

CHAPTER 2

IDENTIFICATION AND CHARACTERISATION OF *BOTRYOSPHERA* SPECIES FROM MANGO IN SOUTH AFRICA



ABSTRACT

Botryosphaeria spp. are well known endophytes and pathogens of many tropical and subtropical fruit crops, including mango. The identity of these species is difficult to determine due to overlapping morphological characteristics of both the teleomorphs and anamorphs. The purpose of this study was to determine the identity of *Botryosphaeria* spp. infecting mango in South Africa. Isolates were obtained from diseased mango plants in the Northern Province of South Africa. They were cultured on potato dextrose agar and cultural and conidial morphology was evaluated. DNA was isolated and the internally transcribed spacer (ITS) and β -tubulin gene regions were amplified and sequenced. Four morphological groups (MGs) were identified among all isolates, based on cultural and conidial morphology. These MGs directly corresponded to four distinct clades with combined ITS and β -tubulin sequence data. The species in these groups were identified as *Fusicoccum parvum*, *Lasiodiplodia theobromae* and two new species, *F. indigiticum* and *F. bacilliforme*, which are described in this study.

Botryosphaeria species are known world-wide for the damage that they cause to various woody hosts (Sutton, 1980; Punithalingham, 1980). These fungi are well known as the causal agents of branch and stem cankers, twig dieback and blossom blight in most of the trees they infect. *Botryosphaeria* diseases also cause severe fruit rots and are responsible for extensive losses to industries that rely on fruit crop export (McPartland & Schoeneweiss, 1984; Pennycook & Samuels, 1985; Ramos *et al.*, 1991; Johnson, 1992).

Mangoes, *Mangiferae indica* Linn., can be severely damaged due to invasion and colonisation by *Botryosphaeria* spp. These fungi cause a variety of preharvest disease symptoms, which are usually expressed when trees are subjected to environmental stress (McPartland & Schoeneweiss, 1984; Ramos *et al.*, 1991; Johnson, 1992; Ploetz, 1994). Recent outbreaks of tree die-back in orchards as well as substantial export losses due to soft brown rot (SBR) and stem end rot (SER) diseases of mango fruit in South Africa, have renewed interest in the taxonomy and epidemiology of *Botryosphaeria* spp. in this country.

A number of different *Botryosphaeria* spp. have been reported to occur on mango, but the taxonomy of these fungi is confusing. Although these fungi are recognised as being *Botryosphaeria* spp. by anamorph association, teleomorph structures have not been recorded on mango (Johnson, 1992; Slippers *et al.*, 2001). As with other *Botryosphaeria* spp., identification of isolates from mango has chiefly been based on morphological characteristics of the anamorphs (Sutton, 1980; Pennycook & Samuels, 1985; Jacobs & Rehner, 1998; Crous & Palm, 1999; Smith & Stanosz, 2001). Much confusion, however, also surrounds the classification of these anamorphs and this has further complicated

accurate treatment of these pathogens, on mango and other crops (Sutton, 1980; Jacobs & Rehner, 1998; Crous & Palm, 1999; Denman *et al.*, 2000). Anamorph genera that have been documented as causal agents of SER and SBR of mango include *Dothiorella dominicana* Pet. et Cif., *D. mangiferae* H. et P. Syd. But., an unnamed fungus that has been referred to as *Dothiorella* 'long', *Nattrassia mangiferae* (Nattrass) Sutton et Dyko and *Lasiodiplodia theobromae* (Pat.) Griff. et Maubl. (Johnson, 1992).

The simplest morphological distinction between *Botryosphaeria* anamorphs is based on the production of either hyaline or pigmented and fusiform to ellipsoid conidia, with or without septation at maturity (Pennycook & Samuels, 1985; Crous & Palm, 1999; Denman *et al.*, 2000). Most species with pigmented conidia are generally treated in the genus *Diplodia* Fr. and those with hyaline conidia in *Fusicoccum*. There are, however, some limitations to the use of anamorph morphological characteristics for identification of *Botryosphaeria* spp. The fact that many species are morphologically similar and that it is sometimes difficult to induce strains to sporulate in culture, has resulted in confusion in the delimitation of species (Pennycook & Samuels, 1985; Smith & Stanosz, 2001; Zhou & Stanosz, 2001).

