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Cercospora agavicola – a new foliar pathogen of *Agave tequilana* var. *azul* from Mexico

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Abstract — *Cercospora agavicola* is newly described and illustrated on *Agave tequilana* var. *azul* from Mexico. Koch's postulates were successfully completed, confirming *C. agavicola* as the causal organism of Agave leaf spot and necrosis. *C. agavicola* is compared to cercosporoid species based on sequence data derived from the ITS nrDNA region, and part of the elongation factor 1-α, actin, calmodulin and histone H3 genes. Its taxonomic position, generic affinity and relatedness to allied species are discussed on the basis of morphological and molecular data.

Key words — mitosporic fungi, *Mycosphaerella* anamorphs, North America

Introduction

Agave cantula Roxb., *A. cupreata* Trel. & A. Berger, *A. longisepala* Tod., *A. palmaris* Trel., *A. pesmulae* Trel., *A. pseudotequilana* Trel., *A. subtilis* Trel. and *A. tequilana* F.A.C. Weber var. *azul* are cultivated in Mexico in the states of Guanajuato, Jalisco, Michoacán, Nayarit and Tamaulipas (Anonymous 2002). The latter species is the most economically significant cultivated agave because of its importance for 'tequila' extraction (Villalvazo 1986, Granados 1993). Penjamo in the state of Guanajuato is one of the regions in which agave was initially planted as crop. In January 2003, collaborators from the Agricultural Department of the State of Guanajuato observed a new disease on *Agave tequilana*. A fungus associated with symptomatic tissue was identified at the Instituto de Fitosanidad, Colegio de Postgraduados (CP) as an undescribed member

of the genus *Cercospora* Fresen. Material was sent to U. Braun in Germany for confirmation of the identification. Additionally, molecular analyses of the ITS nrDNA region, and part of the elongation factor 1-α, actin gene, calmodulin and histone H3 genes were carried out at CP and at the Centraalbureau voor Schimmelcultures in Utrecht, the Netherlands.

Materials and methods

Isolates

Isolates were obtained from symptomatic leaf pieces by placing disinfested necrotic tissue fragments in moisture chambers to enhance sporulation. Monoconidial cultures were subsequently established on water-agar (WA) (20 g agar / 1 L distilled water). Colonies were induced to sporulate on four different media: WA, agave-agar (AA) (40 g of agave leaf fragments boiled for 10 min and then blended with 20 g agar / 1 L distilled water), oatmeal-agar (OA) (15 g of oatmeal, 20 g agar / 1 L distilled water), and potato-dextrose agar (PDA) (200 g potatoes, 20 g dextrose, 20 g agar / 1 L distilled water). Dishes of all media were point inoculated and incubated for 3 wk at ± 24°C under continuous near-ultraviolet light, and inspected for sporulation at 3 d intervals. Morphological observations *in vitro* were based on sporulating cultures on AA. Thirty observations were made of each structure, with extremes given in parentheses.

DNA isolation, amplification and phylogenetic analysis

The protocol of Lee & Taylor (1990) was used to isolate genomic DNA from fungal mycelium of a monoconidial culture grown on MEA in Petri dishes. The primers ITS1 and ITS4 (White et al. 1990) were used to amplify part (ITS) of the nuclear rRNA operon spanning the 3' end of the 18S rRNA gene, the first internal transcribed spacer (ITS1), the 5.8S rRNA gene, the second ITS region and the 5' end of the 28S rRNA gene. To obtain additional sequence information, four other loci were sequenced. Part of the elongation factor 1-α gene (EF) was amplified with primers EF1-728F and EF1-986R, part of the actin gene (ACT) with primers ACT-512F and ACT-783R and part of the calmodulin gene (CAL) with primers CAL-228F and CAL-737R (Carbone & Kohn 1999). Part of the histone H3 gene (HIS) was amplified with primers H3-1a and H3-1b (Glass & Donaldson 1995). The PCR conditions, sequence alignment and subsequent phylogenetic analysis followed the methods of Crous et al. (2004a). To determine the taxonomic position of the fungus, the sequences were added to a subset of the alignment (TreeBASE matrix M2038) of Crous et al. (2004b). Sequence data were deposited in GenBank.

Koch's postulates

Pathogenicity tests were conducted on six-mo-old healthy seedlings of *A. tequilana* var. *azul* that were produced in the region of Tequila, Jalisco. The experiment consisted of six plants, of which 10 leaves per plant were inoculated (five wounded via a sterile toothpick, five unwounded). Agar disks (0.5 cm diam) colonized by the fungus were placed at the base of each leaf. All plants were incubated in a moist chamber (± 90% relative humidity) for 24 h, and subsequently transferred to a glasshouse (± 28°C,

normal daylight) until symptoms appeared. Controls consisted of two plants that were inoculated in a similar fashion with uncolonized agar plugs. Reisolations were made onto PDA to confirm Koch's postulates.

