CHAPTER 5

FIRST REPORT OF *CLADOSPORIUM MUSAE* ON BANANA IN SOUTH AFRICA

AUSTRALASIAN PLANT PATHOLOGY: SUBMITTED

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ABSTRACT

An unknown speckle disease was recently observed on Cavendish banana leaves in Levubu, the most northern of the five banana growing regions of South Africa. Morphological examination of infected material and single conidial isolates of the causal organism revealed that it was *Cladosporium musae*. Isolates of the fungus were subjected to pathogenicity testing and sequencing of the ITS region (ITS-1 and ITS-2) and the 5.8S gene of the rDNA operon, and compared with an authentic strain of *C. musae*. These results verified the identity of the fungus as *C. musae*, and constitute the first confirmed report of Cladosporium speckle on banana leaves in South Africa.

INTRODUCTION

Various fungi are known to cause speckle symptoms on banana (*Musa* spp.) leaves, e.g. *Acrodontium simplex* Mangenor & de Hoog (leaf speckle), *Cladosporium musae* E.W. Mason (Cladosporium speckle), *Mycosphaerella musae* (Spreg.) Syd. & P. Syd (Mycosphaerella speckle), *Veronaea musae* Stahel & M.B. Ellis and *Periconiella musae* Stahel & M.B. Ellis (tropical speckle) (Jones 2000). In South Africa, speckle caused by *M. musae* was reported by Brodrick (1973). However, identification was based on symptomology only and no attempt was made to verify the identity of the causal organism. To compound matters further, Brodrick (1973) was misquoted as ascribing the disease to infection by *C. musae* in a subsequent publication (Gorter 1977), which in turn served as reference for the presence of *C. musae* in South Africa (CMI 1988).

In 2000, symptoms resembling those of Cladosporium speckle were observed on Cavendish banana plants in the Levubu area, the most northern of the five banana growing regions in South Africa (surrounding 23.1° S 30.3° E). Symptoms initially appeared as pale-green flecks on the leaf surface that elongated into brown streaks of about 2 cm and longer. With age these lesions characteristically turned orange in colour, with sparse grey-green blotching becoming evident on the adaxial surface of older leaves. Eventually the orange lesions became dark brown, coalesced and occupied large areas of the photosynthetic leaf surface. Invariably associated with the leaf blade symptoms were dark, sunken, water-soaked lesions, 10–20 mm in diameter, along the midrib of the leaves. Severe infection of older leaves occasionally resulted in death of the entire leaf. This report describes the isolation and identification of the causal organism, and confirmation of its pathogenicity.

MATERIALS AND METHODS

Isolation and conventional identification

Thirty-three samples of Cavendish banana leaves displaying speckle symptoms were randomly collected from eight plantations in Levubu. Samples were placed in envelopes and stored at 5 °C until primary isolations were made. A section containing a lesion was excised from each leaf and immersed in 2 % sodium hypochlorite for 30 sec followed by 1 min in 70 % ethanol, and then rinsed twice in sterile distilled water (SDW). Segments (2 mm x 2 mm) were dissected from the lesion margins, and plated on half-strength potato-dextrose agar (½ PDA) (19 g PDA (Merck) + 10 g agar (Biolab, Midrand, Johannesburg) in 1 l deionized water) supplemented with 0.2 g/l Novobiocin to suppress bacterial growth. Plates were incubated for 3–7 d at 25 °C and hyphal tip isolations were plated on ½ PDA. Isolations were also made by inducing sporulation in moist chambers. A leaf section containing a lesion was excised and sprayed with 70 % ethanol until run-off. It was then placed into a 90–mm Petri dish containing a sterile filter paper disc moistened with SDW. After 1–2 d at 20 °C the leaf section was examined for conidiophores under a dissection microscope. Cultures were obtained by touching a small piece of agar to the conidiogenous apparatus and transferring it to ½ PDA supplemented with Novobiocin. Resultant cultures were identified morphologically. Cultures of representative isolates are maintained at the Centraalbureau voor Schimmelcultures (CBS) in Utrecht, The Netherlands.

The morphology of fungal fruiting structures present on banana leaves was studied using both light and electron microscopy. Conidia and conidiophores were collected from sporulating lesions, suspended in lactophenol, and observed under the light microscope. For scanning electron microscopy, fresh leaf lesions were excised and fixed in 3 % glutaraldehyde

for a minimum of 1 hr, followed by three rinses of 15 min each in 0.075 M phosphate buffer. The samples were then dehydrated for 15 min in 50 %, 70 %, 90 % and 3 x 100 % ethanol, respectively. A critical point drying step followed in liquid carbon dioxide, before mounting the sample on a stub and sputtering it with gold.