DNA sequencing data has begun to provide valuable insights into the natural classification of fungi where traditional characters have been shown to be insufficient for this purpose (Bruns *et al.*, 1991; Mitchell *et al.*, 1995). Recent studies on *Botryosphaeria* using DNA sequence data have provided considerable insight into the taxonomy of these fungi (Jacobs & Rehner, 1998; Denman *et al.*, 2000; Zhou & Stanosz, 2001). For example, where *B. dothidea* and *B. ribis* (now known as *B. parva*) had previously been reduced to synonymy based on morphology, these fungi have clearly been shown to be distinct species based on

sequence data sets for a number of genes (Jacobs & Rehner, 1998; Zhou & Stanosz, 2001; Slippers *et al.*, 2001).

Parts of the rDNA operon have been most useful in resolving taxonomic and phylogenetic questions pertaining to fungi (Hillis & Huesenbeck, 1992; O'Donnell, 1992; Carbone & Kohn, 1993). Thus, the internally transcribed spacers (ITS 1 and ITS 2) of the rDNA operon have been successfully employed to analyse interspecific relationships in various fungi, including *Botryosphaeria* spp. (Smith *et al.*, 1994; Jacobs & Rehner, 1998; Zhou & Stanosz, 2001). Jacobs and Rehner (1998) used ITS sequence and morphological characteristics to relegate several anamorphs to *Botryosphaeria*. Various researchers have, however, warned against basing phylogenies on a single DNA region (O'Donnell & Cigelnik, 1995; Taylor *et al.*, 2000).

The aim of this study was to identify and characterise the *Botryosphaeria* spp. associated with mango diseases in South Africa. Both morphological and molecular data were used to compare isolates from this region with those from other parts of the world where they have been collected from canker, dieback and mango fruit rot symptoms. Sequence data from two gene regions, the ITS and β -tubulin regions, were used for molecular analysis. Conidial and cultural characteristics are considered for morphological comparisons.

MATERIALS AND METHODS

Collection and isolation of fungal isolates

Botryosphaeria spp. used in this study were isolated from mango trees and fruit cultivated in Mpumalanga and the Northern province, South Africa. Isolations were made from

asymptomatic and symptomatic material from various parts of trees and fruit. Prior to isolation, whole twigs, leaves and fruit were surface disinfested twice with 70% (v/v) ethanol and left to air dry for five minutes between treatments. Surface disinfested wood chips (2mm²) and discs (2 – 3mm²) from the edges of lesions on fruit were cut in half and placed on potato dextrose agar (PDA) (Biolab) amended with 100mg chloramphenicol (Centaur Laboratories). Cultures were incubated at 25°C for seven to twelve days. All cultures, with a peripheral morphology resembling that of *Botryosphaeria* spp. were transferred to clean Petri dishes containing PDA.

Twenty isolates were identified from a larger collection, based on colony colour and conidial morphology, to be representative of the *Botryosphaeria* spp. found in the mango industry of South Africa (Table 1; p63-64). All isolates were collected between 1999–2001. Reference isolates obtained from mango in Australia (supplied by Dr. G.I. Johnson) and other hosts were included for comparative purposes (Table 1; p63-64). All the *Botryosphaeria* isolates used in this study are maintained in the culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa (Table 1; p63-64).

Morphological characterisation

Sporulation of putative *Botryosphaeria* isolates were induced by growing isolates on water agar (WA) (Biolab), supplemented with sterile pine needles or mango twigs. Cultures were incubated at 25°C with 12 hour near UV light/dark cycles, to induce sporulation. Conidia produced in this way were also used for morphological characterisation (Sutton, 1980; Johnson, 1992; Crous & Palm, 1999). Conidia were spread on WA and single germinating conidia transferred to PDA after 12 – 24 hours. All *Botryosphaeria* isolates derived from

single conidia were then stored at 4°C on PDA slants and in sterile water to be used for molecular characterisation.

Single spore cultures of *Botryosphaeria* spp. identified in this study, were inoculated in the middle of Petri dishes containing PDA and incubated at five different temperatures ranging from 10°C to 30°C with 5°C intervals. Two to four isolates were used for every species and a total of five replicates were included for every isolate at every temperature. Two perpendicular measurements of colony diameter were taken daily for every isolate at every temperature, from specific marked areas on the Petri dishes. Colony growth and cultural characteristics were recorded for all species.