Results

DNA Phylogeny

Approximately 500, 315, 230, 320 and 410 bases were determined for ITS, EF, ACT, CAL, and HIS, respectively (GenBank accession numbers AY647237, AY966897, AY966898, AY966899, AY966900, respectively). A partition homogeneity test using the sequence data showed that all loci could be combined ($p = 0.073$) into a single analysis.

The data matrix contained 17 taxa (including the two outgroups) and 1702 characters including alignment gaps. Of these characters, 603 were parsimony-informative, 124 were variable and parsimony-uninformative, and 975 were constant. Neighbor-joining analysis using the three substitution models on the sequence data yielded trees with similar topology and bootstrap values. Parsimony analysis of the alignment yielded three most parsimonious trees (TL = 1230 steps; CI = 0.921; RI = 0.960; RC = 0.885), one of which is shown in Fig. 1. The neighbor-joining (using the uncorrected p, Kimura 2-parameter and F84 substitution models) and parsimony analyses provided trees with the same topology (data not shown). All of the *Cercospora* isolates formed a well-supported group (100 % bootstrap support) with the sequences of *Cercospora apii* and *C. beticola* clustering together with a bootstrap support value of 100 %. The isolate from *Agave tequilana* var. *azul* formed a distinct branch in the *Cercospora* clade, separate from the other *Cercospora* species in the tree.

Taxonomy

Cercospora agavicola Ayala-Escobar, **sp. nov.** MB500188 **Figs 2–14**

Differt a C. fourcroyae conidiophoris 20–100 µm longis, conidiis cylindraceis, ad apicem interdum distincte inflatis.

Holotype here designated: on *Agave tequilana* var. *azul* (*Agavaceae*), Mexico, State of Guanajuato, Penjamo, Jan. 2003, V. Ayala-Escobar and Ma. de Jesús Yáñez-Morales (CHAPA # 166), culture ex-type CBS 117292, CPC 11774.

Isotype: HAL 1839 F.

In vivo: Forming irregular necroses of variable size on leaves, dingy gray. Colonies punctiform to pustulate, scattered to dense, dark to blackish brown, later gray-brown to grayish white by abundant conidial formation, scattered to confluent, dense. Mycelium internal. Hyphae sparingly branched, 1.5–5 µm wide, septate, subhyaline to brownish, smooth, hyphae solitary or forming lax to dense ropes or planate aggregations of swollen cells up to 15 µm diam. Stromata well-developed, immersed, often somewhat erumpent, 20–150 µm diam or confluent and larger, composed of swollen hyphal cells,

