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Research Note

First Report of Dieback Caused by *Lasiodiplodia theobromae* in Strawberry Plants in Korea

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Abstract Dieback in strawberry (Seolhyang cultivar) was first observed during the nursery season (June to September) in the Nonsan area of Korea in the years 2012 and 2013. Initial disease symptoms included dieback on runners, as well as black rot on roots, followed by wilting and eventually blackened, necrotic discoloration in the crowns of daughter plants. A fungus isolated from the diseased roots, runners, and crowns is close to *Lasiodiplodia theobromae* based on morphological characteristics. Analysis of a combined dataset assembled from sequences of the internal transcribed spacer and translation elongation factor 1-alpha genes grouped nine fungal isolates with the type strain of *L. theobromae*. The isolates showed strong pathogenicity on strawberry cultivars Kumhyang, Seolhyang, and Akihimae, fulfilling Koch's postulates. Based on these results, the pathogen responsible for dieback on strawberry plants in Korea was identified as *L. theobromae*.

Keywords Dieback, ITS, *Lasiodiplodia*, Strawberry, *tef1*

Strawberry (*Fragaria* × *ananassa* Duch.) is widely cultivated in Korea, occupying some 6,403 ha in 2015, and it is one of the most important fruits grown in the country. During the nursery season, strawberries are grown in plastic houses, using raised beds containing commercial substrates such as cocopeat, peat moss and perlite, loamy sand, and expanded rice hulls [1]. In nursery fields, from June to September in the years 2012 and 2013, severe dieback and black rot on runners and daughter plants of the Seolhyang strawberry cultivar were observed in Nonsan, Chungnam province. Disease symptoms began as dieback on runners and black rot on roots, which led to wilting and eventually to blackened, necrotic discoloration of the crowns of daughter plants. Approximately 5~10% of the plants wilted and eventually died. In particular, the first- and second-generation daughter plants, whose roots dried after temporary irrigation with a short watering period for rooting, were shown to suffer from a high rate of dieback. This disease incidence was highest in August and in nursery beds using commercial substrates and loamy sand.

Dieback in strawberry was first reported to be caused by the pathogen *Lasiodiplodia theobromae* in Turkey [2]. In Korea, *L. theobromae* was reported for the first time in mango by Hong *et al.* [3]. *L. theobromae* occurs mainly in tropical and subtropical regions and can cause stem rot, dieback, and cankers on important commercial crops such as almond [4], blueberry [5], cocoa [6], grapevines [7], mango [3, 8, 9], olive [10], and peanut [11]. *Lasiodiplodia*, a member of *Botryosphaeriaceae*, is a cosmopolitan fungus [12] and a soil-borne saprophyte [13]. To establish the taxonomy of this genus, molecular DNA-based approaches have been widely used [14], and phylogenetic studies of multiple genes including internal transcribed spacer (ITS) and translation elongation factor 1-alpha (*tef1*) have revealed cryptic species within the *L. theobromae* complex [8].

We conducted morphological and phylogenetic analyses using ITS and *tef1* genes to determine the potential causal agent of dieback, and to confirm which isolates were pathogens to strawberry plants.

In the strawberry plants investigated in the present study, disease symptoms began as dieback on runners and black

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Fig. 1. Symptoms of strawberry dieback observed in a nursery field in Korea. A, Wilt and dieback of daughter plants and runners; B, Blackened, necrotic discoloration in the crown; C, Dieback in leaves; D, Black rot on roots.