Fruiting structures formed on mango twigs or pine needles were dissected by hand to observe pycnidia, stromatic locules in cross section, conidia and conidiogenous cells. Sections were mounted in lactophenol and examined using Nomarski differential interference contrast microscopy. Isolates were grouped according to morphological characteristics. The average size (length and width) of 30 – 50 conidia were measured for each isolate. All microscope observations and measurements were made using a light microscope (Carl Zeiss) and photographic images were captured electronically with an Axiovision digital camera system (Carl Zeiss).

Molecular characterisation

DNA isolation

Mycelium from actively growing PDA cultures was used to inoculate 100mL liquid MY (2% Malt Extract and 0.2% Yeast Extract) broth in 250mL Erlenmeyer flasks. These liquid cultures were incubated at 25°C for approximately one week. Mycelium was harvested, filtered and lyophilised. A modified version of the method of Raeder and Broda (1985)



was used for isolation of DNA. Dried mycelium was ground to a fine powder and homogenised in 800 μ l extraction buffer (200mM Tris-HCL pH8.0, 150mM NaCl, 25mM EDTA pH8.0, SDS 0.5%). Phenol and chloroform (ratio 5:3) was added to all samples, shaken and centrifuged (13000 rpm for 60 minutes). Thereafter, chloroform was added, centrifuged and the upper aqueous phase removed repeatedly until the interphase was clear of proteins and contaminating cell debris. Precipitation of nucleic acids was done with 3M NaAc pH5.5 (0.1 v/v) and absolute ethanol (2 v/v). After a 70% EtOH (ethanol) wash step, DNA was vacuum dried to a pellet and resuspended in 50 μ L sterile SABAX water. RNA was degraded by the addition of 3 - 5 μ L RNase (1mg/mL) to the DNA and left at 37°C for three hours, or until all RNA was degraded. DNA concentrations were estimated against a λ -marker standard on a 1.5% agarose gel.

DNA amplification and purification

A portion of the nuclear rDNA operon was amplified using primers ITS1 (5'-TTT CCG TAG GTG AAC CTG C-3') and ITS 4 (5'-TCC TCC GCT TAT TGA TAT GC-3') (MWG Biotech, Germany) (White et al., 1990). The amplified region extended from the 3' end of the 16S (small subunit) rDNA gene, including the first ITS (ITS1), 5.8S gene, the second ITS (ITS2) region and ended at the 5' end of the 26S (large subunit) rDNA gene. Part of the β -tubulin 2 gene region was amplified with primers Bt 2a (5'-GGT AAC CAA ATC GGT GCT TTC-3') and Bt 2b (5'-ACC CTC AGT GTA GTG ACC CTT GGC-3') (Glass & Donaldson, 1995).

Polymerase chain reactions (PCR) contained 0.2mM of each dNTP (Promega, Madison, Wisconsin, U.S.A.), 0.15 μ M of each primer, 0.5U Expand™ High Fidelity Taq polymerase (Roche Molecular Biochemicals, Alameda, CA), 1X Buffer and MgCl₂ (10mM

Tris-HCL, 1.5mM MgCl₂, 50mM KCl). Sterile SABAX water was used to adjust the final volume to 50μL. The following conditions were standardised for all PCR reactions: An initial denaturation at 96°C for one minute followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing for one minute at 56°C for ITS and 58°C for β-tubulin, followed by extension at 72°C for ninety seconds. A step up of five seconds elongation was added with each cycle after the first twenty-five cycles. The process was ended with a final extension at 72°C for ten minutes. All PCR products were stored at 4°C. PCR products were visualised on a 1.5% horizontal agarose gel using a TAE buffer electrophoresis system (Maniatis *et al.*, 1982). PCR products were stained with a 0.5g/mL ethidium bromide (Merck) solution and visualised under UV illumination. PCR product sizes were estimated with a 100bp standard size marker (Promega).

