Mycobiology

Research Note

Cladosporium cladosporioides and *C. tenuissimum* Cause Blossom Blight in Strawberry in Korea

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Abstract Blossom blight in strawberry was first observed in a green house in Nonsan, Damyang, and Geochang areas of Korea, between early January to April of 2012. Disease symptoms started as a grey fungus formed on the stigma, which led to the blossom blight and eventually to black rot and necrosis of the entire flower. We isolated the fungi purely from the infected pistils and maintained them on potato dextrose agar (PDA) slants. To test Koch's postulates, we inoculated the fungi and found that all of the isolates caused disease symptoms in the flower of strawberry cultivars (Seolhyang, Maehyang, and Kumhyang). The isolates on PDA had a velvet-like appearance, and their color ranged between olivaceous-brown and smoky-grey to olive and almost black. The intercalary conidia of the isolates were elliptical to limoniform, with sizes ranging from 5.0~10.5 × 2.5~3.0 μm to 4.0~7.5 × 2.0~3.0 μm, respectively. The secondary ramoconidia of these isolates were 0- or 1-septate, with sizes ranging betweem 10.0~15.0 × 2.5~3.7 μm and 8.7~11.2 × 2.5~3.2 μm, respectively. A combined sequence analysis of the internal transcribed spacer regions, partial actin (*ACT*), and translation elongation factor 1-alpha (*TEF*) genes revealed that the strawberry isolates belonged to two groups of authentic strains, *Cladosporium cladosporioides* and *C. tenuissimum*. Based on these results, we identified the pathogens causing blossom blight in strawberries in Korea as being *C. cladosporioides* and *C. tenuissimum*.

Keywords Blossom blight, *Cladosporium cladosporioides*, *Cladosporium tenuissimum*, Strawberry

Strawberry (*Fragaria* × *ananassa* Duch.) is an important crop in Korea. It has an acreage of more than 6,435 ha and in 2012 this crop brought in the production value of over 900 million dollars. Strawberry cultivar Seolhyang has characteristics such as high yield, low incidences of powdery mildew, and accounts for 70% strawberry production in Korea. During winter, strawberries are grown in green houses in Korea.

Severe blossom blight affected several strawberry fruit production fields in Nonsan, Damyang, and Geochang areas, between January and April of 2012. The fruits showed severe deformities, with increased incidences of the disease found under high-humidity conditions and in strawberry plants planted outside the furrow in green houses. The occurrence of blossom blight in strawberry during the harvesting stage was first reported to be caused by the pathogen *Cladosporium cladosporioides* in California, USA [1]. In Korea, the occurrence of the *Cladosporium* spp. in strawberry was reported as scabs on the leaf, calyx, and runner caused mostly by *C. herbarum* [2]. The occurrence of *C. cladosporioides* and *C. tenuissimum* in Korea was reported in rice and oriental persimmon [3], and pear [4], respectively. The *Cladosporium* spp. have a wide range of host plants, such as grape [5], mandarin [6], and wheat [7]. It commonly persists on host plants as epiphytes or it is dispersed in the air [8].

Cladosporium is one of the largest and most heterogeneous genera of hyphomycetes [9, 10]. To establish the taxonomy of this genus, it is necessary to examine its morphological and molecular features based on ex-type strains. The species can be identified using polyphasic approaches with morphological and molecular markers [11, 12]. In this genus, *C. cladosporioides* is a very common, cosmopolitan, saprobic species and has been applied to several taxa that have been demonstrated as distinct in recent decades [13]. Bensch *et al.* [14] have reported several species, with *C. cladosporioides* being clearly distinguished based on a combination of morphological and biometric features, culture characteristics, and molecular data with neotypes and