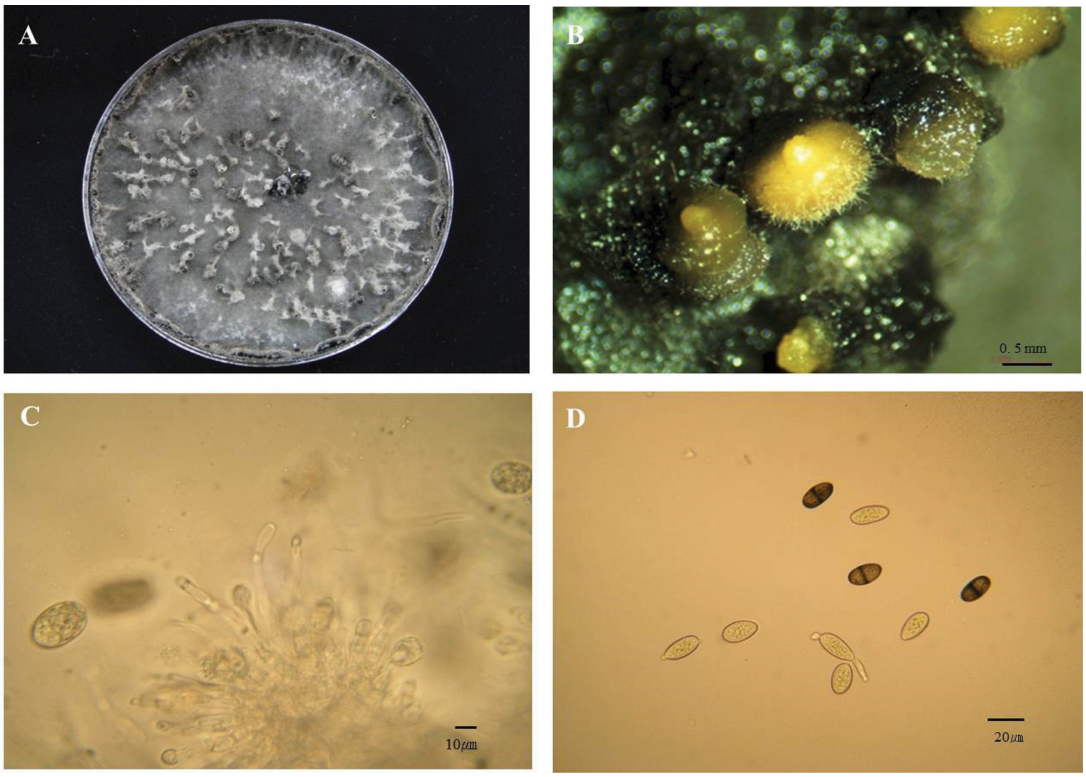

Fig. 2. Colony and conidial morphology of *Lasiodiplodia theobromae*. A, Colony morphology of a 10-day-old sample; B, Pycnidia; C, Paraphyses; D, Immature conidia (whitish) with thin walls, and mature conidia (dark brown) with septa and thick walls (scale bars: B = 0.5 mm, C = 10 μm, D = 20 μm).

Table 1. Morphological characteristics of fungal isolates obtained in this study

Isolate	Conidiomata	Paraphyses	Conidia
LT120901 LT120903 LT120907	Pycnidial, dark brown	Hyaline, cylindrical, septate, not branched, round at apex, up to 55 μm long, 3~4 μm wide	Ellipsoid to obovoid, 1-septate Mean 26.22 ± 5.86 × 13.52 ± 3.04 μm (L/W ratio 1.94)
Lasiodiplodia theobromae [15]	Pycnidial, uniloculate, dark brown to black	Hyaline, cylindrical, septate, occasionally branched, round at apex, up to 55 μm long, 3~4 μm wide	Subovoid to ellipsoid-ovoid, dark brown and 1-septate Mean 26.2 × 14.2 μm (L/W ratio 1.9)

L/W, length/width.

rot on roots, which led to wilting and eventually to blackened, necrotic discoloration in the crown of daughter plants (Fig. 1). Ten fungal strains isolated from the Seolhyang strawberry plants were examined. In 2012, we isolated four strains from roots (LT120701, LT120702, LT120901, and LT120902) and three strains from crowns and runners (LT120903, LT120906, and LT120907). In 2013, the strains LT130701, LT130702, and LT131001 were isolated from crowns.

