CHAPTER 4

DEVELOPMENT AND TESTING OF A PCR-RFLP IDENTIFICATION SYSTEM FOR *BOTRYOSPHAERIA* SPECIES FROM MANGO

ABSTRACT

Botryosphaeria spp. are the primary cause of many diseases of mango fruit and trees. The taxonomy of these species is currently in disarray, because traditional morphological characteristics do not sufficiently distinguish species. In recent years, DNA sequencing has been introduced to resolve identification problems with *Botryosphaeria* spp., but this approach is impractical and inordinately expensive for the rapid identification of large numbers of isolates. The aim of this study was, therefore, to develop a PCR-RFLP system for rapid and reliable identification of *Botryosphaeria* isolates from mango. This technique was then used to identify a large collection of *Botryosphaeria* isolates from the main mango producing regions and cultivars in South Africa. ITS-PCR amplicons were digested with *Cfo*I, *Alu*I or *Bst*71I restriction enzymes (RE) to obtain polymorphic banding patterns. All four *Botryosphaeria* spp. from mango in South Africa were distinguished with *Cfo*I digestion of the PCR products. *Alu*I and *Bst*71I could distinguish Australian isolates *Fusicoccum aesculi* and *F. mangiferum* from the South African isolates. *Botryosphaeria parva* was the most dominant species among the isolates from mango in South Africa, followed by *B. rhodina*.

INTRODUCTION

Botryosphaeria spp. regularly cause stem and branch cankers, twig die-back, blossom blight, leaf spot and fruit rot on various woody hosts, including subtropical fruit crops such as mango (Punithalingam, 1980; Sutton, 1980; Pennycook & Samuels, 1984; Johnson, 1992; Lonsdale, 1993). These disease symptoms, of which stem end rot (SER) and soft brown rot (SBR) are amongst the most serious, are responsible for great losses for mango producers globally (Ramos *et al.*, 1991; Johnson, 1992; Donkin & Oosthuyse, 1996). In South Africa, the seriousness of mango tree diseases has recently been emphasised by wide-spread outbreaks in orchards resulting in tree deaths and substantial economic losses for mango producers (Finnemore, 2000).

Various anamorphs of *Botryosphaeria* are regularly found on trees and tree debri in mango orchards. The species identified as endophytes and opportunistic pathogens of mango in South Africa are the *F. parvum* Pennecook & Samuels (previously known as *Dothiorella dominicana*) (Chapter 2, Table 2; p71), *F. bacilliforme* Jacobs, Slippers & Wingf. (known as *Dothiorella* 'long'*), F. indigoticum* Jacobs, Slippers & Wingf. and *B. rhodina* (Berk. & Curt.) von Arx (Chapter 2). The relative importance of these different species on mango in South Africa is currently not known. Other *Botryosphaeria* spp. that also affect mango in Australia have been identified as *F. aesculi* Corda (known as *D. aromaticum*) and *F. mangiferum* (known as *Nattrassia mangiferae* (Nattrass.) Sutton et Dyko) (Chapter 2, Table 2; p71)(Johnson *et al.*, 1991; Johnson, 1992; Slippers et al., 2001).

Identification of *Botryosphaeria* spp. is mostly based on morphological characteristics of the associated anamorphs. Morphological similarities between the anamorphs, especially

among the *Fusicoccum* spp. has, however, hindered the identification of these fungi in the past (Johnson, 1992). In recent years, the use of molecular techniques, together with morphological characteristics, has proven useful in identifying *Botryosphaeria* anamorphs (Jacobs & Rehner, 1998; Denman *et al.*, 2000; Zhou & Stanosz, 2001; Slippers *et al.*, 2001; Smith & Stanosz, 2001; Smith *et al.*, 2001; Chapter 2). These molecular analyses are, however, mostly based on sequence data, the generation of which can be time consuming and costly to obtain for large numbers of isolates.

