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Identification of the pathogen associated with Sigatoka disease of banana in South Africa

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Abstract. Three species of *Mycosphaerella* are known to cause Sigatoka leaf diseases in banana. *M. musicola* causes yellow Sigatoka, *M. fijiensis* causes black Sigatoka and *M. eumusae* causes eumusae leaf spot. Little is known about the presence of Sigatoka leaf diseases of banana in South Africa. Although yellow Sigatoka has previously been reported, the identity of the causal organism has never been confirmed. Extensive surveys of the five banana-growing areas in South Africa were conducted between 1999 and 2001, which resulted in the collection of various Sigatoka-like leaf spot samples. After morphological examination of the infected material, monoconidial isolates of the causal organism were established from each sample. A molecular confirmation of identity was conducted using species-specific primers for *M. musicola* and *M. fijiensis*. Sequence data of the ITS region was further obtained to compare the South African *Mycosphaerella* isolates from banana with *M. musicola*, *M. fijiensis* and *M. eumusae*. These results confirm that only *M. musicola* is presently associated with yellow Sigatoka symptoms in South Africa.

Introduction

Three Sigatoka leaf diseases have been described on banana (*Musa* spp.). Yellow Sigatoka is caused by *Mycosphaerella musicola*, black Sigatoka by *M. fijiensis* and eumusae leaf spot by *M. eumusae* (Carlier *et al.* 2000; Jones 2000; Crous and Mourichon 2002). Yellow Sigatoka has the widest distribution though black Sigatoka is rapidly replacing it in many tropical coastal regions (Stover 1972; Carlier *et al.* 1994; Jones 2000). Currently, *M. eumusae* has been detected only in Nigeria, Mauritius and South-East Asia (Carlier *et al.* 2000), though its similarity to *M. musicola* and *M. fijiensis* suggests that it could be more widely spread (Carlier *et al.* 2000).

In South Africa, only yellow Sigatoka has thus far been reported (Van den Boom and Kuhne 1969). However, the disease was identified on symptoms only, without any attempt to isolate or identify the causal organism. Primary lesions caused by *M. musicola*, *M. fijiensis* and *M. eumusae* are very similar in appearance, consisting of dark-brown to black specks that develop into streaks several millimetres long, with a grey centre, surrounded by a yellow halo (Jones 2000). Differences in symptoms are mostly related to the presence, nature and position of fruiting structures, but these are not readily discernible macroscopically. Positive identification of the disease, therefore, depends on morphological or molecular confirmation of the causal organism.

Considering the rapid rate at which black Sigatoka is spreading (Jones 2000) and the possibility that eumusae leaf spot may previously have been overlooked in some countries (Carlier *et al.* 2000), a reappraisal of the pathogen status of Sigatoka disease in South Africa was necessary. Severe outbreaks of the disease during the 1999/2000 growing season provided further motivation for such an endeavour. This report provides evidence, based on morphological and molecular studies, that the causal organism of Sigatoka disease in South Africa is indeed *M. musicola*.

Methods

Isolation of Mycosphaerella

Between 1999 and 2001, 163 leaf samples showing disease symptoms of Sigatoka were randomly collected from Cavendish cultivars in the five banana-growing areas of South Africa: Levubu (31 samples), Tzaneen (34), Kiepersol (41), Komatipoort (34) and southern KwaZulu-Natal (23). Samples were taken to the laboratory in envelopes and were maintained at 5°C until processed (2–3 days).

Single-conidial isolations were made from stage 5 leaf spots (Jones 2000) on the respective samples. Leaf spots were ellipsoidal in shape with a yellow halo surrounding a dark outlined area of sunken, white, necrotic tissue in which the sporodochia were found. Fifty microlitres of sterile distilled water were pipetted onto a mature lesion, left for 10 s and then transferred to 50 µL sterile distilled water in an Eppendorf

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tube. The suspension was mixed well and spread over the surface of a 20 g/L water agar (Merck) plate. Plates were incubated at 25°C for 1–2 days to induce conidial germination. Germinating conidia were transferred to half-strength potato-dextrose agar (PDA, Merck, 19 g/L PDA + 10 g/L agar) supplemented with 0.2 g/L novobiocin. All cultures are maintained on PDA slants and under mineral oil at 4°C at the Forestry and Agricultural Biotechnology Institute (FABI) in Pretoria, South Africa.

