First Report of Leaf Spot Caused by Alternaria tenuissima on Black Chokeberry (Aronia melanocarpa) in Korea

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Abstract In July 2015, diseased leaves of black chokeberry (Aronia melanocarpa) were observed in Danyang and Gochang, Korea. The symptoms appeared as circular or irregular brown leaf spots, from which Alternaria tenuissima was isolated. The isolates were cultured on potato dextrose agar, and their morphological characteristics were observed under a light microscope. The colonies were whitish to ash colored. The pathogenicity test on healthy black chokeberry leaves produced circular brown spots, in line with the original symptoms. Molecular analyses of the ITS, GPD, RPB2, and TEF genes were conducted to confirm the identity of the pathogen. The phylogeny of the multi-gene sequences indicated that the causal agent was A. tenuissima. This study is the first report of A. tenuissima leaf spot on black chokeberry (A. melanocarpa).

Keywords Alternaria leaf spot, Black chokeberry leaf spot, Aronia melanocarpa

Black chokeberry (Aronia melanocarpa), a member of the Rosaceae family, is a cold-hardy, deciduous shrub. It comes into bloom in spring and can grow up to 2~3 m in height. Black chokeberries produce dark, purplish blackberries, and the fruits are mechanically harvested between August and September. They contain large amounts of phenolic flavonoid phytochemicals called anthocyanins [1]. The plants are some pest or disease problems owing to the acidic nature of the fruit's anthocyanins [2]. Known fungal pathogens of black chokeberry include Venturia sp., Cercospora spp., Gymnosporangium spp., Isariopsis sp., Mycosphaerella arbutifoliae, Phymatotrichopsis omnivore, and Podosphaera clandestina [3]. Brown leaf spot caused by Alternaria alternata has recently been reported in Korea [4]. In July 2015, diseased leaves on black chokeberry with circular or irregular brown spots were observed at farmhouses in the Danyang region and subsequently in Gochang, South Korea. The disease continued its progression throughout August. The leaf symptoms observed were circular to irregular brown or light brown spots ranging from 4 to 11 mm (Fig. 1B). The five isolates sampled from the two regions of South Korea, where leaf spot first appeared were analyzed for morphological and molecular characteristics, as well as pathogenicity.

To isolate the pathogen, pieces of infected tissues were sterilized in a 1% sodium-hypochlorite solution for 1 min and washed three times with sterilized water. Then, they were placed on potato dextrose agar (PDA) or water agar (WA) and inoculated at 23°C for 3 days. To obtain a pure isolate, the emerging hyphal tips of the fungal colonies were aseptically transferred onto new PDA plates and incubated at room temperature. For long-term preservation of the fungal isolates, 3~4 mycelium-derived plugs of 5 mm in diameter were taken from the edge of a colony of each isolate on the PDA and placed in a tube containing a sterile 20% glycerol aqueous solution. They were stored in a freezer at −70°C until needed.

For the morphological study, three mycelium plugs of each isolate were placed onto the PDA at three points. The plates were incubated for 5 days at 23°C in the dark. The colony diameters were recorded 5 days after inoculation.
The isolates were observed under a light microscope using different phases. The 5-day-old colonies were 50.7 mm in diameter, creamy to ash in color, and had a woolly texture. The conidiophores were sparsely branched. The conidia, typically observed in long chains of 7~17, were egg- or club-shaped, 22–32 × 8–11 µm in size, and septate with 2–4 longitudinal septa and 0–2 diaphragms (Fig. 2). These morphological characteristics were similar to features of other *Alternaria* species, which were reported in *A. melanocarpa* previously (Table 1) [4-6].

The pathogenicity test was carried out on leaves taken from healthy black chokeberries. They were inoculated with conidial suspension containing $1 \times 10^6$ conidia/mL using the spray method. The control leaves were inoculated with distilled water. All the treated branches with leaves were separately placed in two triangular flasks and incubated at 23°C for 5 days. After 5 days, the brown spot symptom appeared on the inoculated leaves, and the spots gradually expanded over the next 5 incubation days (Fig. 3). The pathogen was successfully re-isolated from all the inoculated leaves and confirmed to be identical with the original fungal isolate (fulfilling Koch’s postulate). The leaf spots by *A. tenuissima* were almost totally brown. No typical symptoms appeared on the control leaves.