Various DNA-based techniques, other than DNA sequencing have been applied to identify fungal genera and species. These include random amplified polymorphic DNA (RAPD) (Zimand *et al.*, 1994; Thompson & Latorre, 1999), protein profiles (Lattore *et al.*, 1995), amplified fragment length polymorphisms (AFLPs) (Janssen *et al.*, 1996; Rosendahl & Taylor, 1997) and simple sequence repeats (SSRs) (Tautz, 1989; Weber & May, 1989). These techniques, however, have various limiting factors, such as non-repeatability, high levels of technical difficulty and the need for careful optimisation (Weising *et al.*, 1995; Buscot *et al.*, 1996). A relatively simple, yet reliable technique for distinguishing between strains is restriction fragment length polymorphism (RFLP) fingerprinting of polymerase chain reaction (PCR) products. This technique has often been used for identifying fungi up to species level (Bruns *et al.*, 1991; Buscot *et al.*, 1996; Taylor *et al.*, 1999).

Information regarding the occurrence and relative importance of different *Botryosphaeria* spp. on mango is important when developing effective disease control strategies. The first aim of this study was, therefore, to develop a rapid and effective identification system for *Botryosphaeria* isolates from mango, using PCR-RFLPs. This technique was subsequently utilised in an orchard survey to identify the dominant *Botryosphaeria* spp. in the main

mango producing regions of South Africa. From these data, the relationship between dominant *Botryosphaeria* species, symptoms expressed and cultivars affected, was also considered.

MATERIALS AND METHODS

Development of a PCR-RFLP identification protocol

Sequence data of the internally transcribed spacer (ITS1, 5.8S and ITS 2) region of known *Botryosphaeria* spp. from mango in Australia and South Africa (Chapter 2), were used to identify polymorphic restriction enzyme (RE) sites. This was achieved visually and using the programme Webcutter (http://www.firstmarket.com/cgi-bin/cutter). Restriction enzymes *Cfo*I, *Alu*I and *Bst*71I were identified from sequence data as potentially useful and thus utilised to produce distinguishable polymorphic banding patterns for the different species.

A modified version of the method of Raeder and Broda (1985) was used for DNA isolation from all isolates obtained during this study as described in Chapter 2. A portion of the nuclear rDNA operon was amplified with the polymerase chain reaction (PCR) using primers ITS1 and ITS4 (MWG Biotech, Germany) (White et al., 1990) as described in Chapter 2. All PCR products were digested with the restriction enzymes described above, one per reaction, and visualised on a 3% horizontal agarose gel using a TAE buffer electrophoresis system (Maniatis *et al.*, 1982).

Survey using PCR-RFLP

Botryosphaeria isolates were obtained during an orchard survey of five regions in the Northern province and Mpumalanga, namely Constantia (20%), Hoedspruit (32%), Letsetele Valley (24%), Malelane (20%) and Mariepskop (6%) (Table 1; p135)(Fig. 1; p137). This is the primary mango producing areas of South Africa. To obtain the total number of samples, the commercial cultivars Sensation (26%), Tommy Atkins (37%), Keitt (11%), Kent (19%) and Heidi (6%) were sampled (Table 1; p135). Isolations were made from asymptomatic material (8%) or symptomatic tree trunks (8.5%), branches (29.5%), leaves (5%), blossoms (9%) and fruit (40%) from different orchards during August to September 1999 and 2000 (late winter to early spring) (Table 1; p135) (Fig. 2; p139).

Samples were disinfested twice with 70% (v/v) ethanol and air died for five minutes. Isolations from symptomatic material were made from disks cut at the lesion edge. Disks of asymptomatic plant tissue were cut from all plant parts. All isolates were cultured on potato dextrose agar (PDA) (Biolab) amended with chloramphenicol and were incubated at 25°C for seven to twelve days. Isolates were induced to sporulate on water agar (Biolab) amended with sterile pine needles, and single spore isolates were made, as described in Chapter 2. All single spore isolates were identified by using the PCR-RFLP identification system as described previously. All cultures are maintained at 4°C in the culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria.