Morphological identification

Conidia were collected from stage 5 lesions (Jones 2000), suspended in lactophenol and studied under the light microscope. Fresh leaf lesions were also excised and fixed in 3% glutaraldehyde for a minimum of 1 h, followed by three rinses of 15 min each in 0.075 M phosphate buffer. Samples were dehydrated for 15 min each in 50, 70 and 90% ethanol, and three times in 100% ethanol. Critical point drying was performed in liquid carbon dioxide before mounting the samples on stubs, sputtering them with gold and examining them under a scanning electron microscope.

Artificial inoculation

Young banana plants (cv. Cavendish) used for inoculation were approximately 1 m high with 3–4 leaves, and growing in 5 L bags filled with potting soil. Plants were moved into the greenhouse maintained at 27°C (day/night) 30 days prior to inoculation to allow for acclimatisation. Plants were watered every third day and exposed to normal sunlight hours. The adaxial surface of the leaves was lightly abraded with a syringe needle to remove a portion of the waxy cuticle. An agar plug overgrown with mycelium of a selected culture (CMW 6346, CMW 6347, CMW 6365, CMW 6368, CMW 6373 or CMW 6375) was then placed onto the epidermis, covered with clear strip of Parafilm and marked with the isolate number. Two leaves per plant and three plants per isolate were inoculated. Plants were inspected regularly for symptom development and isolations were made from lesions, as described above, after 3–4 months.

DNA isolation

Molecular diagnosis of the *Mycosphaerella* species associated with Sigatoka disease in South Africa was achieved by PCR and sequencing of the ITS region of the DNA from lesions on all leaf samples and from selected *Mycosphaerella* isolates (Table 1). Initially, DNA was extracted using the method described by Raeder and Broda (1985). DNA extraction from a final selection of leaf samples, and all fungal isolates for sequencing, was achieved using a second method which yielded cleaner DNA in larger quantities. Approximately 1 g of fresh leaf tissue or fungal mycelium was placed in an Eppendorf tube and ground with ca. 10 µg sterile river sand in 500 µL of DNA Extraction Buffer [DEB: 200 mM Tris-HCl (pH 8), 150 mM NaCl, 25 mM EDTA (pH 8), 0.5% SDS]. A further 200 µL DEB were added to the suspension with 500 µL of phenol and 300 µL chloroform, vortexed and centrifuged for 60 min at 10 000 rpm. The supernatant was transferred to a new tube, 500 µL of phenol and 500 µL chloroform added, and centrifuged for 5–10 min at 10 000 rpm. The phenol/chloroform step was repeated until the interphase was clean. A further 500 µL chloroform was added and the tubes centrifuged for 5 min at 11 000 rpm. The supernatant was transferred to a new tube and double the volume of 100% ethanol was added and mixed. The DNA was allowed to precipitate at 4°C overnight and then pelleted by centrifugation for 30 min at 11 000 rpm. Pellets were washed with 500 µL 70% ethanol, dried and resuspended in 100 µL sterile distilled water and 3 µL RNAse (2.5 µM).