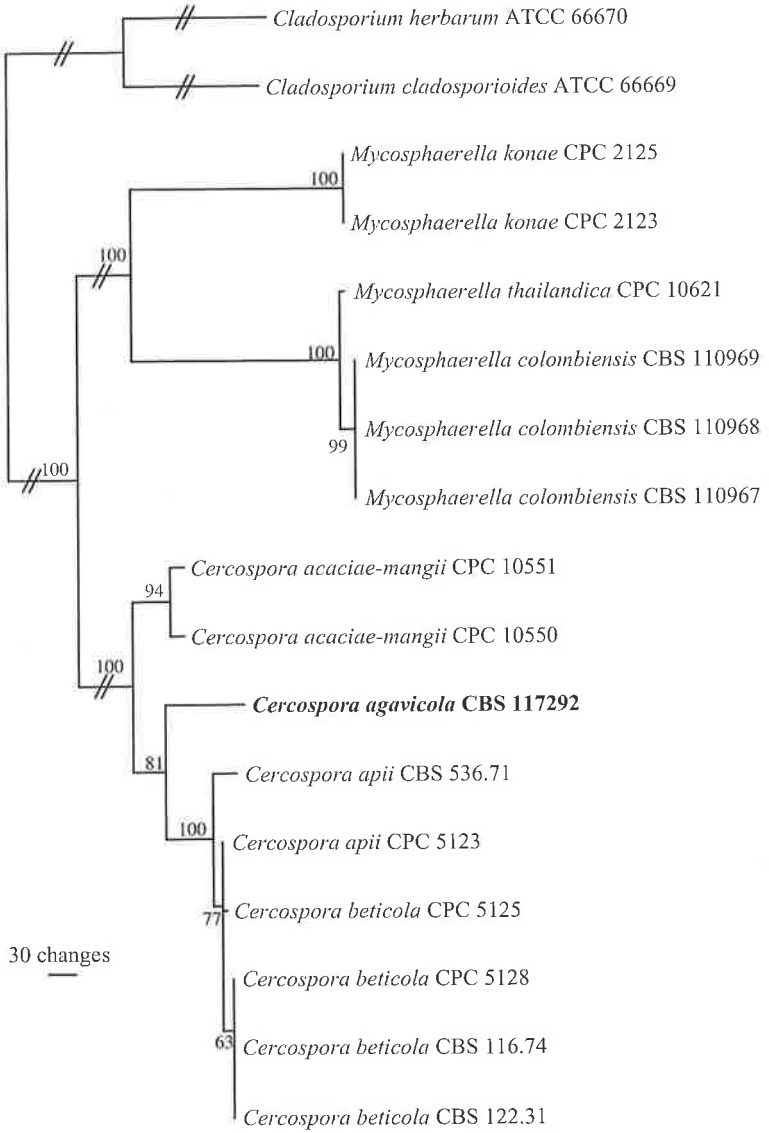
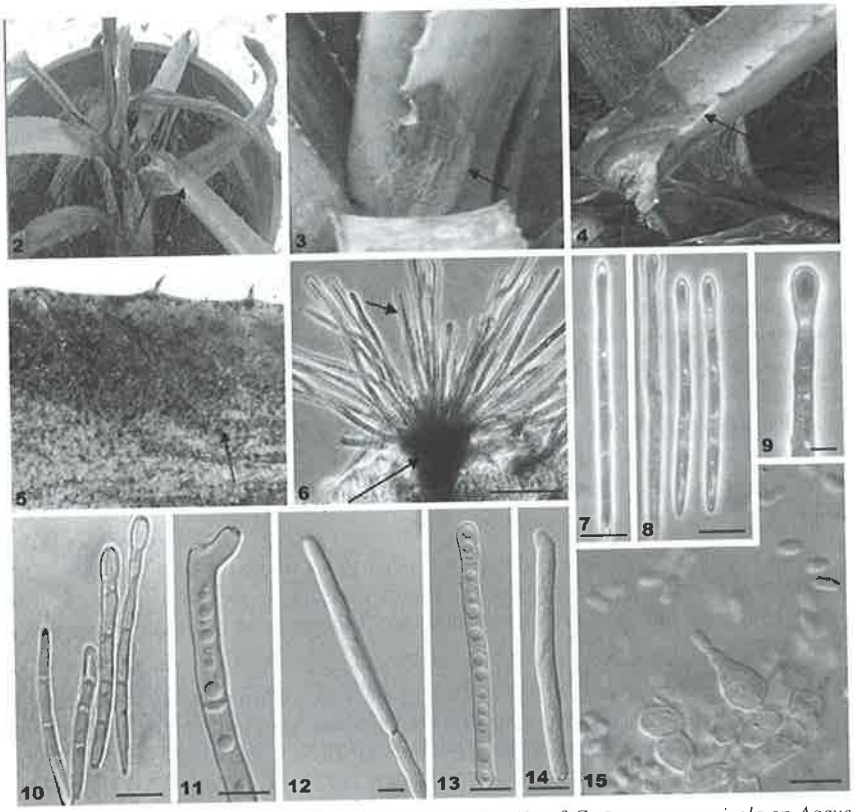


Fig. 1. One of three most parsimonious trees obtained from a heuristic search with 100 random taxon additions of the combined sequence alignment. The scale bar shows 30 changes and bootstrap replicate values from 1000 replicates are shown at the nodes. Strict consensus branches are thickened. The tree was rooted to two *Cladosporium* species. GenBank accession numbers of the sequences from TreeBASE matrix M2038 can be found in Crous et al. (2004b).



Figs 2–14. Disease symptoms and morphological structures of *Cercospora agavicola* on *Agave tequilana* var. *azul*. **2.** Leaf and stem necrosis. **3.** Basal necrosis of a leaf, 3 wk after inoculation. **4.** Basal leaf necrosis 3 mo after inoculation. **5.** Multiple dark stromata covering the basal leaf area. **6.** Fasciculate conidiophores arising from a basal stroma. **7–10.** Hyaline conidia with swollen apical cells *in vivo*. **11.** Brown conidiogenous cells with loci. **12.** Conidiogenous cell giving rise to a conidium. **13–14.** Conidia *in vitro*. **15.** Spermatogenous cells with spermatia *in vitro*. Scale bars: 5 = 100 μm, 6–7, 9 = 15 μm, 10–14 = 10 μm, 8 = 5 μm.