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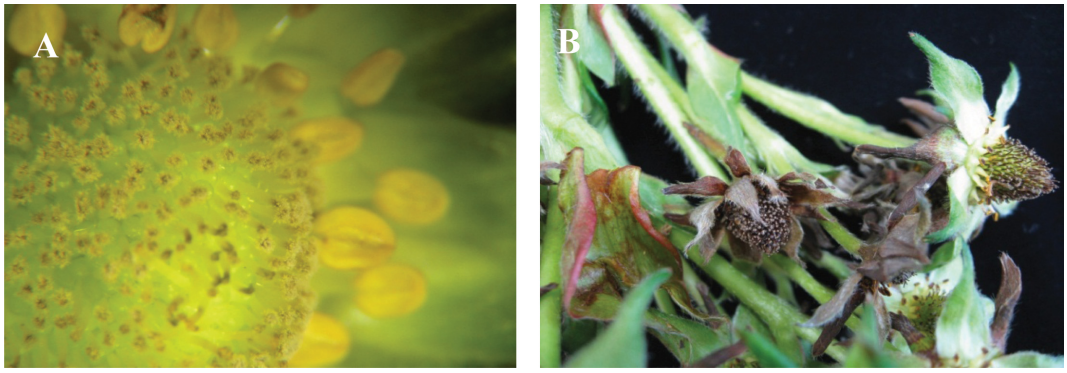

Fig. 1. Symptoms of blossom blight on stigma (A) and flower (B) after natural infection on Seolhyang cultivar.

Table 1. Sources of *Cladosporium* spp. isolates used in this study

Isolate	Isolation year	Cultivar	Plant part	Origin
C120302	2012	Seolhyang	Flower	Damyang, Jeonnam
C130102	2013	Seolhyang	Flower	Geochang, Gyeongnam
C130103	2013	Seolhyang	Flower	Geochang, Gyeongnam
C130104	2013	Seolhyang	Flower	Geochang, Gyeongnam
C130110	2013	Seolhyang	Flower	Geochang, Gyeongnam

epitypes.

In this study, we identified the causal agent using morphological and phylogenetic analysis and confirmed its association with the recent reports of blossom blight in strawberry.

The stigma of the strawberry flower showed the formation of a grey fungus. The entire flower underwent necrosis and turned into black rot (Fig. 1). The pathogen caused green-grey sporulation on dead stigmas and malformed or misshapen fruits. The disease occurred mainly in the Seolhyang cultivar and its incidence was higher in soil cultures than in hydroponic cultures. The average disease incidence was 20%, with higher occurrence in strawberry plants planted outside than in plants planted inside the furrow in green houses (data not shown).

Five fungal strains were isolated from strawberry flowers from 2012 to 2013 (Table 1). Diseased stigma tissues were soaked in sterilized water with shaking for 30 sec, and a drop of the resulting suspension was streaked on water agar and incubated at 25°C. The growing edges of the fungal hyphae developing from the streaks were then aseptically transferred to potato dextrose agar (PDA; Difco, Detroit, MI, USA). Pure cultures were stored on PDA slants at 4°C.

Each isolated pathogen was prepared at a concentration of 1×10^5 conidia/mL, and 1 mL was spayed per flower cluster on Seolhyang, Maehyang, and Kumhyang cultivars of strawberry. Six flower clusters per isolate were used for the Koch's postulates test. The inoculated plants were incubated in a dew plastic box at 25°C and 100% relative humidity for 7 days. After 7 days, the disease severity and black rot on each flower cluster were rated. All isolates caused symptoms on all strawberry cultivars (Table 2). Disease severity was the highest for the C120302 isolate. The disease severity was higher on Kumhyang cultivar than on the other cultivars. The fungal pathogen was re-isolated from the inoculated flowers.

Table 2. Pathogenicity test on strawberry cultivars using strains isolated from the flower *in vivo*

Isolate	Disease severity[a]		
	Seolhyang	Maehyang	Kumhyang
C120302	+++	++	++++
C130102	+++	+	+++
C130103	+++	+++	+++
C130104	++	++	++
C130110	++	++	++
Non-inoculated control	–	–	–

[a]–, no symptom; +, < 10%; ++, 11~30%; +++, 31~50%; ++++, > 50%.