Development of PCR RFLP identification

All four *Botryosphaeria* spp. isolated from mango in South Africa could be differentiated by cleavage of the ITS PCR products with RE *Cfo*I and visualisation of the polymorphic banding patterns (Fig. 3; p141). *Cfo*I digestion of the PCR amplicons did not produce polymorphic banding patterns for *F. indigoticum* and two *Botryosphaeria* spp. isolated from mango in Australia, namely *F. mangiferum* and *F. aesculi* (Fig. 3; p141). Cleavage of the ITS PCR products with RE *Alu*I differentiated *F. aesculi* from *F. indigoticum* and *F. mangiferum* (Fig. 4; p143). *F. indigoticum* and *F. mangiferum* ITS PCR amplicons were then separated with the RE *Bst*71I (Fig. 5; p145). Sizes of the fragments of the ITS PCR products after cleavage with *Cfo*I, *Alu*I and *Bst*71I are indicated on a RE cleavage site map (Fig. 6; p147).

Survey using PCR-RFLP

A total of 156 *Botryosphaeria* isolates were obtained from mango tree trunks (5.1%), branches (35.3%), leafs (0.6%), blossoms (5.1%) and fruit (46.2%) (Table 1). These *Botryosphaeria* spp. were isolated from all five mango producing regions in the Northern province and Mpumalanga, namely Mariepskop (7.7%), Letsetele Valley (34.6%), Constantia (9.6%), Hoedspruit (35.6%) and Malelane (12.2%). The different mango cultivars namely, Sensation (34.6%), Tommy Atkins (49.4%), Kent (1.9%), Keitt (13.5%) and Heidi (1.3%), all yielded *Botryosphaeria* isolates.

All isolates obtained were identified to species level, using the PCR-RFLP technique described previously (Table 1; p135). Results of this survey showed that *B. parva*

represents 82.1% of isolates obtained in this study from diseased fruit, leaves, branches and tree trunk material, as well as asymptomatic tissue (Table 1; p135). This species was, however, isolated most frequently from tree branches and fruit (Table 1; p135). *Botryosphaeria rhodina* was isolated as the second most dominant species (16.0%), mostly from fruit and asymptomatic plant material (Table 1; p135). *Fusicoccum indigoticum* was isolated only from a fruit rot and *F. bacilliforme* from a cankered leaf and discoloured tree branch with a 1.3% isolation frequency each (Table 1; p135).

DISCUSSION

In this study, the four *Botryosphaeria* spp. found on mango in South Africa, namely *F. indigoticum*, *F. bacilliforme*, *B. parva* and *B. rhodina,* were easily identified using the PCR-RFLP technique that was developed. These species could also be distinguished from two species found in Australia, which have not yet been identified from mango in South Africa, namely *F. mangiferum* and *F. aesculi*. This technique overcomes the difficulties experienced using morphological characteristics to identify *Botryosphaeria* spp. from mango. It is simple and rapid and negates problems experienced when needing to sequence DNA from large numbers of isolates in order to confirm their identity.

Botryosphaeria parva was the dominant *Botryosphaeria* spp. found on mango in South Africa. It was isolated from symptomatic and asymptomatic mango tissue from four commercial cultivars in all five production regions surveyed. This species was more frequently obtained than any other *Botryosphaeria* spp., from all plant parts, and was most frequent on fruit and branches. *Botryosphaeria parva* is also the dominant *Botryosphaeria* sp. reported as a pathogen on other woody hosts in various countries, where it causes

diseases that contribute to substantial economic losses (Brown & Britton, 1986; Reckhaus, 1987; Shearer *et al.*, 1987; Darvas, 1991). Furthermore, *B. parva* has been shown to be one of the most pathogenic *Botryosphaeria* spp. on mango (Ramos *et al.*, 1991; Chapter 3). We, therefore, consider *B. parva* to be the main cause of *Botryosphaeria* diseases on mango trees in the orchards and on mango fruit in South Africa. Management practices should thus focus strongly on controlling this species.