Total genomic DNA was extracted from 7-day-old fungal colonies using a HiGene Genomic DNA Prep Kit (BIOFACT, Daejeon, Korea) for molecular identification. Four genetic markers, the glycerol-3-phosphate dehydrogenase (GPD) gene, the internal transcribed spacer (ITS), the RNA polymerase II second largest subunit (RPB2), and the translation

| Table 1. Comparison of mycological characteristics of *Alternaria tenuissima* and *Alternaria* species caused by *Aronia melanocarpa* |
|-----------------|-----------------|-----------------|-----------------|
| Species         | Present study   | *A. tenuissima* | *A. alternata*  | *A. mali*       |
| Colony          | Color           | Creamy to ash gray | Gray, olive green peripheries | Dark olive | Brownish-grey to blackish-grey |
| Conidiophores   | Shape           | Sparsely branched, septate | Branched | - | - |
| Spores          | Shape           | Egg or club shape | Short taper beak | Ellipsoid to ovoid, obclavate to obpyriform | Short and long beak |
| Septa           | Transverse      | 2–4              | 1–6             | 3–6             | 3–8 |
|                 | Longitudinal   | 0–1              | 0–2             | 1–2             | 0–1 |
| Reference       |                 | [6]              | [4]             | [7]             |
elongation factor 1-alpha (TEF) were amplified using genomic DNA as the template [7]. The primer pairs used for the amplification were gpd1 and gpd2 for the GPD gene [8], ITS1F and ITS4 for the ITS region [9, 10], 5F and 7cR for the RPB2 gene [11, 12], and EF1-728F and EF1-986R for the TEF gene [13]. The 50-µL PCR mixture used for the analysis contained 5 µL 10 × Ex Taq buffer, 4 µL of the dNTP Mix, 2 µL of the forward primer (10 pmol), 2 µL of the reverse primer (10 pmol), 2 µL of genomic DNA, 0.4 µL of Ex Taq, and 34.6 µL of sterilized water (TaKaRa, Tokyo, Japan).

Fig. 3. Visual outcome of the pathogenicity test.

Fig. 4. Phylogenetic relationship between Alternaria tenuissima and other Alternaria species, constructed using the maximum likelihood tree analysis, based on the combined GPD, RPB2, and TEF gene sequences. The numbers above/below the branches represent the bootstrap values obtained for 1,000 replicates (values smaller than 80 are not shown). The bar indicates the number of substitutions per position. R, representative strain; T, ex-type strain.
The PCR products were purified by using ExoSAP-IT (Affymetrix, Santa Clara, CA, USA). The PCR products were separated on 1% agarose gels at 130 V for 25 min in 1 x Tris-borate-EDTA (TBE) buffer to confirm whether the PCR reaction had been successful. Sequencing was carried by BIOFACT. The sequences were edited with the help of the SeqMan program within the LASERGENE software package (DNASTAR, Madison, WI, USA) and compared to the NCBI GenBank (http://www.blast.ncbi.nlm.nih.gov/). All sequences were aligned by the MEGA 6.0 software package. The maximum likelihood method with 1,000 bootstrap replicates was used to construct the phylogenetic tree. A sequence of Stemphylium herbarum was used to construct the phylogenetic tree. A sequence of Alternaria tenuissima was aligned by the MEGA 6.0 software package. The GenBank (http://www.blast.ncbi.nlm.nih.gov/). All sequences were separated on 1% agarose gels at 130 V for 25 min in 1 x Tris-borate-EDTA (TBE) buffer to confirm whether the PCR reaction had been successful. Sequencing was carried by BIOFACT. The sequences were edited with the help of the SeqMan program within the LASERGENE software package (DNASTAR, Madison, WI, USA) and compared to the NCBI GenBank (http://www.blast.ncbi.nlm.nih.gov/). All sequences were aligned by the MEGA 6.0 software package. The maximum likelihood method with 1,000 bootstrap replicates was used to construct the phylogenetic tree. A sequence of Stemphylium herbarum was selected as an outgroup (Fig. 4) [5, 13-15]. The fungal pathogen isolated from leaf spot on Aronia melanocarpa was closely related to Alternaria tenuissima. DNA sequences of five strains were registered in the NCBI GenBank (accession numbers: GPD, LC134315~9; ITS, LC134320~4; RPB2, LC134325~9; TEF, LC136861~5). Alternaria tenuissima is a well-known pathogen, which causes leaf spot on a variety of crop plants, including blueberry [16], eggplant [17], and pepper [18] in Asia. The brown leaf spot caused by Alternaria alternata has previously been reported on Aronia melanocarpa [4], and the present study describes Alternaria tenuissima as another leaf spot pathogen on black chokeberry. In conclusion, this is the first report about leaf spot on black chokeberry caused by Alternaria tenuissima.

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