Polymerase chain reaction

Species-specific primers described by Johanson and Jeger (1993) were used to distinguish between *M. fijiensis* and *M. musicola*. Primer MM137 was specific for *M. musicola*, and MF137 for *M. fijiensis*. R635 served as a reverse primer for both species. Authentic *M. fijiensis* DNA (courtesy Dr H. Hayden, University of Queensland) and DNA isolated from a morphologically confirmed South African isolate of *M. musicola* were included as positive controls. A modification of the Johanson and Jeger (1993) method of PCR was used to amplify the DNA. Each PCR tube contained a total volume of 25 µL; 18.7 µL sterile distilled Sabax water, 2.5 µL PCR buffer with $MgCl_2$ (10×), 2 µL dNTPs (2.5 µM), 0.5 µL primer 1 (50 µM), 0.5 µL primer 2 (50 µM), 0.5 µL DNA (27 ng/µL) and 0.3 µL Expand Taq (5U/µL). DNA amplification was performed in a PCR thermal cycler using the following program: 3 min at 94°C, 40 cycles of 1 min at 94°C, 1 min at 60°C and 3 min at 72°C, followed by 5 min at 72°C, and then held at 4°C. The PCR product was analysed on a 1% agarose gel.

Sequencing of the ITS region

Eight randomly selected *Mycosphaerella* isolates were subjected to a standard ITS PCR with primers ITS1 and ITS4 (White *et al.* 1990). The ITS PCR products were purified with a 'High pure PCR product purification kit' (Roche). A sequencing reaction was performed, with each tube containing 1 µL clean PCR product, 2 µL 'Big Dye' (Roche) sequence mix, 0.32 µL primer and 1.68 µL sterile Sabax water. The PCR product was cleaned by adding 15 µL sterile Sabax water, transferring the entire volume to a sequencing tube, adding 2 µL of 3 M sodium acetate and 50 µL 95% ethanol, and allowing it to stand on ice

Table 1. Collection and sequence details of the *Mycosphaerella* isolates included in the phylogenetic analysis

Mycosphaerella sp.	Isolate no.	Locality	Date	Collector	Host cultivar	GenBank accession no.
M. musicola	CMW 6375	Komatipoort	19-May-00	A. Viljoen	Williams	AF509728
M. musicola	CMW 6373	Kiepersol	17-Mar-00	A. Viljoen	Williams	AF509729
M. musicola	CMW 6314	Kiepersol	24-Jun-00	A. Viljoen	Grande Naine	AF509730
M. musicola	CMW 6325	Komatipoort	16-Mar-00	A. Viljoen	Grande Naine	AF509731
M. musicola	CMW 6368	KwaZulu Natal	24-Jun-00	A. Viljoen	Grande Naine	AF509732
M. musicola	CMW 6340	Kiepersol	14-Jun-00	A. Viljoen	Williams	AF509733
M. musicola	CMW 6346	Kiepersol	14-Jun-00	A. Viljoen	Williams	AF509734
M. musicola	CMW 6365	Tzaneen	24-Jun-00	A. Viljoen	Chinese Cavendish	AF509735
M. fijiensis	ATCC 36054	Honduras	—	R.H. Stover	*Musa* AAA	AF297225
M. fijiensis	ATCC 22117	Hawaii	—	D.S. Meredith	Gros Michel	AF297234
M. fijiensis	ATCC 22116	Philippines	—	D.S. Meredith	Giant Cavendish	AF181705
M. musicola	ATCC 22115	Philippines	—	D.S. Meredith	Lacatan	AF181706

for 10 min. Sequencing tubes were then centrifuged at 10000 rpm for 30 min. The ethanol solution was removed, the pellet rinsed in 150 µL 70% ethanol and the tubes centrifuged at 10000 rpm for 5 min. The ethanol was aspirated and the pellet dried under vacuum for approximately 10 min. Tubes were transferred on ice to the sequencer. DNA sequences were determined using the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq DNA Polymerase (Applied Biosystems, UK). ITS2 and ITS3 (White *et al.* 1990) were included as internal primers to confirm the sequence data obtained.