DNA isolation

DNA was extracted from three South African isolates (CBS 110961, CBS 110962 and CBS 110965), as well as a verified strain of *C. musae* (CBS 161.74), as described by Surridge *et al.* (2003).

Polymerase chain reaction

DNA from each isolate was subjected to an ITS-PCR using primers ITS1 and ITS4 (White *et al.* 1990). The PCR product resulting from this was purified with a "High pure PCR product purification kit" (Roche). DNA sequences were determined using the ABI PRISM ™ Dye Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq® DNA Polymerase (Applied Biosystems, UK).

Sequence analysis

Sequences of the ITS region (ITS-1 and ITS-2) and the 5.8S gene of the rDNA operon were manually aligned with inserted gaps treated as missing data. Ambiguously aligned regions were excluded from the data set before analysis. Phylogenetic analysis was based on parsimony using PAUP 4.0b8 (Phylogenetic Analysis Using Parsimony) (Swofford 2000). Heuristic searches were done with random addition of sequences (1000 replicates), tree bisection-reconnection (TBR), branch swapping and MULPAR effective and MaxTrees set to auto-increase. Phylogenetic signal in the data sets was assessed by evaluating tree length

distributions over 100 randomly generated trees. The consistency (CI) and retention indices were determined for all data sets. Other *Cladosporium* species (*C. cladosporioides* (Fresen.) G.A. de Vries, *C. herbarum* (Pers.) Link and *C. sphaerospermum* Penz.) were included for comparison and resolution purposes (Table 1). Phylogenetic trees were rooted with *Mycocentrospora acerina* (R. Hartig) Deighton as an outgroup to the remaining taxa. Bootstrap analyses were conducted to determine confidence in branching points (1000 replicates) for the most parsimonious trees generated.

Pathogenicity

Pathogenicity of eight isolates (CBS 110958, CBS 110960, CBS 110961, CBS 110962, CBS 110963, CBS 110964, CBS 110965, CBS 110966) was confirmed by inoculating leaves of potted Cavendish banana plants approximately one year old and 1 m tall. Plants were transferred to a greenhouse and maintained at 27 °C for 30 d prior to inoculation to allow them to acclimatise. The adaxial surface of the leaves was lightly abraised with a hypodermic needle to remove a portion of the waxy cuticle. An agar plug punched from the periphery of a two-week-old culture on ½ PDA was placed onto the abraded epidermis and secured with clear 50-mm-wide adhesive tape. Each isolate was inoculated onto two leaves on each of three plants. Symptom development was observed for 90 d.

RESULTS

Morphology

The fungus associated with leaf speckle of banana in Levubu conformed to the description of *C. musae* (David 1988). It produced erect colourless to brown conidiophores, 4–6 µm in diameter and up to 500 µm long that were readily visible under a hand lens (x10). The basal cell of the conidiophore had a conspicuously thickened wall (Fig. 1A). Conidiophores occurred either singly or in groups of four to six. Terminal or intercalary conidiogenous cells were produced on branches (3–4 x 50 µm) at the apex of the conidiophore (Figs. 1A, B). Conidia were borne singly or in chains of up to three. They were 3–5 µm wide and 6–22 µm long, smooth, thin-walled, 0–1-septate, subhyaline, and ellipsoidal or fusiform in shape, with a protuberant scar often visible at each end (Fig. 1C).

On ½PDA, colonies were white at first and then turned olivaceous and sometimes rosy buff in colour. The superficial mycelium comprised thin-walled and hyaline hyphae. When viewed with the electron microscope, superficial constrictions around hyphal septa could be observed. Fructifications in culture corresponded with those observed on infected plant material.

Molecular identification

Parsimony analysis of the ITS-1 and ITS-2 regions and the 5.8S gene of the rDNA operon determined the phylogenetic placement of South African *Cladosporium* isolates from banana leaves in relation to other *Cladosporium* species isolated from different hosts. Alignment by inserting gaps resulted in a total of 551 characters used in the comparison of the different species. Inserted gaps were treated as missing data. A total of 258 constant

characters, 62 parsimony-uninformative characters and 231 parsimony-informative characters were obtained. Heuristic searches on the data generated eight most parsimonious trees. The consensus tree presented in Fig. 2 indicated that the South African *C. musae* isolates were the same as the reference strain obtained from CBS. The clade containing South African isolates of *C. musae* and the reference strain showed differences of one to three base pairs. The high CI and RI values of 0.978 and 0.989, respectively, support the validity of this tree.