DNA sequencing and analysis

Twenty isolates, representative of all morphological groups from mango in South Africa, were used for sequencing (Table 1; p63-64). All PCR products were cleaned prior to sequencing with a High Pure PCR Product Purification kit (Roche Molecular Biochemicals, Alameda, CA) according to manufacturers specifications. PCR products were sequenced in both directions using the primers ITS1, ITS4, Bt 2a or Bt 2b. Sequencing reactions were performed using the ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer Applied Biosystems, Foster City, CA). Twenty-five sequencing PCR cycles were performed with the following standardised conditions: a denaturation step at 96°C for ten seconds, annealing of primers at 50°C for 30 seconds and elongation at 60°C for four minutes. All sequence reactions were run on an ABI PRISM 377 Autosequencer (Perkin-Elmer Applied BioSystems, Foster City, CA). Sequences were analysed using Sequence Navigator version 1.0.1™ (Perkin Elmer Applied BioSystems,

Foster City, CA) and manually aligned by inserting gaps. Phylogenetic analyses were done using PAUP (Phylogenetic Analysis Using Parsimony) version 4.0 (Swofford, 1998). All characters were given equal weight and gaps were treated as newstate (fifth base). ITS and β -tubulin datasets were analysed separately and combined. A partition homogeneity test (PAUP 4.01b) was used to test the congruence and combinability of the ITS and β -tubulin sequence data sets (Huesenbeck *et al.*, 1996). Subsequently, the datasets were analysed together. Most parsimonious trees were identified with heuristic searches using random stepwise addition and tree bisection and reconstruction (TBR) as branch swapping algorithm. Branches with a length of zero were collapsed and all multiple equally parsimonious trees were saved. Bootstrap consensus trees were obtained with PAUP for all equally parsimonious trees saved, with 1000 bootstrap replicates (Felsenstein, 1985). Eight sequences representing the most commonly recognised *Botryosphaeria* spp. were obtained from Genbank (Table 1; p63-64). Isolates known to infect mango in Australia and other woody hosts in South Africa were also included for comparative purposes (Table 1; p63-64) (Slippers *et al.*, 2001). Trees were rooted to the outgroup *Guignardia philoprina* (Ellis) Viala & Ravaz, a genus known to be closely related to *Botryosphaeria*.

RESULTS

Morphological characterisation

All isolates included in this study had multilocular and eustromatic conidiomata. Conidiophores were hyaline, cylindrical, smooth and 0-1 septate. Conidiogenous cells were hyaline and smooth. Conidia were produced holoblastically on the conidiogenous cells. Conidia were hyaline, thin-walled, smooth and ellipsoid to fusiform. Aseptate,

(immature) and uni- to biseptate (mature) conidia were observed for all but one group of isolates, which the conidia were aseptate at all times (Fig. 1; p 65) (Table 1; p 63 - 64).

Analysis of colony morphologies and conidial dimensions for single conidial isolates on PDA, gave rise to four morphologically distinct groups which were designated as MG1 – MG4 (Fig. 2; p67) (Table 1; p63 - 64). Three of these groups of isolates resembled the characteristics of *Fusicoccum* spp. Conidia in the fourth group (Fig. 1; p65) resembled those of *L. theobromae*.

Botryosphaeria isolates residing in morphological group MG1, readily produced fluffy, white aerial mycelium, which become pale olivaceous grey (21''''d) to olivaceous grey (21''''') (Rayner, 1970) (Fig. 2; p65) from the middle of the colony within three to four days of incubation on PDA. The reverse side of the Petri dishes reflectes an olivaceous grey (21''''') to iron grey (24''''k) colour. For this fungus, an average growth of 10.8 ±1mm per day is measured. The optimum temperature for growth was between 25°C and 30°C. Conidiomata were readily produced at the edge of the colony and were generally covered with tough greenish grey (33''''i) hyphae. Immature conidia were hyaline, smooth, aseptate and fusiform, but become uni- to biseptate and light brown pigmented with age and prior to germination (Fig. 1, A–B; p 65). Conidial apices were sub-obtuse and the bases truncate or rounded. Width of conidia was measured over the widest part of conidia (middle to upper third of conidia) (Fig. 1, A-B; p65). Average of 50 conidia per isolate was (16-)17.5 – 19.7(-22) x 4.5 – 4.7(-6.2)µm [l/w = 4.3]. Based on these morphological characteristics, isolates assigned to MG1 resembled *F. parvum* (the *Fusicoccum* anamorph of *B. parva* previously known as *D. dominicana*) (Table 2; p71).

