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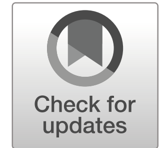

Cercospora brachiata on slender amaranth (*Amaranthus viridis*) in Brazil

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Abstract

Cercospora leaf spot of slender amaranth *Amaranthus viridis,* caused by *Cercospora brachiata,* is reported for the first time in Brazil. The identity of the etiological agent was confirmed by a combination of morphological and molecular information. Koch's postulates were fulfilled with a selected isolate of this fungus obtained at Monte Carmelo, state of Minas Gerais, Brazil.

Keywords Fungal pathogen · Ocurrence · Amaranthaceae · Leaf spots

Slender amaranth (*Amaranthus viridis* - Amaranthaceae) is a widespread herbaceous species, regarded as having originated from the Caribbean. It is presently found as a weed in tropical and subtropical regions throughout the world being associated with more than 50 crops (Francischini et al. 2014). It is also broadly found as a weed in agricultural areas of Brazil (Maluf 1999; Lorenzi and Matos 2002). Slender amaranth is known to be highly adaptable for a range of environmental conditions and to be a prolific seed producer (Francischini et al. 2014). Controlling *A. viridis*, especially in cotton growing areas, is reputed to be difficult because of the lack of registered herbicides available for its control and because of the progressive occurrence of biotypes resistant to acetolactate synthase inhibiting herbicides (Francischini et al. 2014). Exploratory surveys for fungal pathogens with potential for use as biocontrol agents of *A.viridis* were conducted in the municipality of Monte Carmelo (state of Minas Gerais, Brazil). Slender amaranth individuals were found in a ruderal area bearing abundant leaf spots. Samples were collected, dried in a plant press, and a representative specimen was selected and deposited in the herbarium at the Universidade Federal de Viçosa under the Accession number VIC 47138.

Samples were examined under a dissecting microscope Nikon Eclipse E 100 and a dematiaceous fungus was consistently found in association with the leaf spots. Leaf spots were amphigenous, circular or irregular, 2–5 mm diam., coalescent, necrotic, light brown, with distinct dark brown margin, sometimes surrounded by a chlorotic area. Structures of this fungus were scraped from the infected leaves and mounted in lactophenol. Morphology was observed and fungal structures were measured and photographed with a light microscope Olympus BX 53, fitted with an Olympus EVOLT E330 digital camera. Conidia were taken from sporulating colonies and transferred to potato dextrose-agar (PDA) plates. Pure cultures were obtained and deposited in the culture collection of the Universidade Federal de Viçosa under the accession number COAD 2593.

The fungus had the following morphology: Stromata absent to very poorly-developed, sub-epidermal, pale brown. Internal hyphae 3–4 μm wide, branched, septate, pale brown. Conidiophores reduced to conidiogenous cells, monoblastic or polyblastic (up to 5 loci), loosely fasciculate (3–17), unbranched, straight to flexuous, cylindrical to slightly attenuated towards a truncate tip, 42–120 × 4–5.5 μm, geniculate, light brown, paler at the apex, thin-walled, smooth, conidiogenous cells proliferating sympodially. Conidiogenous loci conspicuous, thickened and darkened, 2.0–2.6 μm. Conidia solitary, straight to curved, fusiform, 40–150 × 2.5–4.5 μm, apex acute to subacute, obconically truncate to truncate at the base, 1–8-septate, hyaline, thin-walled, smooth; hilum thickened and darkened, 2.0–2.3 μm.