Pathogenicity

All isolates inoculated onto banana leaves produced symptoms similar to those observed in the field (Figs 3A, B). Typical orange speckling was observed around the point of inoculation (Fig. 3A). Necrosis occurred at the site of inoculation from where symptoms radiated outwards. Lesion size varied between approximately 20 mm in diameter and, occasionally, entire leaf death.

DISCUSSION

This study is the first to confirm the presence of Cladosporium speckle caused by *C. musae* on banana in South Africa. Siboe (1994) recently transferred *C. musae* to the genus *Periconiella* as *P. sapientumicola* G. Siboe on the basis of its short conidial chains and complex conidiophore branching pattern. Although the present phylogenetic analysis supports the removal of the speckle pathogen from *Cladosporium* s.str., its placement in *Periconiella* remains unclear. The established name, *C. musae*, is therefore retained in this report. Morphological and sequence data of the ITS region of the rDNA operon, indicate that South African isolates are similar to the verified isolate of *C. musae* isolated from Honduras

(CBS 161.74). Symptoms in the field also corresponded with those described by Jones (2000), particularly for *C. musae* on AAA cultivars in East Africa.

Cladosporium musae is regarded as a minor pathogen causing loss of photosynthetic area mainly on mature banana leaves in humid climates (Stover 1972), and is not considered to affect yield and fruit quality significantly. Banana cultivars in the Cavendish group do, however, seem to be relatively susceptible (Frossard 1963). Older leaves develop symptoms first, transferring inoculum to younger ones via aerially dispersed conidia, which germinate under high humidity conditions (Jones 2000; Jones 1994).

Cladosporium musae has been reported from Australasia-Oceania, Asia, the Latin-American Caribbean region, and in Africa as far South as Zimbabwe (Jones 2000). In South Africa, it appears to be confined to the Levubu area as it has not been isolated from any other of the banana growing regions (Chapter 3). The occurrence of *C. musae* in Levubu, which is situated just North of the Tropic of Capricorn, is in accordance with the pathogen's preference for tropical climates (Wardlaw 1961, CMI 1988). While conditions were conducive to disease development and spread during the past growing seasons, Cladosporium speckle remained confined to the Levubu area. The most probable explanation for the presence of *C. musae* in South Africa is that diseased vegetative material may have been introduced from neighbouring countries. As *C. musae* is more adapted to a tropical climate, this pathogen may prove to be climatically contained within this region.

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Table 1: Collection and sequence details of the fungi included in the phylogenetic analysis.

Fungus	Culture number	Location	Date	Collector	Host Cultivar	GenBank accession number
C. musae	CBS 161.74	Honduras	Feb 1974	R.H. Stover	Musa sp.	AY186199
C. musae	CBS 110962	Levubu	17-Mar-00	A. Viljoen	Williams	AY186200
C. musae	CBS 110965	Levubu	24-Jun-00	A. Viljoen	Grand Nain	AY186201
C. musae	CBS 110961	Levubu	16-Mar-00	A. Viljoen	Grand Naine	AY186202
C. cladosporioides	-	-	-	-	-	AF455535
C. cladosporioides	-	-	-	-	-	AF455442
C. cladosporioides	-	-	-	-	-	AF455472
C. cladosporioides	-	-	-	-	-	AF455525
C. cladosporioides	-	-	-	-	-	AF455519
C. herbarum	-	-	-	-	-	AF455517
C. herbarum	-	-	-	-	-	AF455479
C. herbarum	-	-	-	-	-	AF455404
C. sphaerospermum	-	-	-	-	-	AF455481
M. acerina	ATCC 34539	Norway	-	K. Arsvol	Carrot	-