The isolates were analyzed for colony characteristics, namely the shape and size of the conidia and ramoconidia, after 7~10 days of incubation on PDA. The colony radii were measured daily for 7 days, and the growth rate over the 7-day period was calculated as the mean daily growth (in millimeters per day). The colors of the conidial masses and zonation were recorded after 10 days.

The C130102, C130103, C130104, and C130110 isolates on PDA formed olivaceous-green to olivaceous-brown, velvet-like colonies with apically and laterally branched conidiophores and lemon-shaped conidia, which were usually smooth but sometimes textured (Fig. 2). The intercalary conidia of these isolates were elliptical to limoniform with sizes ranging 5.0~10.5 × 2.5~3.0 µm, and secondary ramoconidia were cylindrical-oblong with sizes ranging 10.0~15.0 × 2.5~3.7 µm (Table 3). The C120302 isolate formed smoky-grey and olive colonies. The intercalary conidia of these isolate were ovoid to elliptical with sizes ranging 4.0~7.5 × 2.0~3.0 µm, and secondary ramoconidia were cylindrical with sizes ranging 8.7~11.2 × 2.5~3.2 µm.

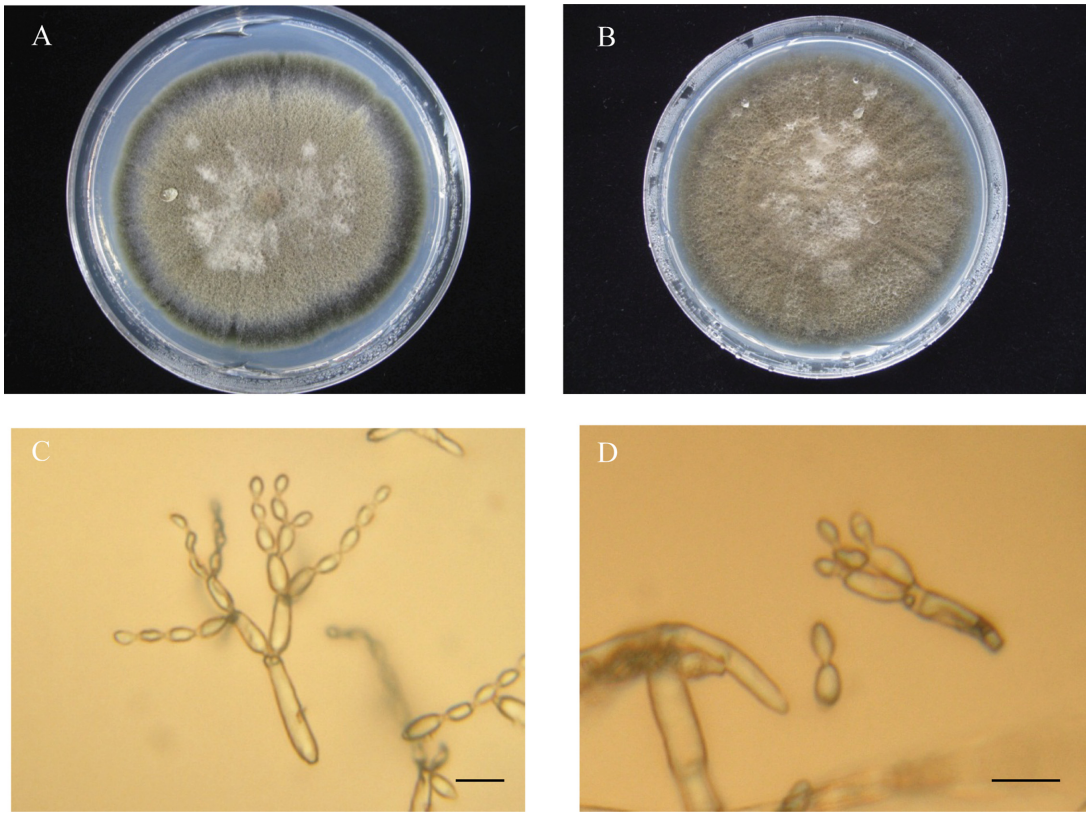

Fig. 2. Colony characters (A, B), and conidia and ramoconidia (C, D) of *Cladosporium cladosporioides* (A, C) and *C. tenuissimum* (B, D) cultured on potato dextrose agar at 20°C for 10 days in the dark (scale bars = 10 μm).

