

Identification and Characterization of *Cercospora malayensis* Causing Leaf Spot on Kenaf

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Abstract In September 2013 and 2014, a significant number of kenaf plants showing symptoms of leaf spots with approximately 50% incidence were found in experimental plots in Iksan and Namwon, Korea. Leaf spots were circular to irregular, more or less vein-limited, reaching to 10 mm in diameter. The spots were initially uniformly brown to reddish brown, turning pale brown with a purplish margin and showing grayish patches on the lesion due to heavy fructification. The causative agent of the leaf spot disease was identified as *Cercospora malayensis*. The pathogenicity test was conducted with similar results, which fulfilled Koch's postulates. This is the first report of *C. malayensis* infection of kenaf in Korea.

Keywords *Cercospora malayensis*, *Hibiscus cannabinus*, Kenaf, Leaf spot, Multigene phylogenetic analysis

Kenaf (*Hibiscus cannabinus* L., family *Malvaceae*) is a herbaceous annual fiber crop widely cultivated in the tropics and subtropics as a source of cotton, jute, and seed oils. Kenaf can be grown under a wide range of weather conditions and grows to more than 3 m within 3 months even under moderate ambient conditions [1]. Historically, kenaf has been used as a cordage crop to produce twine, rope, and sackcloth. Currently, there are various new applications for kenaf, including paper products, building materials, absorbents, and animal feeds [2]. In Korea, kenaf has been actively evaluated in recent years as an alternative agricultural crop for animal feed and as a source of high-quality cellulose fibers. The crop can utilize soil nitrogen

and phosphorus and accumulate atmospheric carbon at a very high rate.

The genus *Cercospora* (anamorphs of *Mycosphaerella* Johanson) represents one of the largest genera of hyphomycetes and its species are regarded as major pathogens of a wide variety of plants. In a monograph of this genus *Cercospora* Fresen., Chupp [3] accepted 1,419 species. More than 3,000 species of *Cercospora* have been described, of which 659 are recognized [4]. Species of *Cercospora* are considered host-specific at the level of the plant genus or family; this concept has led to the description of a large number of species [5]. Recently, the phylogeny of several *Cercospora* species was evaluated using protein-coding genes, such as translation elongation factor 1- α (EF-1 α), actin (ACT), calmodulin (CAL), and histone 3 (HIS). Multigene analyses have provided a more robust identification of *Cercospora* species and most *Cercospora* species appear to be host-specific with a few exceptions [6].

Cercospora leaf spots of the 17 species of *Hibiscus* are a serious problem wherever this crop is grown. It is one of the most common and destructive *Hibiscus* diseases, often resulting in complete crop loss [7]. More than 200 fungal species are associated with various diseases of kenaf worldwide [7]. Among these, *Cercospora* leaf spots include *C. abelmoschi* (China, India, and Japan), *C. abelmoschi-cannabini* (India), *C. cannabina* (China), *C. hibisci-cannabini* (Taiwan), *C. hibiscina* (China and India), *C. hibiscivora* (China), and *C. malayensis* (Cambodia, China, South Africa, Tanzania, Zambia, and Zimbabwe) [7]. However, in Korea,

