT	22

LSU: 28S large subunit of the nrDNA gene; ITS: internal transcribed spacer regions 1 and 2 including the 5.8S nrDNA gene; *TUB2*: ß-tubulin; *RPB2*: RNA polymerase II second largest subunit.

Aanalysis of phylogenetic characteristics

Isolates were sequenced using SeqMan II (DNASTAR Inc., Madison, WI, USA), and manually adjusted, if necessary. Relevant sequences of *Didymella* spp. were retrieved from previous studies [23–25]. MUSCLE within the MEGA 7 software [26] was used to evaluate the performance of multiple sequence alignments. The outgroup taxa, *Ascochyta koolunga* (CBS 373.84) and *Ascochyta rabiei* (CBS 534.65) were used to construct the phylogenetic tree. The phylogenetic tree was constructed from the concatenated datasets using the maximum-likelihood method (ML) in RAxML 8.2.4 [27], employing a general time-reversible gamma-distributed rate variation model. Branch support was assessed using 1,000 bootstrap replicates, and only values ≥ 50% were shown at the nodes.

The most suitable substitution models were selected using MrModelTest v.2.4 [28] and implemented in MrBayes v.3.2.4 [29] for Bayesian analysis, which was continued until the average standard deviation of the split frequencies fell below 0.01. Generated trees underwent a 25% burn-in procedure to calculate posterior probabilities (PP). Probabilities ≥ 0.9 were displayed at the nodes. The phylogenetic tree was visualized using FigTree version 1.4.4 software [30].

Pathogenicity test on host plants

Aqueous suspensions of the isolates were prepared from 3-week-old cultures on 25% OA. After filtration through a double layer of Miracloth (Sigma-Aldrich), the conidial density of the suspensions was adjusted

to 1–2 × 10^7 conidia/mL using sterile distilled water. For pathogenicity tests, coastal hogfennel plants were cultivated in 15-cm-diameter plastic pots containing bedding materials under greenhouse conditions. The leaves of each 55-day-old plant were inoculated by spraying with 50 mL of conidial suspension per isolate. The inoculated plants were put in plastic boxes with relative humidity exceeding 95%, and the plastic boxes were placed in a room at 24–26°C for 4 days. The same amount of sterile distilled water was used for control plants, which were maintained under identical conditions. After 4 days, the inoculated plants were removed from their boxes and placed in a room. The pathogenicity rating of the isolates was determined by assessing the formation of lesions on leaves 7 days following inoculation. All pathogenicity tests were triplicated.

RESULTS AND DISCUSSION

Disease occurrence and symptoms

Leaf spot symptoms were found on coastal hogfennel plants grown in a field during the crop disease survey. The symptoms began as small, brown, and circular to irregular spots exhibiting yellow halos. As the disease progressed, the spots coalesced into irregular dark-brown lesions (Fig. 1A and B). The incidence of disease symptoms in the field ranged from 5 to 10%.