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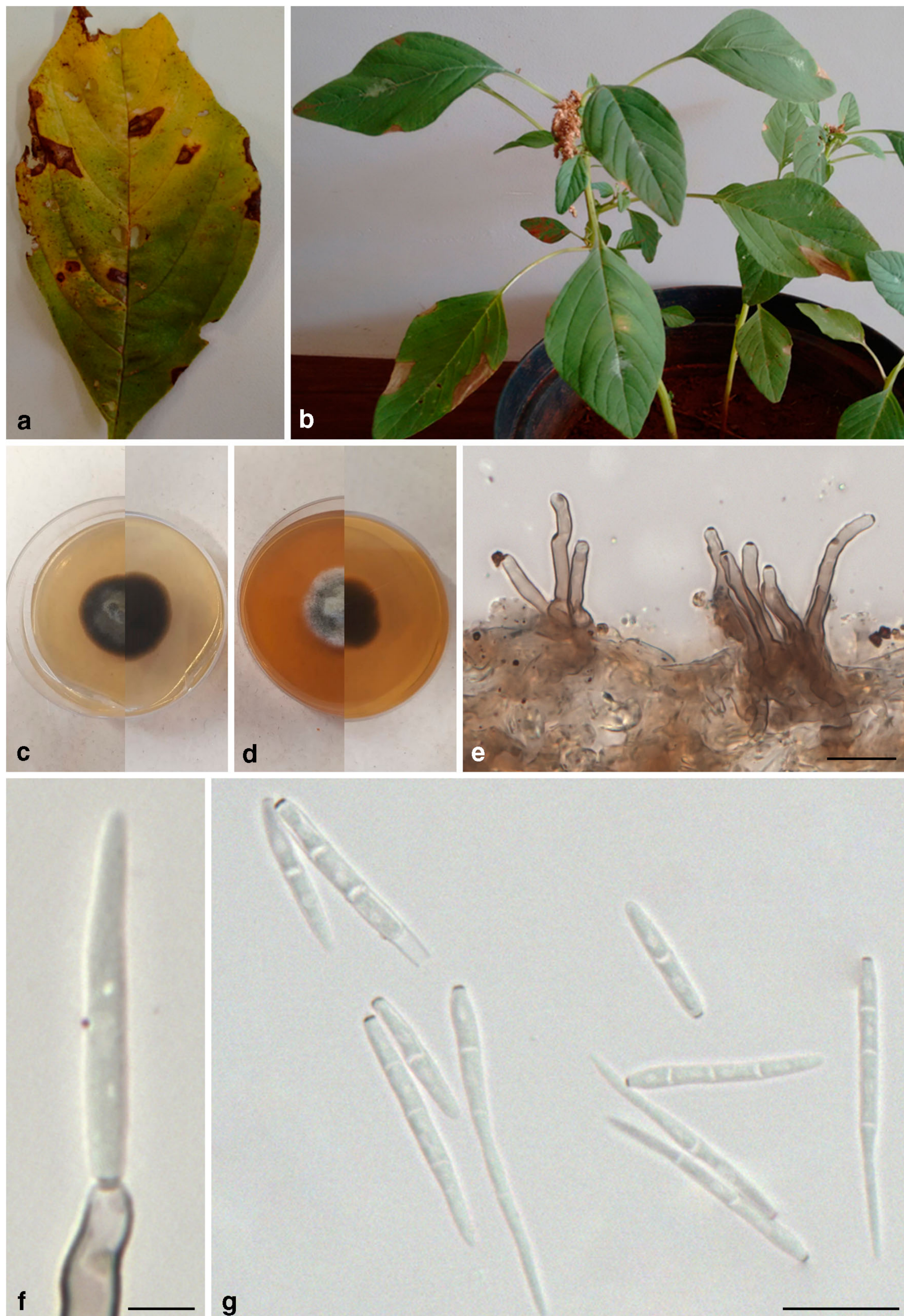

Fig. 1 *Cercospora brachiata* on *Amaranthus viridis* **a** Leaf spots on an *Amaranthus viridis* leaf collected in the field. **b** Numerous leaf spots on *A. viridis*15 days after inoculation of adaxial surface disks taken from 15-day-old *C. brachiata* cultures grown on potato dextrose-agar (PDA). **c** Colony on PDA after 7d (surface/reverse). **d** Colony on MEA after 7d (surface/reverse). **e** Loosely fasciculate conidiophores. **f** Conidium attached to conidiogenous cell. **g** Conidia (note thickened and darkened hila). Scale bars: E and G = 20 μm; F = 5 μm