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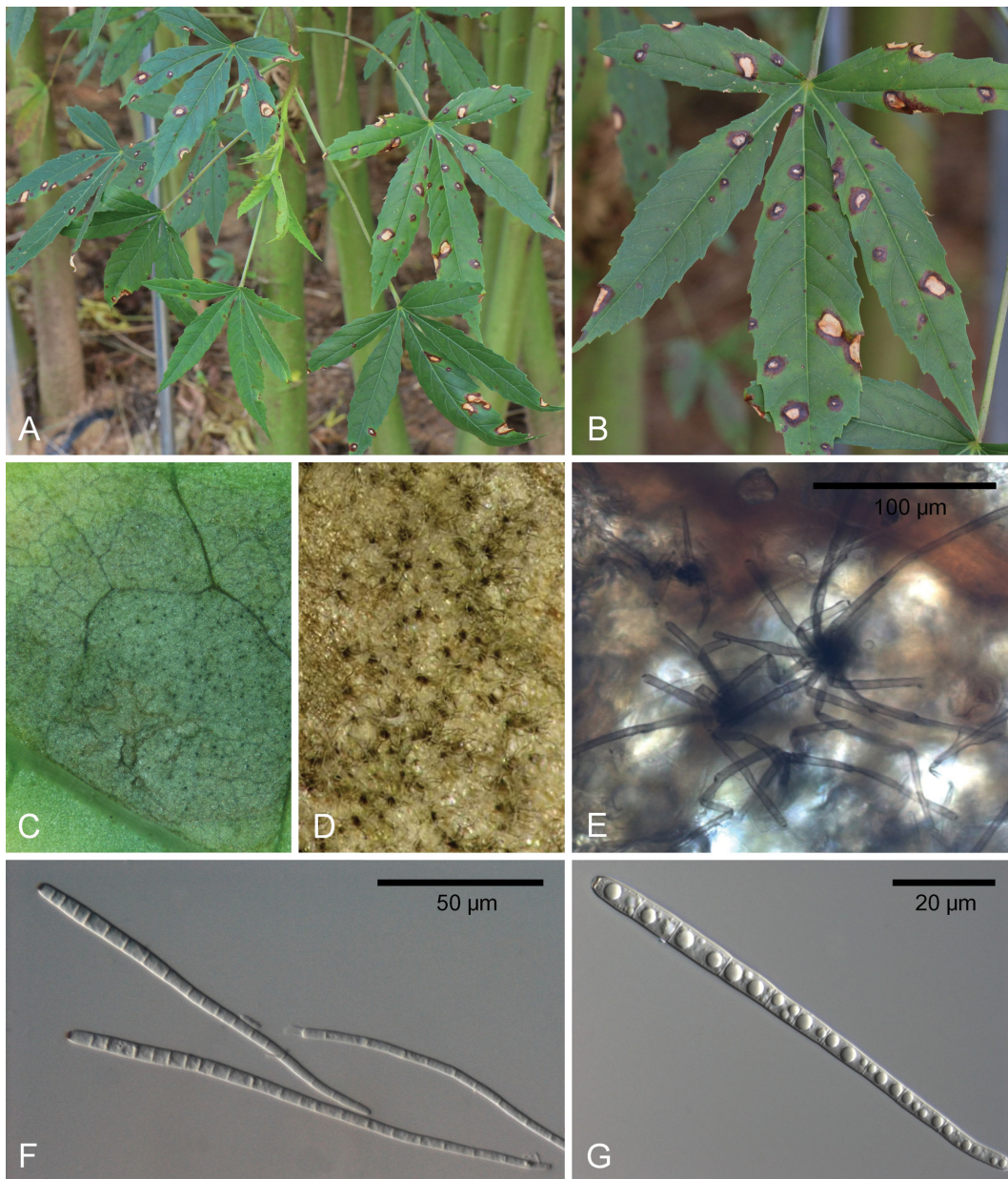


Fig. 1. Leaf spots caused by *Cercospora malayensis* on kenaf. A, Leaves were infected, decreasing vigor of the plants; B, Circular to irregular lesions with purplish margin; C, D, Close-up view of a leaf spot, showing grayish patches on the lesion due to heavy fructification; E, Conidiophores; F, G, Conidia (scale bars: F = 50 µm, G = 20 µm).

only anthracnose caused by *Colletotrichum* sp. [8] and sooty mould disease caused by *Leptoxylum kurandae* [9] have been described in association with kenaf.

In September 2013, hundreds of kenaf plants ‘Jangdae’ showing symptoms of leaf spots with approximately 50% incidence were found in experimental plots in Iksan, Korea. In September 2014, the same symptoms were also found on kenaf ‘Jangdae’ in a farmer’s field in Namwon, Korea. Initially, symptoms on leaf surfaces were circular to irregular, typically vein-limited, and reached 10 mm in diameter. The spots were initially uniformly brown to reddish brown, turning pale brown with purplish margins. A cercosporoid

fungus was consistently found to be associated with disease symptoms (Fig. 1A and 1B). Because *Cercospora* leaf spot disease on kenaf has not been previously recorded, we identified and characterized the causal agent based on morphological characteristics and molecular analyses.

Morphological characteristics of *Cercospora malayensis*.

To examine morphological characteristics, fungal structures from fresh samples were mounted on a glass slide with a drop of water and examined using bright-field and differential interference contrast light microscopy with an Olympus BX51 microscope (Tokyo, Japan) for measurements and

Zeiss AX10 microscope equipped with an AxioCam MRc5 (Carl Zeiss, Göttingen, Germany) for imaging. Thirty measurements were taken at 100× and 1,000× magnification for each sample.