Table 3. Morphological characteristics of *Cladosporium* spp. isolated from strawberry

Isolate	Colony	Conidia (intercalary, μm)	Ramoconidia (secondary, μm)
C130102, C130103, C130104, C130110	Olive	5.0~10.5 × 2.5~3.0	10.0~15.0 × 2.5~3.7
C120302	Smoky-grey, olive	4.0~7.5 × 2.0~3.0	8.7~11.2 × 2.5~3.2
C. cladosporioides (CBS 112388) [15]	Grey-olivaceous to dull green	5.0~12.0 × 2.5~3.0	10.0~33.0 × 2.5~4.0
C. tenuissimum (CBS 125995) [15]	Smoke-grey to grey-olivaceous	4.0~12.0 × 2.0~3.0	7.0~25.0 × 2.5~4.0

The cultural and morphological characteristics of C130102, C130103, C130104, and C130110 isolates were consistent with the published descriptions of *C. cladosporioides*, whereas C120302 was similar to *C. tenuissimum* [14]. These two species have distinct morphological characteristics. The C130102 and C120302 isolates were deposited in Rural Development Administration (RDA) Genebank Information Center, assigned with Korean Agricultural Culture Collection (KACC) 47995 and KACC 47996, respectively.

The mycelia of isolates grew between 10°C and 30°C, whereas no growth occurred after 7 days at 5°C on PDA (Fig. 3). The C120302 colonies were significantly larger in radius than that of the other isolates after 7 days at both 20°C and 25°C. For all isolates, optimal growth rate was attained after 7 days of incubation at 20°C. This result was similar to that reported by Tashiro *et al.* [6], who reported optimal growth of *C. cladosporioides* at 20~22°C.

The sequences of five strains from strawberry and the

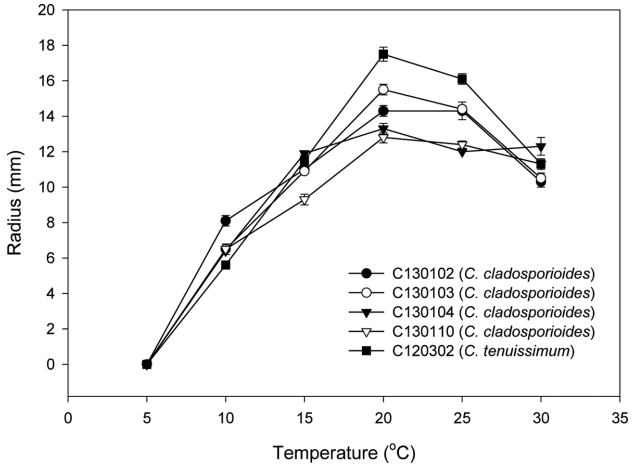

Fig. 3. Mean colony radii of *Cladosporium cladosporioides* and *C. tenuissimum* isolated from strawberry flowers grown on potato dextrose agar plates for 7 days at various temperatures.

Table 4. Reference sources of *Cladosporium* spp. isolates used in this study

Species	Source	GenBank accession No.		
		ITS	*TEF*	*ACT*
C. acalyphae	CBS 125982[T]	HM147994	HM148235	HM148481
C. angustisporum	CBS 125983[T]	HM147995	HM148236	HM148482
C. australiense	CBS 125984[T]	HM147999	HM148240	HM148486
C. basiinflatum	CBS 822.84[T]	HM148000	HM148241	HM148487
C. cladosporioides	CBS 112388[T]	HM148003	HM148244	HM148490
C. cladosporioides	CPC 14705	HM148050	HM148291	HM148537
C. cladosporioides	CPC 15038	HM148051	HM148292	HM148538
C. cladosporioides	CPC 15167	HM148052	HM148293	HM148539
C. colocasiae	CBS 386.64[T]	HM148067	HM148310	HM148555
C. funiculosum	CBS 122129[T]	HM148094	HM148338	HM148583
C. gamsianum	CBS 125989[T]	HM148095	HM148339	HM148584
C. pini-ponderosae	CBS 124456[T]	FJ936160	FJ936164	FJ936167
C. pseudocladosporioides	CBS 125993[T]	HM148158	HM148402	HM148647
C. rectoides	CBS 125994[T]	HM148193	HM148438	HM148683
C. subuliforme	CBS 126500[T]	HM148196	HM148441	HM148686
C. tenuissimum	CBS 125995[T]	HM148197	HM148442	HM148687
C. verrucocladosporioides	CBS 126363[T]	HM148226	HM148472	HM148717
C. xylophilum	CBS 125997[T]	HM148230	HM148476	HM148721