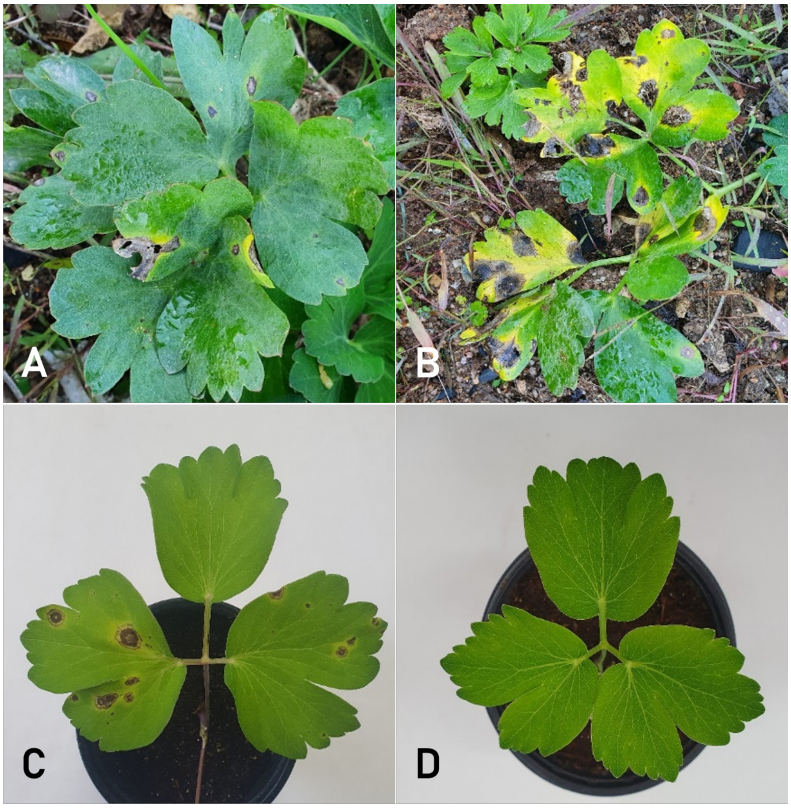

Fig. 1. Leaf spot symptoms of coastal hogfennel plants. (A, B) Symptoms observed in the investigated field. (C) Induced symptoms by artificial inoculation with the isolate (PEJA-2207) of *Didymella acutilobae*. (D) A non-inoculated control plant.

Phylogenetic characteristics

Three single-conidium isolates (PEJA-2203, PEJA-2205, and PEJA-2207) from coastal hogfennel were analyzed for their phylogenetic characteristics. The optimal models for the LSU, ITS, *TUB2*, and *RPB2* alignments were identified as HKY + I + G, SYM + I + G, GTR + I + G, and GTR + I + G, respectively, based on model tests. Overall, 2,470 characters, including alignment gaps (LSU, 958; ITS, 558; *RPB2*, 600; and *TUB2*, 354), were obtained from the concatenated four-gene sequence alignments of the 24 ingroup taxa. The ML and Bayesian trees showed no significant topological differences. Therefore, an ML tree with bootstrap and PP values at the nodes in the phylogenetic tree was presented. Phylogenetic analyses confirmed that the three isolates clustered with the reference strain KACC 410302 of *Didymella acutilobae* G.B. Lee and W.G. Kim [31] (Fig. 2). The isolates formed a well-supported cluster with acceptable bootstrap and PP values, distinguishing them from other closely related *Didymella* spp. [11,13,25,32]. The sequence data of LSU, ITS, *TUB2*, and *RPB2* obtained from the isolates were deposited in the NCBI GenBank database (Table 2).