Isolates assigned to MG2 produces fluffy to appressed mycelium, becoming olivaceous grey (21''''') to olivaceous black (27''''m) (Fig. 3; p69) within three to four days of incubation. Aerial mycelium become appressed with culture maturity. The Petri dish reverse side became indigo blue (47''m) to black. The optimum temperature for growth was 25°C and the average colony growth rate is 4.7 ± 1 mm per day. Conidiomata were small, iron grey (24''''k) and were rarely produced in culture. Immature conidia were hyaline, aseptate, smooth and fusiform, but became uni- to biseptate with darker pigmentation in some instances, prior to germination (Fig. 1, C-D; p65). Conidial apices were sub-obtuse and bases truncate to rounded. Width, taken at the widest part (middle to upper third), and length measurements of conidia are (17.5-)19.5 – 21(-24) x (5-)5.5 – 6.5(-7.1) μ m [l/w = 3.4]. Based on these characteristics, isolates assigned to MG2, appeared to represent an unidentified *Fusicoccum* sp. (Table 2; p71).

Isolates assigned to MG3 produce white, sparse aerial mycelium clustered in concentric rings on PDA. A yellow [pale luteous (18f)] (Fig. 3; p69) colour pigment was produced in young cultures, diffusing into the medium. Mycelium became pale olivaceous grey (21''''d) to olivaceous grey (21''''') within five to seven days of incubation. The reverse side of Petri dishes displayed a grey olivaceous (21''''') to olivaceous grey (21''''') colour with a visible dendritic pattern. One isolate [BOT2421] remained light coloured at all times. The optimum temperature for growth of this group was 25°C and the average colony growth rate was 7 ± 1 mm per day. Small conidiomata were produced sparingly in concentric rings. Conidia were hyaline, aseptate, smooth and cylindrical to bacilliform (Fig. 1, E-F; p65). Conidia rarely became uniseptate at maturity. Conidial measurements of MG3 isolates were (18.8-)20.8 – 23(-24.9) x (3.7-)4.1 – 5.2(-5.7) μ m [l/w = 4.6]. Isolates

assigned to MG3 resembled the unnamed *Fusicoccum* sp. that has previously been referred to as *Dothiorella* 'long' (Johnson *et al.*, 1991; Johnson, 1992) (Table 2; p71).

Isolates residing in MG4 were typical of *L. theobromae*. This identification is based on the following characteristics. Isolates produce very fluffy and white aerial mycelium that rapidly covered the surface of Petri dishes within two days of incubation. The optimum temperature for growth is 30°C and the average growth rate was 19±1mm per day. White mycelium rapidly became pale olivaceous grey (33''''e) to iron grey (24''''k) (Fig. 3; p69) and submerged mycelium gave rise to an olivaceous grey (21''''') to iron grey (24''''k) colour viewed from the underside of the Petri dishes. Conidiomata occurred scattered in mycelial mat and at the edges of the colonies. Conidiomata were covered with smooth hyphae. Mature conidia oozed from ostioles of conidiomata within nine to fifteen days incubation at 30°C. Immature conidia were hyaline, aseptate, ovoid to rounded (Fig. 1, G-H; p65). They became uniseptate, thick walled, light brown pigmented with longitudinal striations when mature. Averaged width of conidia were taken at the widest part (middle) and the average length and width was (8-)10 – 18(-20) x 4 – 5.2(-6)µm [l/w = 3.6].

Molecular characterisation

DNA amplification and sequence analysis

ITS and β-tubulin gene regions were highly conserved in all species examined based on the size of the amplified PCR product fragments. Fragment sizes of approximately 550bp and 450bp in length for the ITS and β-tubulin regions respectively, were obtained for all isolates used in this study. Approximately 515bp of the ITS sequence data were used in the phylogenetic analysis, amounting to 560 characters after alignment. Only 430bp for β-

tubulin were used in the phylogenetic analysis, amounting to 469 characters after alignment. The partition homogeneity test indicated that the datasets were combinable ($P < 0.06$; $g1 = -0.753$). The total alignment of the combined data sets amounted to 1009 characters. Of the total combined data set after alignment for the ITS and β -tubulin regions, 725 characters were parsimony-uninformative and were, therefore, excluded from the heuristic searches. The variable and parsimony-informative characters amounted to 284. After heuristic searches in PAUP, 226 most parsimonious trees of 100 steps were retained (consistency index (CI) = 0.752; retention index (RI) = 0.918) (Fig. 2; p67).