Sequence analysis

ITS sequences for *M. musicola*, *M. fijiensis* and *M. eumusae* were obtained from GenBank (Table 1). DNA sequences were manually aligned and 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, Version 4.0b8; 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 accessed by evaluating tree length distributions over 100 randomly generated trees. The consistency (CI) and retention (RI) indices were determined for all data sets. Phylogenetic trees were rooted with *Mycocentrospora acerina* (Stewart *et al.* 1999) as a monophyletic sister outgroup to the remaining taxa. Bootstrap analyses were conducted to determine confidence in branching points (1000 replicates) for the most parsimonious trees generated.

Results

Morphological characterisation

The morphology of all isolates from banana leaves in South Africa appeared to be consistent with that of *Pseudocercospora musae* (Jones 2000), anamorph of *M. musicola*. Masses of conidia were produced in sporodochia on both sides of the leaf, though more abundantly on the upper surface. Some sporodochia developed in sub-stomatal chambers and emerged through the stomatal pore (Fig. 1*A*). Conidiophores were straight, sometimes slightly curved and bottle-shaped. They lacked septa and were unbranched. The conidiophore apex was rounded with no significant scarring (Fig. 1*A*). Conidia were pale brown, smooth, cylindrical and varied in shape from straight to curved, ranging from 50–120 µm in length and 2–6 µm in width (Fig. 1*B*). Conidial apices were obtuse and the base without a thickened hilum. No conidia were produced in culture.

Artificial inoculation

Inoculated banana plants developed typical yellow Sigatoka symptoms within 3–4 months of inoculation for all isolates tested and the fungus could be readily re-isolated.

PCR with species-specific primers

DNA from Sigatoka lesions on South African leaf samples produced a 1018 bp band on a 1% agarose gel with the *M. musicola* primer MM 137 (Fig. 2) in accordance with

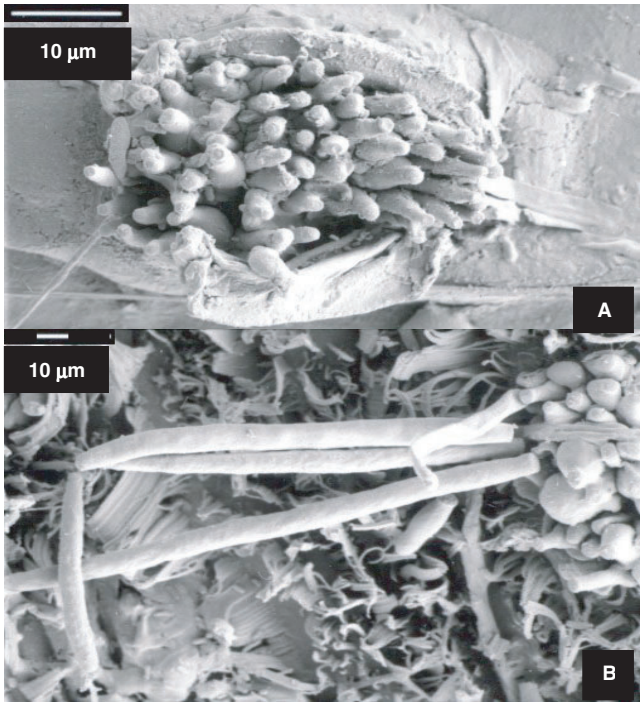

Fig. 1. Electron micrographs of *Mycosphaerella musicola*. (**A**) Pustule on the banana leaf surface formed by conidiophores in a sporodochium, emerging through a stomatal pore. (**B**) Conidia.