In culture: Slow-growing (22–25 mm diam. After 7 days), aerial mycelium sparse, dark to light olive green to dark gray with gray center, reverse cracked, not sporulating. This combination of features was recognized as typical of *Cercospora brachiata* Ellis & Everh., as described in Chupp (1954), which is a common pathogen of *A. viridis* (Braun and Urtiaga 2012; Urtiaga and Braun 2013). COAD 2593 was grown in potato dextrose-agar (PDA) plates and DNA was extracted from this isolate as following: for DNA extraction the mycelial mass of the fungus was collected and filtered using a filter paper. Then, the mycelium was macerated in the presence of liquid nitrogen and placed in 1.5 mL tubes. 500 μL extraction buffer (200 mmol Tris HCl pH 8.5, 250 mmolNaCl, 25 mmol EDTA, 0.5% SDS) was added and shaken by hand. Subsequently, 350 μL phenol and 150 μL chloroform were added and stirring was continued. The material was centrifuged at 11.300 rpm at room temperature for 30 min. After centrifugation 700 μL of the upper aqueous phase was transferred to a 1.5 mL tube in which 10 μL of 10 mg/mL RNase was added and the material was incubated for 30 min at 37 °C. 700 μL chloroform was added and stirred, centrifuged for 1 min and all the supernatant was discarded. Following centrifuging the sample at 11,800 rpm for 10 min, the upper aqueous phase was transferred to another 1.5 mL tube. The DNA was precipitated with 700 μL ice cold isopropanol and stirred. Centrifuged for 1 min and discarded all the supernatant. 200 μL 70% ethanol was added and centrifuged at 11,800 rpm for 1 min. The ethanol was discarded, leaving only the DNA the pellet. After repeating the procedure once more, the precipitate was resuspended in 50 μL TE. The samples were then quantified in NanoDrop® spectrophotometer (ND-1000) (Thermo Scientific, Asheville, NC, USA). Amplification reactions were performed in a final volume of 12.5 μL containing 1.5 mM $MgCl_2$; 0.5 mM dATP, dCTP, dGTP and dTTP; 2.5 μM primer; 45 ng of genomic DNA; 2.5 units of Taq DNA polymerase and 1X PCR buffer. The DNA fragment corresponding to the alpha 1-elongation factor (TEF-1α) was amplified with the EF1-728F primers: CATCGAGAAGTTCGAGAAGG (forward) and EF1-986R: TACTTGAAGGAACCCTTACC (reverse); and the actin gene (act) with the primers ACT-512F: ATGTGCAA GGCCGGTTTCGC and ACT-783R: TACGAGTCCTTCTG GCCCAT (Carbone and Kohn 1999). PCR reactions were performed in an Amplitherm Thermal Cyclers thermal cycler under the following conditions: 94 °C for 5 min, followed by 40 cycles of 30 s at 94 °C, 30 s at 52 °C and 30 s at 72 °C, and a final step for extension of 7 min at 72 °C. The amplified fragments were separated on agarose gel (1%) in 1X TBE buffer (1 M Tris base, Boric Acid and 500 mM EDTA) using fluorescent dye for Blue Green LoadingDye I DNA (LGC Biotechnology) at 1:10 concentration. To estimate the size of the fragments, a 100 base pair marker was used. The gels were subjected to ultraviolet light in a L-PIX (Loccus Biotechnology) gel photodocumentation system for visualization and analysis. PCR products were sequenced on an ABI-Prism 3500 Genetic Analyzer (Applied Biosystems) using 60 ng of template DNA and 4.5 pmol of the primers used in the amplification by ACTGene Análises Moleculares, Alvorada, RS. The sequences were deposited in GenBank under the accession numbers MK118087 (act) and MK118086 (TEF-1α). These were compared through a BLASTn search with other entries in GenBank, and the closest match were *Cercospora alchemillicola* (KP164575), *C. kikuchii* (KP860260) and *C. physalidis* (JX143146) with 100% nucleotide homology (100% query coverage) for act, and *C. flagellaris* (JX143362) and *C. piaropi* (DQ835101) with 99% nucleotide homology (100% query coverage) for TEF-1α. No sequences of *C. brachiata* were available for comparison in GenBank. The present concept of *C. brachiata* is preliminary, but as several other species of the *C. apii* complex (Crous and Braun 2003), even molecular data have been missing for *C. brachiata* in present report was given an important contribution with their protein gene sequences. Hitherto it is absolutely not possible to resolve many *Cercospora* species even with multigene analyses (Groenewald et al. 2013). Since there is no barcode available for *Cercospora* species, the species identification in this report is the currently best possible one.

In order to demonstrate pathogenicity, three healthy leaves on each of five *A. viridis* plants were inoculated by using surface disks (5 mm in diameter) taken from 15-day-old *Cercospora brachiata* cultures grown on potato dextrose-agar (PDA), being inoculated 5 disks per leaf), followed by their transfer of all plants to a humid chamber for 48 h and then to a greenhouse bench. Control treatment consisted of plants inoculated with agar discs. Typical symptoms appeared after 15 days of inoculation and sporulation appeared over necrotic tissues after 20 days on adaxial surface leaves (Fig. 1).

Cercospora brachiata is already known for *Amaranthus* species (Braun et al. 2015; Crous and Braun 2003), however this is the first record of *C. brachiata* in Brazil on *A. viridis*.

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