After phylogenetic analyses, all isolates considered in this study could be grouped into ten clades (I – X) based on ITS and β -tubulin sequence data (Fig. 3; p67). Clades I – VII represent *Botryosphaeria* spp. with hyaline *Fusicoccum*-like conidia, and clades VIII – X represent species with pigmented or darker *Diplodia*-like conidia. All the South African mango isolates grouped into one of four clades (clade I, IV, VI or VIII) and these corresponded to identifications based on morphology and the assignment of isolates to four morphological groups MG1 – MG4. Clade I [BOT2413, BOT2302, BOT2398, BOT2353, BOT2331, BOT2339, BOT2382, BOT2363, BOT2345, BOT2291, BOT2405, BOT7799, BOT7026, BOT7025, BOT2352] corresponded to *F. parvum* (MG1). Isolates in clade IV [BOT2351, BOT2355] did not group with any *Botryosphaeria* spp. currently known (MG2). Clade VI [BOT2417, BOT2421, CMW7802, CMW7022] is a separate clade which is represented by isolates assigned the informal name *Dothiorella* ‘long’ isolated from Australia and South Africa (MG3). The isolates from these countries very closely related, but the variation between them is supported by very strong bootstrap values. The fourth species, isolated from mango in South Africa, reside in clade VIII [BOT2399, BOT2376, BOT2422, BOT2430] and represents *L. theobromae* (*B. rhodina*) (MG4).

All isolates in clades II [CMW7801, CMW7024] and VII [CMW7803, CMW7020, CMW7027] were collected in Australia and represent the species *F. mangiferum* and *F. aesculi* respectively (Table 1; p63 - 64). Clade V [BOT945 (plum) and BOT931 (pear)] included *Botryosphaeria* isolates from other fruit trees in South Africa and represents the anamorph species, *F. luteum* (Slippers *et al.*, 2001; Phillips *et al.*, 2002). Clade III [BOT11, BOT32] represents *F. eucalyptorum* isolates from *Eucalyptus* trees in South Africa (Smith *et al.*, 2001). Clades VIII and IX represent sequence data for *B. obtusa* and *Sphaeropsis sapinea* isolates obtained from Genbank.

TAXONOMY

Results of morphological comparisons and DNA sequence comparisons have clearly shown that two undescribed species of *Fusicoccum* occur in South Africa. One is equivalent to the fungus previously known as *Dothiorella* 'long' in Australia and the other has not been isolated previously. These fungi are, therefore, described as new species in *Fusicoccum* as follows:

These preliminary descriptions are presented only for the purpose of this thesis and formal descriptions will be published in the mycological literature.

Anamorph. *Fusicoccum indigoticum* R. Jacobs, B. Slippers et M.J. Wingf. sp. nov.

(Fig. 1C-D)

Colonies initially white with appressed to fluffy mycelium, becoming olivaceous grey (21''''') to olivaceous black (27''''m) within three to four days after inoculation, and

mycelium on reverse side of petri dish indigo blue (47''m) to black. Quick growing on PDA at 25°C, little to no growth below 15°C or above 30°C.

Conidiomata small, eustromatic, immersed in host sub-epidermally, iron grey (24''''k), covered with thick, dark hyphae, rarely produced in culture on PDA. Conidiomata are multilocular, locules totally embedded in some instances without distinct ostioles. Locule walls consist of dark *textura angularis*, becoming thinner and hyaline towards conidiophores and conidiogenous cells.

Conidiophores hyaline, smooth, cylindrical, aseptate, unbranched, 9.2 – 18.5 x 0.5 – 1.0, formed from cells of locule wall.

Conidiogenous cells hyaline, cylindrical, granulate, produce the first formed conidia holoblastically, subsequent conidia formed enteroblastically, proliferating precurently with two to three precurent proliferations and formation of annellations, (10.3-)10.5 – 13.6(-15.9) x (0.8-)1.3 – 1.7(2.4).