The first growth phase for the isolates LT120901, LT120903, and LT120907, documented on potato dextrose agar (PDA), gave rise to white colonies, followed by a dense, black mycelium (Fig. 2A). Each organ was measured four times, and at least seven organs were measured each time. Pycnidia of dark brown to black color were formed after 30 days of culture (Fig. 2B). Paraphyses were hyaline, cylindrical, septate, and not branched, and they were round at the apex, up to 55-μm-long, and 3~4 μm wide (Table 1, Fig. 2C). The length/width ratio of the conidia was 1.94. The conidia of isolates were unicellular when young, and 1-septate, thick-walled, and ellipsoid to obovoid in shape when mature, with dimensions of 26.22 ± 5.86 × 13.52 ± 3.04 μm (Table 1, Fig 2D). *L. theobromae* isolated from mango in Korea [3] had conidia with a size of 17.5~26.8 × 12.3~17.1 μm, and were either unicellular or 1-septate. The shape and size of the conidia reported by Hong *et al.* [3] were thus similar to the present observations. Additionally, the morphology of the strains isolated from strawberry was similar to that of *L. theobromae* as reported by Alves *et al.* [15].

Temperatures suitable for growth were determined using the LT120901 and LT130701 isolates. Each isolate was incubated in the dark at 15°C, 20°C, 25°C, 30°C, 35°C, and 40°C on PDA, and growth rates (mm/day) were then measured for each isolate. The mycelia of the isolates grew at temperatures between 15°C and 40°C, and the optimal growth temperature was 30°C (Fig. 3); this agrees with one previous study [16], and is very similar to another that reported maximum radial growth of *L. theobromae* at 29.4°C [17].

For molecular phylogenetic analysis, we sequenced nine of the strains isolated from the strawberry plants in the present study, and compared them with ex-type sequences of *Lasiodiplodia* species retrieved from GenBank (Table 2). Genomic DNA was extracted using the method of Park *et al.* [18]. The ITS and *tef1* genes were amplified to identify each strain at the species level. The PCR amplifications of

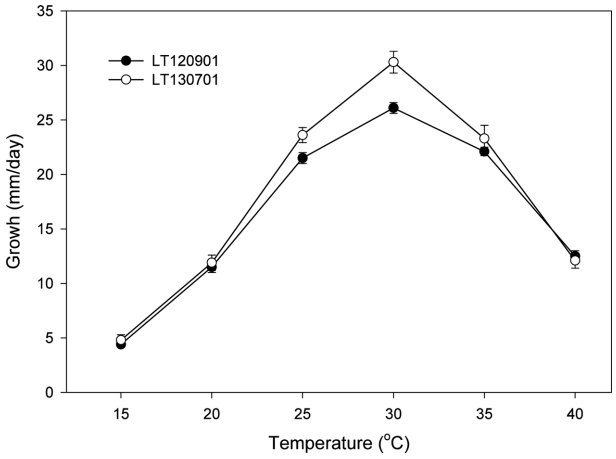

Fig. 3. Effect of temperature on mycelial growth in *Lasiodiplodia theobromae*.

ITS and *tef1* were performed using ITS5 and ITS4 [19] and EF1-728F and EF1-986R [20], respectively. Each PCR reaction was performed on a CFX96 thermal cycler (Bio-Rad, Hercules, CA, USA) under the PCR conditions described by Crous *et al.* [21]. PCR products were purified using the PCRquick-spin PCR Product Purification Kit (iNtRON BioTechnology, Seongnam, Korea). The PCR amplicons were sequenced at Bioneer Corporation (Chungwon, Korea). Sequences were edited using MEGA 5 software [22] and aligned using the default settings of MAFFT v7 [23]. A neighbor-joining tree using a combined data set (ITS + *tef1*) was constructed in MEGA 5 using the Kimura 2-parameter model and 1,000 bootstrap replicates. ITS and *tef1* gene sequences from the isolates LT120701, LT120702, LT120907, and LT120901 were deposited in GenBank under accession Nos. KX506781~KX506788.