[T], ex-type.

ex-type strains of *Cladosporium* available from GenBank (Table 4) were used in this study. Genomic DNA was extracted following the method of Park *et al.* [15]. For the amplification of the internal transcribed spacer (ITS), partial actin (*ACT*), and translation elongation factor-1 alpha (*TEF*) genes, three different primer sets were used: ITS5 and ITS4 [16], ACT-512F and ACT-783R [17], and EF1-728F and EF1-986R [18], respectively. The amplification was performed using Maxime PCR PreMix (I-Taq; iNtRoN Biotechnology, Seongnam, Korea) in a final volume of 20 μL containing 10 pmol of each primer set, under the PCR conditions described by Crous *et al.* [19]. The PCR products were electrophoresed on a 1% agarose gel, stained with ethidium bromide, and purified using the PCR quick-spin PCR Product Purification Kit, according to the manufacturer's instructions (iNtRON Biotechnology). The nucleotide sequences were determined by Bioneer Corporation (Daejeon, Korea). Sequences were assembled, proofread, and edited using the MEGA 5 software [20]. Multiple DNA sequence alignments were performed with the default settings of MAFFT v7 [21] and were checked visually, with ambiguously aligned positions adjusted manually. Maximum likelihood phylogenetic analyses were performed for the combined data set (ITS + *ACT* + *TEF*) using RAxML [22] under the GTRGAMMA model of evolution for tree inference and 1,000 bootstrap replicates. The sequences of ITS, *ACT*, and *TEF* from three isolates C130102, C130110, and C120302, were deposited in GenBank (Nos. KJ558393~ KJ558401).

The alignment length of the isolates for each of the four data sets varied: ITS, 476 bp; *ACT*, 221~224 bp; *TEF*, 179~182 bp; and for the combined data set (ITS + *ACT* + *TEF*), 876~882 bp. For ITS analysis, the isolates from strawberry plants were not identified due to poor resolution of the ITS phylogeny (data not shown), while the analyses of other data sets showed high resolution. For *ACT*, C130102, the C130103, C130104, and C130110 isolates showed sequence similarities of 99.1~100% with the *C. cladosporioides* strains CPC 14705 and CPC15038. For *TEF*, the C130102, C130103, C130104, and C130110 isolates revealed sequence similarities of 97.8~99.4% with the *C. cladosporioides* strains CPC 14705 and CPC15038. Whereas the C120302 isolate showed a sequence similarity of 100% and 99.5% for *ACT* and *TEF*, respectively, with *C. tenuissimum* strain CBS 125995. For the combined data set, C130102, C130103, C130104, and C130110 isolates, and *C. cladosporioides* formed a monophyletic group supported by 65% bootstrap values, with two subgroups (Fig. 4). The first subgroup included an ex-type strain (CBS 112388 from indoor air) and one strain (CPC 15167 from a living mite inhabiting a strawberry leaf) of *C. cladosporioides*. The second subgroup included four isolates (C130102, C130103, C130104, and C130110) and two strains (CPC 14705 from chasmothecia of *Phyllactinia* sp. on *Fraxinus rhynchophylla* and CPC 15038 from *Eucalyptus* sp.). Phylogenetic analysis based on the combined data set of ITS, *ACT* and *TEF* showed that *C. cladosporioides* formed a monophyletic group with various subgroups supported by a high bootstrap value [14].