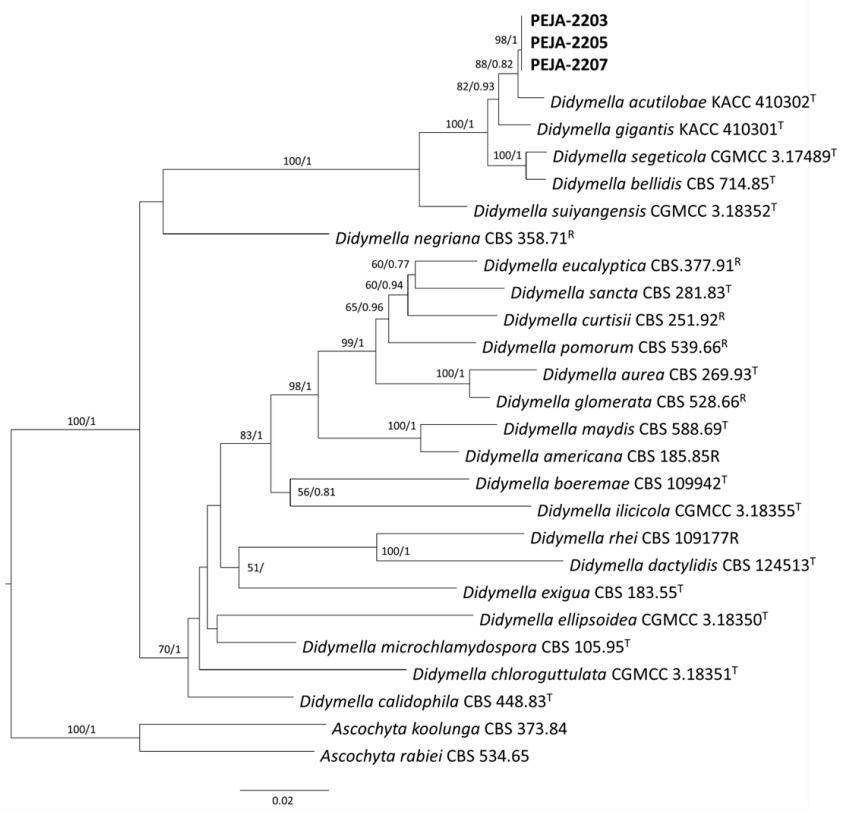

Fig. 2. Phylogenetic tree inferred from the maximum-likelihood analysis with general time-reversible model based on concatenated alignments of 28S large subunit of the nrDNA gene, internal transcribed spacer regions 1 and 2 including 5.8S nrDNA gene, β-tubulin, and RNA polymerase II second largest subunit sequences of three isolates (PEJA-2203, PEJA-2205, and PEJA-2207) from coastal hogfennel and *Didymella* spp. strains retrieved from the NCBI GenBank database. The phylogenetic tree was rooted to *Ascochyta koolunga* (CBS 373.84) and *Ascochyta rabiei* (CBS 534.65). RAxML bootstrap values (BS) and Bayesian posterior probabilities (PP) are provided at nodes (BS/PP). Values of BS/PP more than 50/0.5 are shown at the nodes. The bar at 0.08 represents the number of nucleotide substitutions per site. T: type or ex-type strains; R: reference strains.

Table 2. Accession number of sequence data obtained from *Didymella acutilobae* isolates and reference species of *Didymella* and outgroup taxa deposited in GenBank

Species	Strain/Isolate	Host/Substrate	Locality	Genbank accession number			
				TUB2	*RPB2*	LSU	ITS
D. acutilobae	PEJA-2203	*Peucedanum japonicum*	Korea	PX555481	PX555484	PX578144	PX593789
	PEJA-2205	*Peucedanum japonicum*	Korea	PX555482	PX555485	PX578145	PX593790
	PEJA-2207	*Peucedanum japonicum*	Korea	PX555483	PX555486	PX578146	PX593791
D. acutilobae	KACC 410302	*Angelica acutiloba*	Korea	OQ744073	OQ744071	OQ749983	OQ749981
D. americana	CBS 185.85	*Zea mays*	Russia	KT389594	FJ427088	GU237990	FJ426972
D. aurea	CBS 269.93	*Medicago polymorpha*	New Zealand	KT389599	GU237557	GU237999	GU237818
D. bellidis	CBS 714.85	*Bellis perennis*	The Netherlands	KP330417	GU237586	GU238046	GU237904
D. boeremae	CBS 109942	*Medicago littoralis*	Australia	KT389600	FJ427097	GU238048	FJ426982
D. calidophila	CBS 448.83	Desert soil	Egypt	MT018170	FJ427168	GU238052	FJ427059
D. chloroguttulata	CGMCC 3.18351	Air	China	KY742142	KY742299	KY742211	KY742057
D. curtisii	CBS 251.92	*Nerine* sp.	The Netherlands	MT018131	FJ427148	GU238013	FJ427038
D. dactylidis	CBS 124513	*Dactylis glomerata*	USA	MT018173	GU237599	GU238061	GU237766
D. ellipsoidea	CGMCC 3.18350	Air	China	KY742145	KY742302	KY742214	KY742060
D. eucalyptica	377.91	*Eucalyptus* sp.	Australia	KT389605	GU237562	GU238007	GU237846
D. exigua	CBS 183.55	*Rumex arifolius*	France	EU874850	GU237525	EU754155	GU237794
D. gigantis	KACC 410301	*Angelica gigas*	Korea	OQ731405	OQ731407	OQ746316	OQ746336
D. glomerata	CBS 528.66	*Chrysanthemum* sp.	The Netherlands	GU371781	FJ427124	EU754184	FJ427013
D. ilicicola	CGMCC 3.18355	*Ilex chinensis*	Italy	KY742150	KY742307	KY742219	KY742065
D. maydis	CGMCC 3.17489	*Zea mays*	USA	GU371782	FJ427190	EU754192	FJ427086
D. microchlamydospora	CBS 105.95	*Eucalyptus* sp.	UK	GU238143	GU237787	GU237657	MT018177
D. negriana	CBS 358.71	*Vitis vinifera*	Germany	KT389610	GU237635	GU238116	GU237838
D. pomorum	CGMCC 3.18352	*Polygonum tataricum*	The Netherlands	KT389618	FJ427166	GU238028	FJ427056
D. rhei	CBS 109177	*Rheum rhaponticum*	New Zealand	KP330428	GU237653	GU238139	GU237743
D. sancta	CBS 281.83	*Ailanthus altissima*	South Africa	MT018132	MT005619	GU238030	FJ427063
D. segeticola	CGMCC 3.17489	*Cirsium segetum*	China	KP330414	KP330399	KP330455	KP330443
D. suiyangensis	CGMCC 3.18352	Air	China	MT018174	MT005643	MN943747	MN973540
Ascochyta koolunga	CBS 373.84	*Pisum sativum*	Australia	KT389775	KT389560	KT389698	KT389481
A. rabiei	CBS 534.65	*Cicer arietinum*	India	GU237533	KP330405	GU237970	GU237886