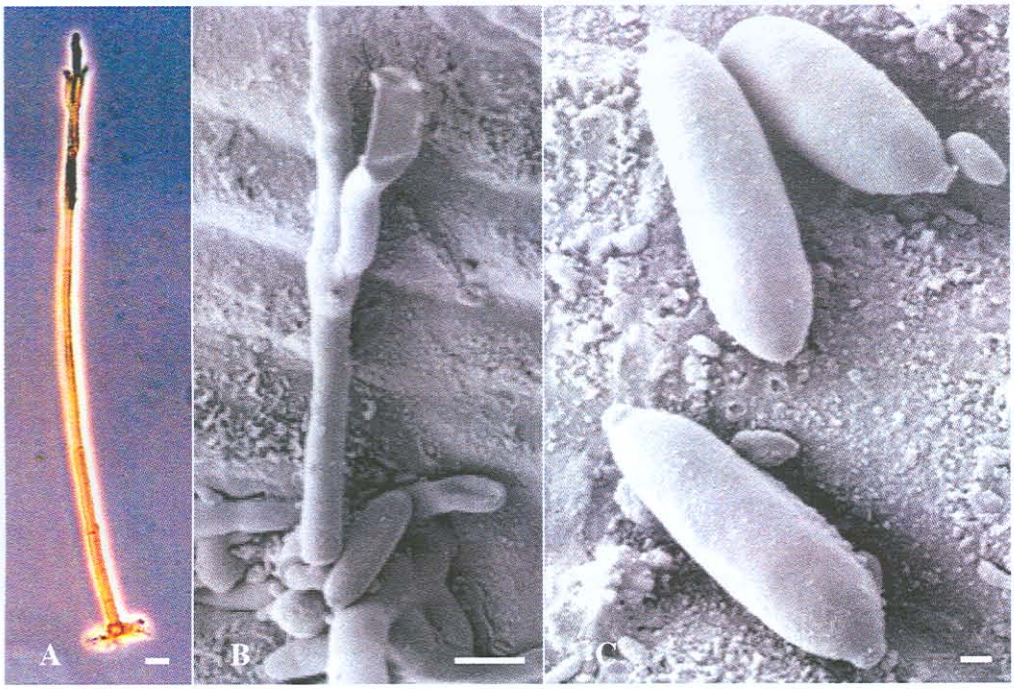

Figure 1: Light and electron micrographs of *Cladosporium musae*. A. Excised conidiophore showing thickened basal cell (Bar = 10 µm). B. Conidiophore on the banana leaf surface (Bar = 10 µm). C. Conidia (Bar = 1 µm).