A voucher specimen was submitted to the Korea University Herbarium (KUS) under accession number KUS-F27687. Pathogenic agents were collected from the leaves naturally infected with *Cercospora*. To obtain a pure isolate, conidia were collected from infected leaf tissues using sterile forceps under a dissecting microscope and placed on a drop of sterile water on a glass slide. The conidial suspension was streaked onto 2% water agar plates supplemented with 100 mg/L of streptomycin sulfate and incubated at 25°C for 4 days. Suitable germinated conidia were transferred to potato dextrose agar plates using a sterile needle under a dissecting microscope. These single-spore isolates were used to evaluate colony characteristics and colors. An isolate from KUS-F27687 was deposited with the Korean Agricultural Culture Collection, Rural Development Administration, Wanju, Korea (accession No. KACC47655).

After 7 days incubation at 25°C on potato dextrose agar in the dark, raised fungal colonies had formed and showed moderate aerial mycelium with smooth and erose or dentate margins. Fruitings were amphigenous. Stromata were not present or weakly developed, subimmersed, globular, dark brown, small to moderate, and composed of swollen hyphal cells (Fig. 1C and 1D). Conidiophores emerged through the cuticle, were fasciculate ($n = 3-12$), olivaceous to brown, straight to mildly curved, and geniculate, 100–230 µm long, 3.0–5.0 µm wide, had 1- to 5-septate, and showed conspicuous conidial scars (Fig. 1E). Conidia were hyaline, acicular, subacute to obtuse at the apex, and truncate to obconically truncate at the base, had 3- to 20-septate, were 50–240 × 4.0–5.5 µm, guttulate or aguttulate, and showed thickened, darkened hila at the base (Fig. 1F and 1G). These morphological characteristics were consistent with those of *Cercospora malayensis* F. Stevens & Solheim [3].

Phylogenetic analysis of *Cercospora malayensis*.

Genomic DNA was extracted from 200–400 mg of fungal mycelia (KACC47655) harvested from 7-day potato dextrose agar cultures grown at 25°C using a DNeasy Plant Mini Kit (Qiagen Inc., Hilden, Germany). A sterile blade was used to scrape the mycelia from the surface of the plate. Five nuclear gene regions were targeted for PCR amplification and subsequent sequencing. The primers ITS4 and ITS5 [10] were used to amplify the internal transcribed spacer areas and 5.8S rRNA gene (ITS). Part of the EF-1 α gene using the primers EF728F [11] and EF2Rd [6] and part of the ACT gene was amplified using ACT512F [11] and ACT2Rd primers [6]. The CAL228F and CAL737R primers [11] were used to amplify part of the CAL gene, the primers CylH3F and CylH3R [12] to amplify part of the HIS gene, and 28S nrDNA using primers described previously [6]. All PCR mixtures and conditions were as described by

Hunter *et al.* [13]. The PCR products were separated by electrophoresis at 100 V for 40 min on a 0.8% (wt/vol) agarose gel containing ethidium bromide at 0.1 µg/mL in 1× Tris-acetate-EDTA buffer (0.4 M Tris, 0.05 M NaAc, and 0.01 M EDTA, pH 7.85) and visualized under UV light, followed by purification with a PCR purification kit (Core-one; Core-Bio, Seoul, Korea). The amplicons were sequenced in both directions using the same PCR primers used for the initial amplification according to the manufacturer's recommendations. The reactions were monitored using BigDye Terminator Cycle Sequencing Kits (Applied Biosystems, Foster City, CA, USA) as indicated by the manufacturer and analyzed on an ABI 3130 automated DNA sequencer (Applied Biosystems). The possible identity of the isolates was established by comparing their ITS, EF-1 α , ACT, CAL, and HIS sequences with those in the GenBank database (National Center for Biotechnology Information [NCBI] US National Institute of Health, Bethesda, MD, USA; <http://www.ncbi.nlm.nih.gov/BLAST>). Selected *Cercospora* spp. sequences, including ITS, EF-1 α , ACT, CAL, and HIS, were retrieved from GenBank for phylogenetic analysis. These retrieved sequences were included in the *Cercospora* phylogenetic tree constructed by Crous *et al.* [14]. The obtained sequences were edited and assembled using SeqMan software (Lasergene; DNASTAR, Madison, WI, USA). A neighbor-joining phylogenetic tree was constructed using the maximum composite likelihood method by MEGA6 [15].