Conidia hyaline, ovoid to slightly ellipsoid, straight, granulate, thin walled, immature conidia aseptate. Conidia are evenly tapered at both ends with a bluntly rounded to obtuse base and truncate apex, widest part at the middle or upper third of conidia. In most instances, conidia become light brown and uni- to biseptate at maturity prior to germination. Long, sparingly branched germ tubes grown from one or more of the individual cells of the conidia. Conidia (17.5-)19.5 – 21(-24) x (5-)5.5 – 6.5(-7.1)µm [l/w = 3.4].

Teleomorph. Unknown *Botryosphaeria* sp. (not seen in this study)

Etymology. Name refers to the indigo-black colour of the reverse side of colonies on PDA.

Host. *Mangiferae indica* Linn.

Distribution. Mpumalanga, South Africa.

Specimens examined. Hoedspruit and Letsetele Valley, Mpumalanga, South Africa, 2000-2001, R. Jacobs.

Holotype: PREM 57316 (BOT 2355), isolated from canker lesion on mango leaf.

Paratype: PREM 57317 (BOT 2351), isolated from soft brown rot lesion on mango fruit.

Anamorph. *Fusicoccum bacilliforme* R. Jacobs, B. Slippers et M.J. Wingf. sp. nov.

(Fig. 1E-F)

Colonies initially white with sparse aerial mycelium clustered in concentric circles, becoming pale olivaceous grey (21''''d) to olivaceous grey (21''''') within five to seven days after inoculation. Mycelium colour on reverse side of petri dish grey olivaceous (21''''') to olivaceous grey (21'''''), dendritic pattern visible. Colonies quick growing on PDA at 25°C, with little to no growth below 15°C or above 30°C. A pale luteus (18f) pigment is produced in young cultures, which readily diffuses into the medium.

Conidiomata small, eustromatic, immersed in host sub-epidermally, covered with thick, pale white to smoke grey (21''''f) hyphae at all times, produced in concentric circles in culture on PDA. Conidiomata are multilocular, locules totally embedded in some instances, with ostioles. Locule walls consist of dark *textura angularis*, becoming thinner and hyaline towards conidiophores and conidiogenous cells.

Conidiophores hyaline, smooth, cylindrical, aseptate to uniseptate, unbranched, formed from cells of locule wall, 13.4 – 21.8(-22) x 0.4 – 0.9.

Conidiogenous cells hyaline, clavate to cylindrical, granulate, produce the first formed conidia holoblastically, subsequent conidia formed enteroblastically, proliferating precurrently with two to three precurent proliferations, 18.7 – 20.8(-25.2).

Conidia hyaline, bacilliform to cylindrical, straight to slightly curved, smooth, thin walled, aseptate. Conidia are evenly rounded at both ends with a bluntly rounded to obtuse

base and truncate apex. Conidia not dark or septate prior to germination. Conidia (18.8-20.8 – 23(-25.5) x (3.7-)4.1 – 5.2(-5.7)µm [l/w = 4.6].

Teleomorph. Unknown Botryosphaeria sp. (not seen in this study)

Etymology. The name refers to the distinctive bacilliform conidia in this fungus.

Host. *Mangiferae indica* Linn.

Distribution. Mpumalanga, South Africa.

Specimens examined. Malelaan, Mpumalanga, South Africa, 2001, R. Jacobs.

Holotype: PREM 57318 (BOT 2417), isolated from canker lesion on mango stem.

Paratype: PREM 57319 (BOT 2421), isolated from canker lesion on mango stem.

DISCUSSION

Results of this study show clearly that four *Botryosphaeria* spp. occur on mango in South Africa. This is the first time that the taxonomy of these fungi on mango has been studied in South Africa and results will facilitate more effective management of the various diseases associated with mango. In the past, at least three of the fungi found in this study, have been indiscriminately assigned to species. From a taxonomic point of view, names used in previous South African publications should be viewed with a level of disgression. The four species of *Botryosphaeria* occurring on mango in South Africa can be identified relatively easily based on morphological characteristics, especially those pertaining to conidia. These morphological species could also consistently be separated based on ITS and β -tubulin gene sequences. They represent *F. parvum*, *L. theobromae* (teleomorph *B. rhodina*) and two undescribed *Fusicoccum* spp., for which names are provided here.