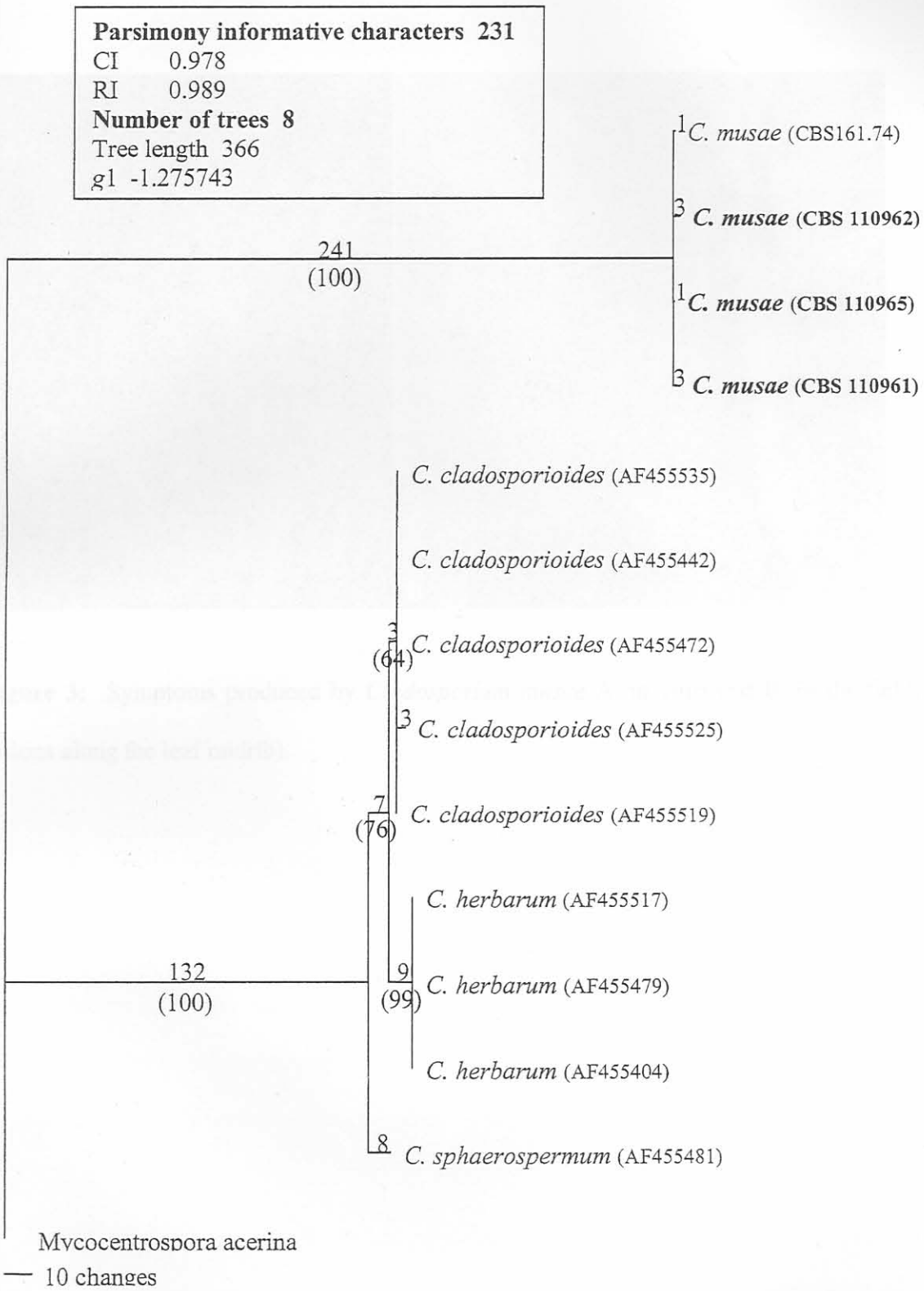

Figure 2: Phylogeny of the internal transcribed spacer sequences of *Cladosporium musae* (CBS 161.74) and South African isolates causing speckle disease on banana leaves.

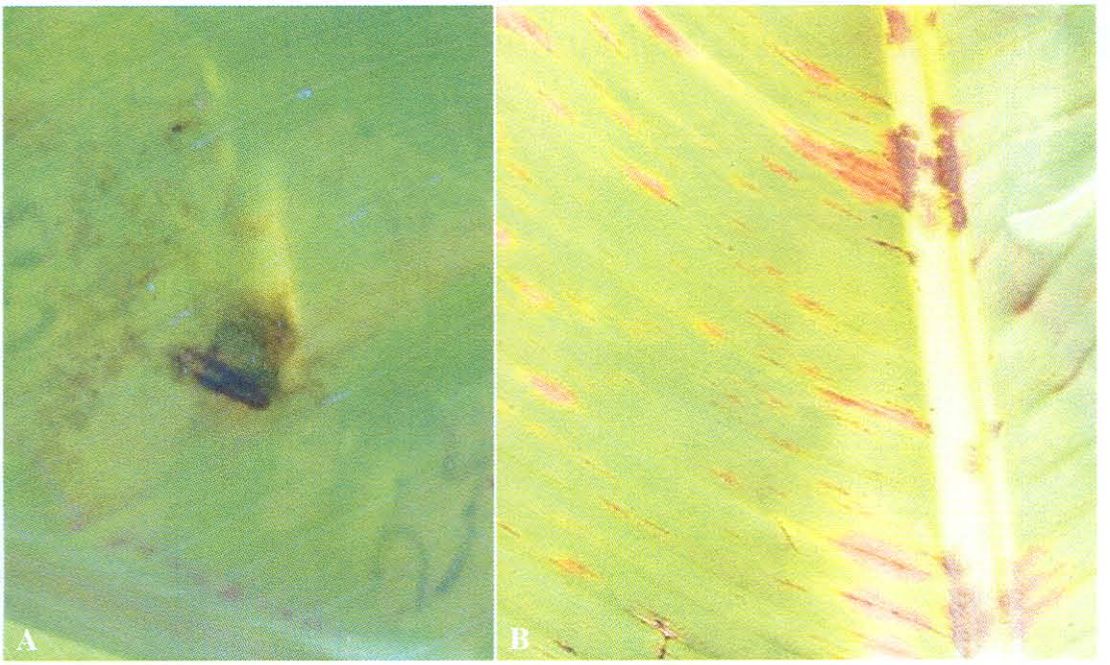

Figure 3: Symptoms produced by *Cladosporium musae* A. in vitro and B. in the field, (note lesions along the leaf midrib).