Based on the combined dataset of ITS and *tef1* sequences, the Korean isolates formed a monophyletic group with *L. theobromae* CBS 164.96 (type strain) and CBS 111530 with 92% bootstrap support (Fig. 4). The isolates and *L. theobromae* showed 99.8~100% sequence similarity for ITS and 99.7~100% for *tef1*. Although *L. theobromae* has been previously isolated from diseased strawberry in Turkey, there was no report of this disease in Korea. All Korean isolates related to dieback were determined to be *L. theobromae* based on these morphological and molecular phylogenetic analyses.

Table 2. Reference sources for the *Lasiodiplodia* isolates used in this study

Species	Source	GenBank accession No.	
		ITS	*tef1*
Diplodia mutila	CBS 112553	AY259093	AY573219
Diplodia seriata	CBS 112555^{T}	AY259094	AY573220
Lasiodiplodia citricola	CBS 124707^{T}	GU945354	GU945340
	CBS 124706	GU945353	GU945339
Lasiodiplodia crassispora	CBS 118741^{T}	DQ103550	EU673303
	CBS 110492	EF622086	EF622066
Lasiodiplodia egyptiacae	CBS 130992^{T}	JN814397	JN814424
Lasiodiplodia gilanensis	CBS 124704^{T}	GU945351	GU945342
	CBS 124705	GU945352	GU945341
Lasiodiplodia gonubiensis	CBS 115812^{T}	AY639595	DQ103566
	CBS 116355	AY639594	DQ103567
Lasiodiplodia hormozganensis	CBS 124709^{T}	GU945355	GU945343
	CBS 124708	GU945356	GU945344
Lasiodiplodia iraniensis	CBS 124710^{T}	GU945346	GU945334
	CBS 124711	GU945347	GU945335
Lasiodiplodia margaritacea	CBS 122519^{T}	EU144050	EU144065
	CBS 122065	EU144051	EU144066
Lasiodiplodia mahajangana	CBS 124927^{T}	FJ900597	FJ900643
	CBS 124925^{T}	FJ900595	FJ900641
Lasiodiplodia missouriana	CBS 128311^{T}	HQ288225	HQ288267
	CBS 128312	HQ288226	HQ288268
Lasiodiplodia parva	CBS 456.78^{T}	EF622083	EF622063
	CBS 494.78	EF622084	EF622064
Lasiodiplodia plurivora	CBS 120832^{T}	EF445362	EF445395
	CBS 121103	AY343482	EF445396
Lasiodiplodia pseudotheobromae	CBS 116459^{T}	EF622077	EF622057
	CBS 447.62	EF622081	EF622060
Lasiodiplodia rubropurpurea	CBS 118740^{T}	DQ103553	EU673304
	WAC 12536	DQ103554	DQ103572
Lasiodiplodia theobromae	CBS 164.96^{T}	AY640255	AY640258
	CBS 111530	EF622074	EF622054
Lasiodiplodia venezuelensis	CBS 118739^{T}	DQ103547	EU673305
	WAC 12540	DQ103548	DQ103569
Lasiodiplodia viticola	CBS 128313^{T}	HQ288227	HQ288269
	CBS 128315	HQ288228	HQ288270

T, ex-type.

Three of the isolated pathogens (LT120701, LT120901, and LT131001) were prepared in a suspension at 1×10^{5} conidia/mL, and daughter plants of the Seolhyang strawberry cultivar were drenched with 50 mL of conidia suspension. A pathogenicity test was conducted using six daughter plants per isolate. The inoculated plants were sealed in plastic boxes with wet paper towels. These plants were incubated at 25°C and 100% relative humidity for three days. After three days, the inoculated plants were transferred to a greenhouse. Disease index and severity on each plant were rated after 10 days. Disease index was rated using a 0~3 scale, where 0 = no symptoms, 1 = one leaf wilted, 2 = two or more leaves wilted, and 3 = necrosis in the crown. Disease severity was measured as the percentage of wilted plants out of the total number of plants. All three isolates caused disease symptoms on healthy strawberry plants, and the identical pathogen was re-isolated from the inoculated plants. Non-inoculated plants showed no symptoms.