The majority of isolates collected in this study reside in clade I, which represents *B. parva* (Slippers *et al.*, 2001). *Fusicoccum parvum*, the *Fusicoccum* anamorph of this species is the form most frequently encountered in nature. Conidia of this *Fusicoccum* sp. assigned to MG1 typically become uni- to bisepate and darker with maturity. In this sense, the conidia are similar than those of morphological group two (MG2). However, those of *B. parva* are more tapered at the ends with more truncate bases than those of MG2. The fluffy cultural morphology is also very distinctive for this species. On mango, this fungus has commonly been treated under the name *D. dominicana* (Johnson, 1992), but has been shown to be *F. parvum* by Slippers *et al.* (2001).

Botryosphaeria parva (previously known as *B. ribis*) is a well-known pathogen of many woody plants world-wide (Von Arx, 1987; Punithalingham, 1980). It is also recognised as existing in healthy plants as latent pathogens. Our isolates were from both healthy and symptomatic tissues, confirming the endophytic nature of this fungus on mango. *Fusicoccum parvum* has been isolated regularly from mango in various countries and is considered the primary causal agent of pre- and postharvest disease (Darvas, 1991; Ramos *et al.*, 1991; Johnson, 1992). Although pathogenicity tests are required, the frequency of collection of this fungus in the present study, tends to support results of previous pathological studies.

A unique *Fusicoccum* sp. was isolated from fruit and leaves of mango from South Africa, as part of this study. Both molecular and morphological data confirmed that the fungi represents a previously undescribed taxon and it was thus assigned the name *F. indigoticum*. The closest related species, based on ITS and β -tubulin sequence data is *F. luteum* (clade V). The conidial morphology of this new species resembles that of *F.*

parvum, but it remains distinct in cultural morphology. Colonies are darker and more appressed than seen for other species. Mature conidia of *F. indigoticum* also tend not to become pigmented prior to germination, although this characteristic is not consistently useful. Conidial morphology alone may be confusing in defining this species and we recommend combining molecular and morphological data for identification.

Two isolates obtained in this study resided in a discrete clade (clade VI). The two isolates in this clade were recognised by Johnson *et al.* (1991) as an unknown species, from a mango SER pathogen survey in Australia. The fungus was not formally described, but referred to as *Dothiorella* 'long' (Johnson *et al.*, 1991; Johnson, 1992). *Dothiorella* 'long' has, however, been shown to belong to the genus *Fusicoccum* (Slippers *et al.*, 2001). In our study, clade VI isolates made up morphological group MG3, in which conidia are cylindrical to bacilliform and a yellow pigment is produced in the growth medium. Mycelial clumps are also produced in concentric rings, which is very different to any of the other *Botryosphaeria* spp. studied here. Sequence data separated the pairs of isolates from Australia and South Africa in clade VI. These groups were, however, not treated as distinct species due to the limited number of isolates and the lack of further distinction between them. We have, therefore, provided the name *Fusicoccum bacilliforme* for all isolates in clade VI. This species is made up of morphologically similar isolates from Australia and South Africa.

The only *Botryosphaeria* spp. with thick walled, dark conidia collected in this study was *L. theobromae* (*B. rhodina*). Isolates of this fungus were easily identified based on morphological characters and identifications were confirmed using DNA sequence data. *Lasiodiplodia theobromae* is known to cause SER of various fruit crops (Punithalingam,



1980) and infections are thought to occur both pre- and postharvest on mango. Although this species is commonly isolated together with *Botryosphaeria* spp. having hyaline conidia (eg. *B. ribis*), it tends to dominate the same niche only in warm, tropical regions (Brown & Britton, 1986; Johnson, 1992).

Results of this study lead us to conclude that four *Botryosphaeria* spp. occur on mango in South Africa. Two are new species of *Fusicoccum* of which one occurs on mango in South Africa and the other on mango in South Africa and Australia. Other *Botryosphaeria* spp. have, however, also been implicated as causal agents of diseases on mango in other countries, such as *F. mangiferum* (known as *D. mangiferae*) and *F. aesculi* (known as *D. aromatica*) (Table 2; p71), which are commonly collected in Australia (Johnson, 1992; Slippers *et al.*, 2001). These species are endophytes and care should be taken not to introduce them on vegetative growing material, which is often transported between countries.

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