3–8 μm wide, brown. Conidiophores in small to moderately large, lax fascicles, erect, divergent, subcylindrical-filiform to flexuous-sinuous, barely geniculate, unbranched, occasionally with constrictions and swellings, 20–100 × 3–6(–7) μm (up to 200 μm in length and strongly branched under high humidity in moisture chambers), pluriseptate, at first subhyaline, later pale olivaceous, olivaceous-brown or pale brown, often paler towards the apex, thin-walled, smooth; conidiogenous cells integrated, terminal, 10–40 μm long; with a single or up to three conidiogenous loci, terminal and lateral, 2–3 μm diam, thickened and darkened. Conidia solitary, subcylindrical, (35–)40–100(–120) × 3–5.5 μm, (0–)3–8-septate, hyaline, thin-walled, smooth, apex obtuse, sometimes with distinctly swollen tips, base truncate to slightly obconically truncate, 1.5–2.5 μm wide, hila somewhat thickened and darkened.

***In vitro*:** Colonies on all culture media green to grayish, reaching 2 cm diam within 3 wk. Sporulation only observed on AA medium after 21 d. Conidiophores dense, 231–960 μm long, pluriseptate, with a single terminal conidiogenous locus or up to five slightly protruding lateral loci. Conidia straight, cylindrical, 25–120 μm long, 2–8-septate, hyaline, apical cells often swollen, subglobose to clavate. Spermatogonia formed on OA, exuding masses of hyaline, rod-shaped spermatia, 3–6 × 1–2 μm.

Koch's postulates

Disease symptoms were observed after 10 d, and consisted of pale to dark brown spots. Lesions were 3–4 cm long and 2 cm wide after 21 d, while the whole leaf (15 cm long) turned necrotic after 3 mo, also extending to the stem (Figs 2–4). *C. agavicola* was successfully re-isolated from the margins of symptomatic leaves within 2–3 wk after inoculation. No disease developed on unwounded leaves. Control plants remained healthy. Inoculated, wounded leaves developed stromata (Fig. 5), which, when placed in moist chambers, produced conidiophores within 5 d and conidia after 8 d.

Discussion

Cercospora leaf spot and necrosis is a new disease of *Agave* in Mexico and, as such, it is not known if this fungus also occurs in other states where the crop is grown. The common occurrence of spermatogonia on host material, as well as *in vitro* on agar media, suggests that it is very likely that this pathogen also has a *Mycosphaerella* teleomorph.

The generic affinity of this fungus to *Cercospora* was confirmed by BLASTn results obtained with the sequence data. The ITS sequence was highly similar to sequences of '*Cercospora sorghi* var. *maydis* Ellis & Everh.' (AF 297229, Goodwin et al. 2001; 99.8 % similarity), *C. nicotianae* Ellis & Everh. (AF 297230, Goodwin et al. 2001; with the same similarity as pervious taxon) and *C. asparagi* Sacc. (AF 297229, Goodwin et al. 2001; 2 bp different). '*Cercospora sorghi* var. *maydis*' is quite distinct from and not conspecific with *C. sorghi s. str.* (Chupp 1953, Goodwin et al. 2001) and, together with *C. nicotianae* and *C. asparagi*, belongs to the *C. apii* complex (Crous & Braun 2003). *C. agavicola* is genetically close to *C. apii s. lat.* based on ITS sequence data, but morphologically quite distinct by having very large stromata and consistently cylindrical conidia, often with swollen tips. From the phylogenetic tree obtained using the combined sequence data of five genomic loci, it is clear that *C. agavicola* is also genetically distinct from *C. apii s. lat.* *C. floricola* Heald & F.A. Wolf on *Yucca* spp. and *C. fourcroyae* Obreg.-Bot. on *Alstroemeria* sp. (*Alstroemeriaceae*) and *Fucraea* (*Fourcroya*) *gigantea* are two *Cercospora* species on hosts belonging to the *Agavaceae*. The latter species is morphologically close to *C. agavicola*, but differs in having very long conidiophores (up to 350 μm *in vivo*) and cylindrical-obclavate conidia without any apical swellings (Chupp 1953). *C. floricola* is characterized by its uniformly short conidiophores, 10–35 μm long, and cylindrical-obclavate conidia, 15–60(–70) × 3–6 μm, with 1–3(–5) septa, and non-swollen tips (type material of *C. floricola* examined: BPI 436450). *C. haemanthi* Kalchb. on *Haemanthus* and *Scadoxus* species of the allied family *Amaryllidaceae* is morphologically also close to the new species, but

distinguished by having larger conidiogenous loci, 3–4 μm diam, obclavate-cylindrical to subacicular wider conidia, (20–)40–120(–220) × 4–8 μm, without any swollen tips (type material of *C. haemanthi* examined: B).

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