TUB2: β-tubulin; *RPB2*: RNA polymerase II second largest subunit; LSU: 28S large subunit of the nrDNA gene; ITS: internal transcribed spacer regions 1 & 2 including 5.8S nrDNA gene; CBS: Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands; CGMCC: China General Microbiological Culture Collection, Beijing, China; KACC: Korean Agricultural Culture Collection, National Institute of Agricultural Sciences, Wanju, Korea.

Mycological characteristics of the isolates

The colony diameter of a representative isolate (PEJA-2207) grown on MEA, OA, and PDA for 7 days was 66–69, 67–69, and 71–72 mm, respectively. Colonies on MEA displayed white floccose and fawn mycelium with pycnidia in the aged zones (Fig. 3A). Colonies on OA displayed white floccose and buff mycelium in the middle (Fig. 3B). Colonies on PDA displayed rosy buff-to-buff mycelium with dark brown concentric rings covered with whitish mycelia (Fig. 3C). An NaOH spot test on the MEA showed negative reaction.

No teleomorphs were produced in culture. Pycnidia were non-papillate or papillate, globose, brown to black, solitary (Fig. 3D), and measured 67–223 μm in diameter. They possessed 1–3 ostioles and sometimes exhibited elongated necks. Pycnidial walls were pseudoparenchymatous, comprising round

cells in 2–3 layers, and measured 7–17 μm in thickness (Fig. 3E). Conidiogenous cells were hyaline, phialidic, and varied in shape from globose to ampulliform or flask-shaped, and measured 3.3–4.0 × 3.7–4.3 μm (Fig. 3E). Conidia were smooth-walled, ellipsoidal, aseptate, typically containing multiple guttules (Fig. 3F), and measured 3.5–6.4 × 1.7–2.7 μm (av. 4.5 × 2.1 μm). The conidial matrices were saffron to cinnamon in PDA culture. Chlamydospores were not detected.

The mycological characteristics of the *D. acutilobae* isolate from coastal hogfennel were generally consistent with the descriptions of the holotype species [31] (Table 3), although they differed slightly in pycnidial neck shape and growth rates. The slight differences in pycnidial neck shape and growth rate highlight the intraspecific variation among *D. acutilobae* strains, suggesting that further comparative studies are needed.