The representative resulting 497-bp ITS, 525-bp EF-1 α , 603-bp ACT, 304-bp CAL, and 391-bp HIS sequences obtained from KACC47655 were deposited in GenBank (accession Nos. KR400012, KY082663, KY082664, KY082665, and KY082666, respectively). A BLAST search in GenBank using the ITS sequences revealed that the sequences showed over 99–100% identity with several sequences of *Cercospora* species, including *C. cyperina*, *C. zebrina*, *C. kikuchii*, *C. capsici*, and *C. malayensis*. Importantly, ITS sequences shared 99% identity with an isolate from leaf spot of okra (*Abelmoschus esculentus* (L.) Moench), a plant belongs to the family *Malvaceae*. Furthermore, CAL and HIS sequences showed 99–100% identity with *C. cf. sigesbeckiae* from *Paulownia coreana* and *C. cf. richardiicola* from *Ajuga multiflora*, respectively. Although the ITS, CAL, and HIS sequences of the fungus were the same as several *Cercospora* sequences, the ACT and EF-1 α sequences showed that the isolate was a distinct species. The phylogenetic tree, created using a five combined sequence ITS, EF-1 α , ACT, CAL, and HIS dataset, showed that *C. malayensis* from *H. cannabinus* formed a well-supported clade that was sister to a clade consisting of *Cercospora* spp., as well as revealed a separate clade distinct from other genera such as *Septoria*, with bootstrap values greater than 98% for each clade. Furthermore, the *C. malayensis* pathogen isolated from leaf spot on *H. cannabinus* was closely related to *C. cf. sigesbeckiae* on *Malva verticillata*, *Cercospora* sp. on *Hibiscus sabdariffa*, and *C. fagopyri* on *H. syriacus* (Fig. 2).