Although *C. cladosporioides* has been previously isolated from diseased strawberries in California, USA [1], *C. tenuissimum* has not yet been reported. *C. cladosporioides* and *C. tenuissimum* are two quite common saprobic species isolated from numerous substrates. These species are morphologically very similar and are, therefore, often misidentified [14]. *C. cladosporioides* has been reported as a pathogen of scab in papaya [23], sooty mold in persimmon [24], blossom blight in strawberry [25, 26],

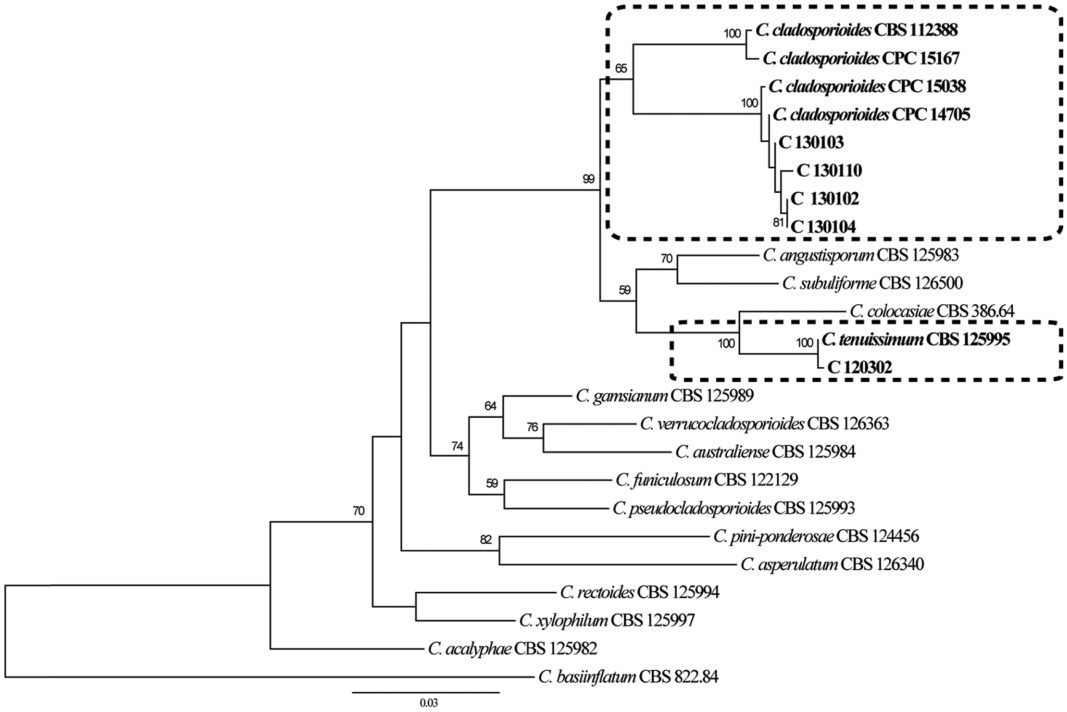

Fig. 4. Maximum-likelihood tree inferred from a combined dataset of internal transcribed spacer, *ACT*, and *TEF* sequences showing phylogenetic relationships among *Cladosporium* spp. from strawberry plants in Korea and representative species. Bootstrap scores are presented at the nodes only if they are greater than 50. The scale bar indicates the number of nucleotide substitutions per site.

and raceme blight in macadamia nuts [27]. *C. tenuissimum* has been reported as a pathogen of skin sooty and decay disease in pear [4], dry rot in tomato [28], leaf spot in banana [29], and leaf blight in watermelon [30], and cucumber [31], and as a hyperparasite of several rust fungi [32]. Infection by *C. tenuissimum* has been often reported in tropical regions.

In the present study, the blossom blight fungi that were isolated from strawberry in Korea were identified as *C. cladosporioides* and *C. tenuissimum* based on their morphological and molecular phylogenetic characteristics.

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