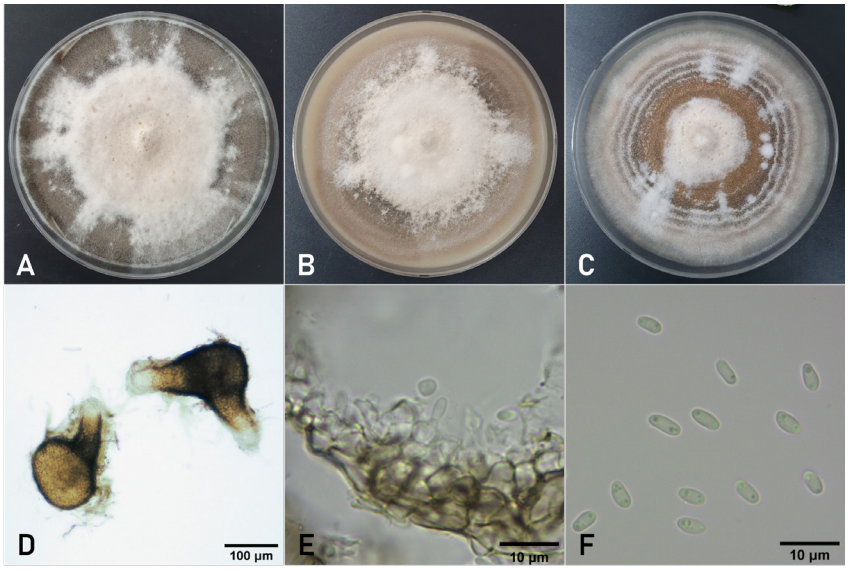

Fig. 3. Cultural and morphological features of *Didymella acutilobae* isolated from coastal hogfennel. Fourteen-day-old colonies of the fungus on malt extract agar (A), oatmeal agar (B), and potato dextrose agar (C). (D) Pycnidia produced in oatmeal agar. (E) Conidiogenous cells and conidia on a pycnidial wall. (F) Conidia.

Table 3. Morphological and cultural characteristics of *Didymella acutilobae* isolate from coastal hogfennel and the holotype species

Isolate (Reference)	Mo rphological char acteristics		Colony on media[a] and result of NaOH spot tests
	Pycnidia	Conidiogenous cells and conidia	
PEJA-2207 (Present study)	67–223 μm in diameter. Globose, brown to black, solitary, 1–3 ostioles, elongated neck, non-papillate or papillate.	Conidiogenous cells: 3.3–4.0 × 3.7–4.3 μm, globose to ampulliform or flask-shaped, hyaline, phialidic. Conidia: 3.5–6. 4 × 1.7–2.7 μm (av. 4.5 × 2.1 μm), ellipsoidal, aseptate, multiple guttules mostly. Conidial matrices saffron to cinnamon in PDA. Chlamydospores absent.	MEA: white floccose, fawn mycelium; 66–69 mm. OA: white floccose, buff mycelium; 67–69 mm. PDA: white spots, rosy buff-to-buff mycelium with dark brown concentric rings; 71–72 mm. NaOH spot test: negative.
KACC 410302 (32)	70–240 μm in diameter. Solitary or confluent, globose, brown to black, with 1–5 ostioles, non-papillate or papillate.	Conidia: 2.9 –6.5×1.6–3.0 μm, ellipsoidal or slightly curved, aseptate with usually 2 bipolar guttules. Conidial matrices white. Chlamydospores absent.	MEA: brown to black with light concentric rings; 54–61 mm. OA: brown to dark olivaceous; 53–54 mm. PDA: white to light brown with concentric rings; 53–55 mm. NaOH spot test: negative.

[a]Diameter of colonies on MEA, OA, and PDA was measured after incubation at 22 ℃ for 7 days. Colony morphology was investigated after incubation at 22 ℃ for 14 days. MEA: malt extracted agar; PDA: potato dextrose agar; OA: oatmeal agar.

Pathogenicity

The pathogenicity tests confirmed that the three *D. acutilobae* isolates caused leaf spot symptoms identical to those occurred in the field (Fig. 1C). Control plants were asymptomatic (Fig. 1D). Re-isolation of the fungus from induced lesions confirmed the identity of the inoculated isolates.

D. acutilobae was first reported to cause leaf spot and stem rot in *Angelica acutiloba* (Siebold & Zucc.) Kitag. [31], and a subspecies of the fungus was recently reported to cause leaf spot in East Asian hogweed [33]. To date, several diseases have been reported in coastal hogfennel in Korea and Japan, including powdery mildew, Fusarium wilt, damping-off, Sclerotinia rot, and leaf spot [10,34]. In Korea, *Alternaria peucedani* and *Ascochyta* sp. have been documented as the causal fungi of leaf spot in coastal hogfennel [10], with *D. acutilobae* having never been documented, until now, to act as the etiologic agent for this disease. Thus, this is the first study to report on *D. acutilobae*-induced leaf spot in coastal hogfennel.

CONFLICT OF INTERESTS

No potential conflict of interest was reported by the authors.

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