To examine the resistance of strawberry plants to *L. theobromae*, the cultivars Akihimae, Jukhyang, Kumhyang, Maehyang, Redpearl, Santa, Seolhyang, and Sukhyang were used. Three daughter plants of each cultivar were subjected to pathogenicity tests carried out in the same manner as described above. The cultivars Kumhyang, Seolhyang, and Akihimae were found to be highly susceptible to *L. theobromae,* whereas Jukhyang and Sukhyang did not show high disease susceptibility (Table 3). A different pathogenicity test of *L. theobromae* on strawberry plants [2] revealed that the strawberry cultivar Festival, which is grown in the United States and in Central and South America, developed wilting and dieback symptoms. In Korea, the Seolhynag cultivar is widespread, constituting up to 81.3% of all cultivated varieties, so dieback caused by *L. theobromae* is an issue that requires considerable attention from the strawberry nursery industry.

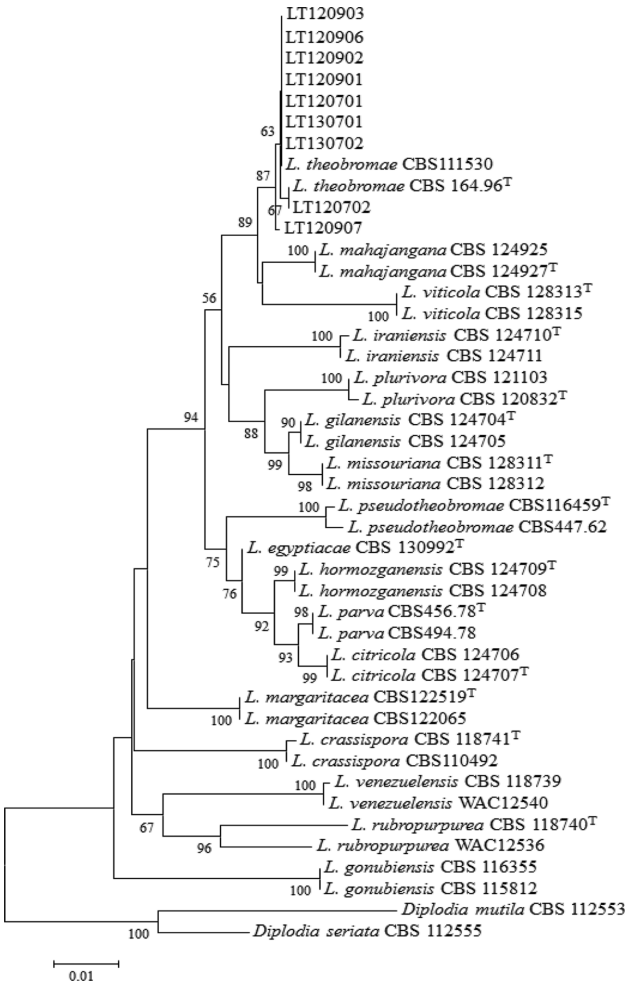

Fig. 4. Phylogenetic tree of *Lasiodiplodia* isolates from strawberry plants in Korea and related species based on a neighbor-joining analysis of a combined internal transcribed spacer and translation elongation factor 1-alpha dataset. Numbers beside each branch represent bootstrap values obtained after a bootstrap test with 1,000 replications. Bar indicates the number of nucleotide substitutions.

Table 3. Pathogenicity test of fungal isolates on different strawberry cultivars

Cultivar	Disease index[a]	Disease severity[b]
Keumhyang	3.0 a[c]	100.0
Seolhyang	2.8 a	91.7
Akihimae	2.8 a	91.7
Santa	1.8 b	58.3
Maehyang	1.5 bc	50.0
Redpearl	1.0 c	33.3
Jukhyang	0.3 d	8.3
Sukhyang	0.0 d	0.0

[a]Disease index: 0, no symptoms; 1, one leaf wilted; 2, two or more leaves wilted; and 3, necrosis in the crown.
[b]Disease severity: the percentage of wilted plants out of the total number of plants.
[c]Means followed by the same letter are not significantly different ($p = 0.05$) according to Duncan's multiple range test.

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