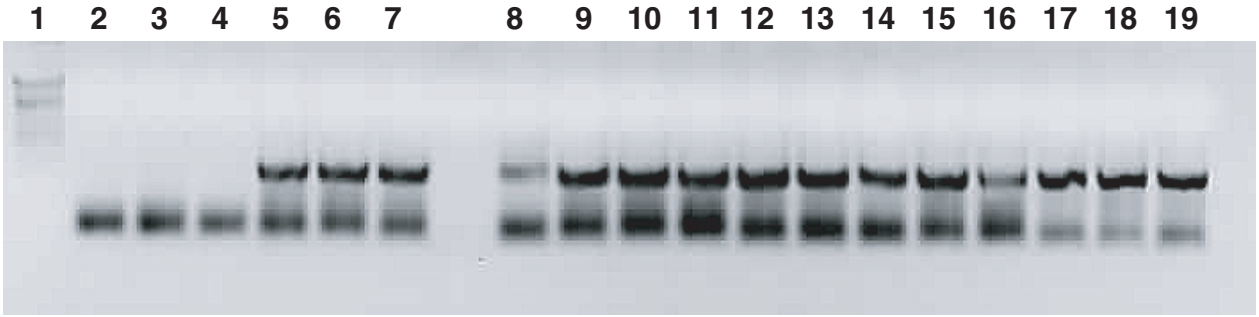

Fig. 2. 1% Agarose gel of PCR products from amplification of infected leaf and fungal DNA with primers MM137 and R635 stained with ethidium bromide. Lanes 1, λ Marker; 2–4, water controls; 5–7, positive control *Mycosphaerella musicola* DNA (from South African fungal cultures); 8–19, positive test for *M. musicola* (infected leaf DNA).