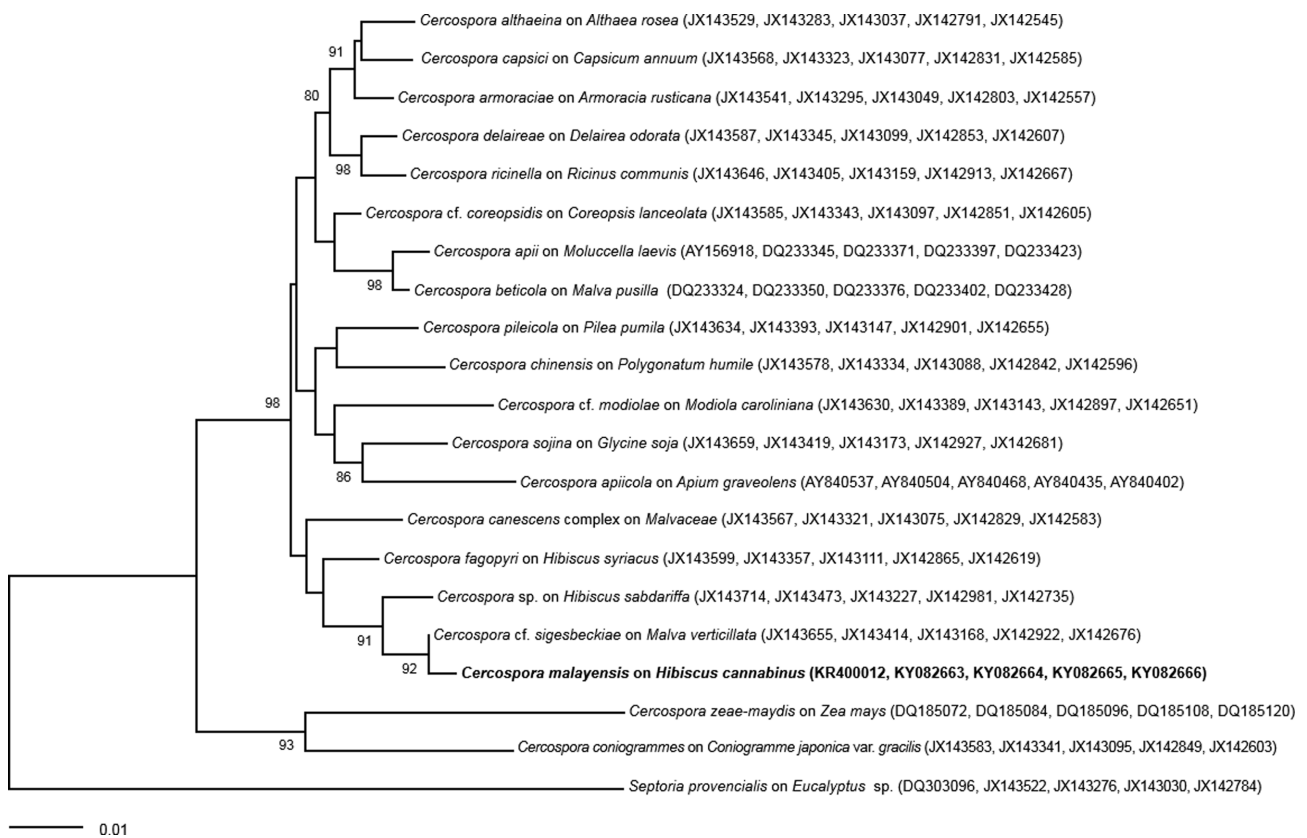


Fig. 2. Neighbor-joining tree of *Cercospora malayensis* based on the multigene dataset (internal transcribed spacer [ITS], translation elongation factor 1-alpha [EF-1 α], actin [ACT], calmodulin [CAL], and histone 3 [HIS]). The numbers above the nodes are the bootstrap values obtained from 1,000 replicates. The GenBank numbers are represented in order of ITS, EF-1 α , ACT, CAL, and HIS, respectively. The isolate obtained in this study is shown in boldface.

Pathogenicity test. Koch's postulates were evaluated to establish the pathogenicity of the isolated fungus. An isolate of KACC47655 was used in the pathogenicity tests. To prepare hyphal suspensions, 3-week-old colonies grown on potato dextrose agar plates at 25°C were homogenized in distilled water. Ten leaves (2 per plant) were inoculated with hyphal suspensions and ten leaves were sprayed with sterile distilled water on the young leaves of 1-year-old healthy kenaf plants 'Jangdae' until the water began to run off. The leaves were individually covered with plastic bags to maintain a relative humidity of 100% for 24 hr and then maintained in a greenhouse at 28 \pm 2°C with a 12-hr photoperiod. Typical symptoms of necrotic spots appeared on the inoculated leaves 10 days after inoculation and were identical to those observed in the field. *Cercospora malayensis* re-isolated from symptomatic leaf tissues was morphologically identical to the original isolate. The pathogenicity test was conducted twice and showed similar results, fulfilling Koch's postulates. No symptoms were observed on control plants.

Identification and discussion. The classification of this *Cercospora* was mainly based on a combination of characteristics such as morphological characteristics, host

specificity, and molecular analyses. Although morphological characteristics are frequently used to identify newly isolated fungi, it is not possible to distinguish *Cercospora* spp. based solely on morphology. Molecular techniques are commonly used to overcome taxonomic problems posed by the limitations of morphological characteristics or in cases where morphological characteristics are in conflict, ambiguous, or missing [16]. Extensive studies of *Cercospora* and related genera in Korea have generated records of numerous species. However, *C. malayensis* has not been detected in Korea [7]. Leaf spot caused by *C. malayensis* was previously been reported on *Abelmoschus*, *Hibiscus*, *Lavatera* (Rosaceae), and *Hyptis* (Lamiaceae) plants. Leaf spot caused by *C. malayensis* is common among *Hibiscus* species, including *H. manihot*, *H. cannabinus*, *H. esculentus*, *H. meeusei*, *H. mutabilis*, *H. rosa-sinensis*, *H. sabdariffa*, *H. syriacus*, and *H. tiliaceus* [7]. However, these reports were mainly reliant on a combination of morphological characteristics and host specificity without an analysis of molecular characteristics of the pathogen. As these sequences of *C. malayensis* were not previously reported in phylogenetic studies, they will provide a platform for future studies of *Cercospora* taxonomy and further analyses of *Cercospora-Hibiscus* associations.

Kenaf leaf spots associated with *C. malayensis* have been

reported in Africa (South Africa, Tanzania, Zambia, and Zimbabwe) and South Asia (Cambodia and China) [7]. However, this is the first report of *C. malayensis* infection of kenaf in Korea. Our observations in the fields of several farmers showed that the presence of *C. malayensis* on kenaf in Korea is a potentially serious threat to this plant.

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