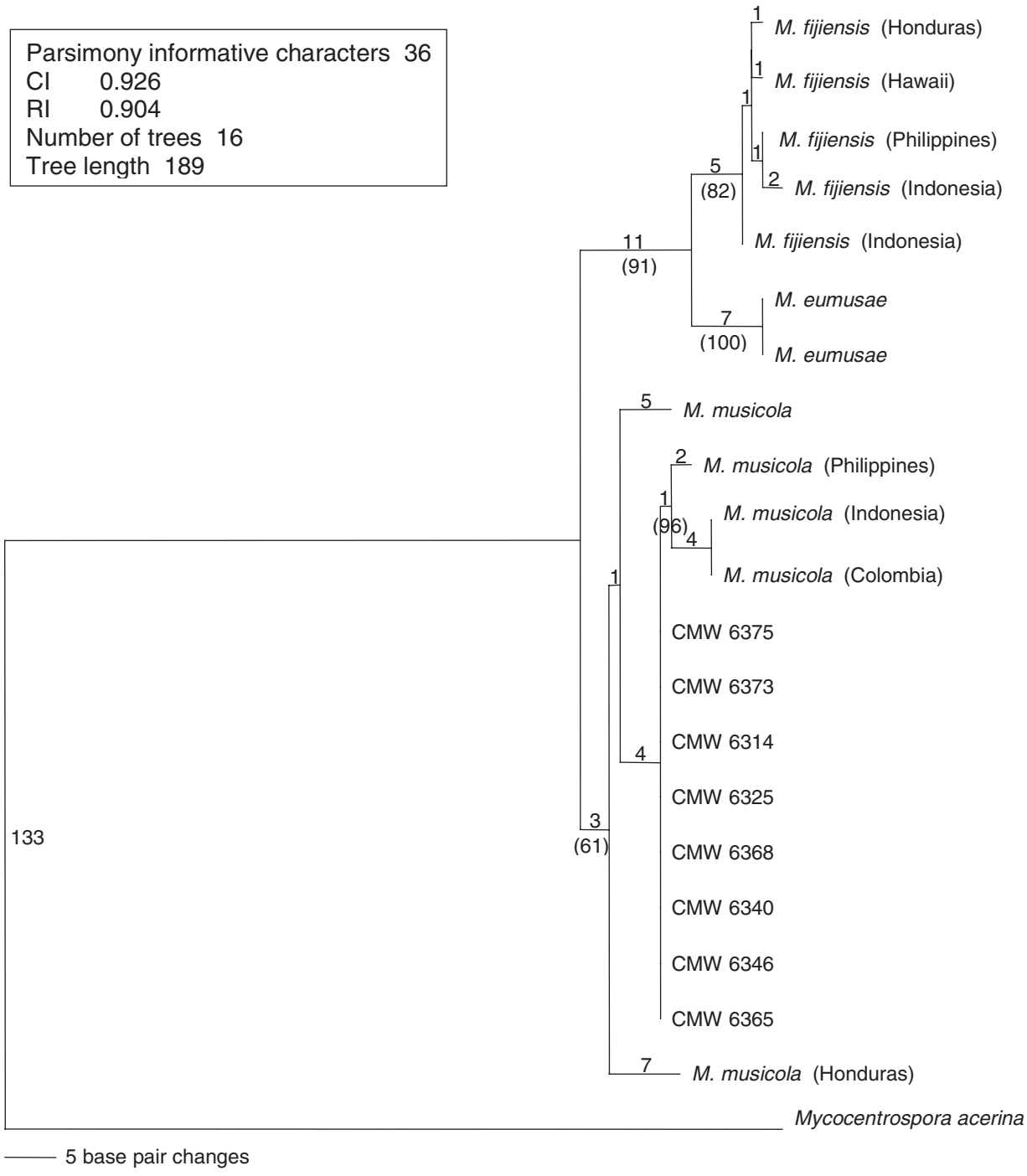

Fig. 3. Phylogeny of *Mycosphaerella fijiensis*, *M. eumusae*, *M. musicola* and South African isolates of *Mycosphaerella* causing Sigatoka leaf disease of bananas.

Johansen and Jeger (1993). The samples produced banding patterns corresponding with the positive control sample of DNA of *M. musicola*. No bands were produced when leaf DNA samples were tested with the *M. fijiensis* primer, MF 137. When the *M. fijiensis* DNA was tested with MF137, a 1018 bp band was produced, confirming the validity and accuracy of the PCR reactions.

Sequencing of the ITS region

Comparison of the ITS region of the South African isolates with those of *M. musicola*, *M. fijiensis* and *M. eumusae* indicated that the local isolates are phylogenetically similar to *M. musicola* (Fig. 3). However, the South African isolates appeared to have greater similarity to each other than to isolates of *M. musicola* from other

countries. The clade containing isolates of *M. fijiensis* and *M. eumusae* could be differentiated from the *M. musicola* clade by at least 8 base pairs, and the two former species from each other by 11 base pairs. Base pair differences were also evident between isolates of the same species from different geographic regions. The high CI and RI values of 0.926 and 0.904, respectively, support the validity of this tree. These eight sequences were deposited in Genbank (Table 1).

Discussion

The morphological, molecular and phylogenetic studies reported here confirmed that the Sigatoka disease on banana in South Africa is caused by *M. musicola*, hence verifying the identification by Van den Boom and Kuhne (1969) of the disease as yellow Sigatoka. Although the teleomorph was not present, conidial and conidiophore morphology of the fungus present in the Sigatoka lesions conformed to the description of the anamorph of the yellow Sigatoka pathogen, *M. musicola*. The identity of the fungus was further confirmed by PCR with species-specific primers for the identification of *M. musicola* (Johanson and Jeger 1993), and by proving that the ITS sequences of local isolates were similar to *M. musicola*, rather than *M. fijiensis* or *M. eumusae*. Isolates of *M. musicola* from South Africa appear to be closely related and sequencing of the ITS region revealed a 100% homology. The South African isolates, however, differ from the isolates from South-East Asia and Central America with at least 4 base pairs.

The occurrence of *M. musicola* in South Africa is consistent with the belief that yellow Sigatoka is more prevalent in subtropical areas (Jones 2000), and the high incidence of the disease in the 1999/2000 season could probably be ascribed to the above-average rainfall and warm winters that prevailed during this period. The results furthermore indicate that black Sigatoka and eumusae leaf spot have not yet reached South Africa, despite the ability of *M. fijiensis* to adapt to cooler climates (Jones 2000) and the presence of *M. eumusae* in Mauritius (Carlier *et al.* 2000), which is situated at the same latitude and not too distant from the banana-growing areas of South Africa.

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