In this survey, *B. rhodina* was the second most dominant species isolated. This species was mostly obtained from fruit rots and asymptomatic plant tissue. These findings are similar to those from previously published literature, where *B. rhodina* is well-documented as endophyte and the most common fruit rot pathogen of many fruit crops, including mango (Punitalingham, 1980; Sanchote, 1991; Johnson, 1992). The fact that this species was infrequent or absent from any symptomatic plant parts other than the fruit, suggests that it is probably insignificant in causing tree diseases. This is despite the fact that it has been shown to be able to cause significant lesions on inoculated trees (Chapter 3).

No isolates of *F. indigoticum* and *F. bacilliforme* were identified, other than those included for reference purposes from a previous study (Chapter 2). These species have recently been described from South Africa for the first time in Chapter 2, and have been shown to be pathogenic and weakly pathogenic, respectively (Chapter 3). *Fusicoccum indigoticum* was isolated from diseased fruit and leaves, while *F. bacilliforme* isolates were obtained only from diseased branches. Our survey suggests that these species are relatively unimportant in causing disease on mango in South Africa.

Botryosphaeria spp. were isolated from all mango producing regions of South Africa surveyed, but were isolated more commonly in certain regions. The highest frequency of *Botryosphaeria* spp. present was in the regions, Letsetele Valley, Hoedspruit and Mariepskop. These regions contributed to over 75% of all isolates obtained, although only 62% of the samples were collected from the areas. These results can be due to different environmental stress conditions on mangoes in the different regions (Johnson, 1992). Regions with higher rainfall (such as the Letsetele Valley) are usually more severely affected by *Botryosphaeria* spp. due to water stress (Johnson *et al.*, 1992).

Botryosphaeria spp. were isolated in varying frequency from the different commercial mango cultivars grown in South Africa. For example, *Botryosphaeria* spp. were isolated from Sensation and Tommy Atkins with a higher frequency (63% of total samples yielding 80% of total isolates) than was the case with Keitt and Kent (30% of total samples yielding 18% of total isolates). This correlates with the fact that Keitt and Kent are more disease tolerant under field conditions than Tommy Atkins and Sensation (Finnemore, 2000). Very few isolates were obtained from cultivar Heidi, but this might also be due to the fact that very few samples of this cultivar were available.

Botryosphaeria diseases symptoms were most common on mango tree branches and fruit. By far the highest number (80%) of all *Botryosphaeria* isolates obtained in this study were isolated from these symptoms, while 12% were obtained from diseased tree trunks, leaves and blossoms. A very small number (8%) of all isolates were from asymptomatic tissue, but these come from all different plant parts. These data correspond with findings of Johnson *et al.*, (1992), in which the endophytic colonisation of healthy mango tissue by *Botryosphaeria* spp. was evident in all mango plant parts. The fact that endophytic

colonisation is found in all healthy plant parts can be explained by movement of *Botryosphaeria* spp. between plant parts through the vascular system or by individual infections on the same tree (Johnson *et al.*, 1992; Ramos *et al.*, 1991; Johnson *et al.*, 1992; Lonsdale, 1993).

The PCR-RFLP identification system developed in this study was used successfully to identify a large number of *Botryosphaeria* isolates to species. In future, this technique could be used to identify *Botryosphaeria* pathogens responsible for disease outbreaks and will thus influence the information used to implement the appropriate control measures. This RFLP technique should also be useful in quarantine measures, enabling screening of samples to prevent introduction of mango pathogens, e.g. *F. aesculi* and *F. mangiferum* that currently do not occur in South Africa.

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Table 1 Distribution of four *Botryosphaeria* spp. from mango obtained in this study

Species	Area	Total samples	Total isolates	*B. ribis*	*F. indigoticum*	*F. bacilliforme*	*B. rhodina*
Isolates		**250**	**156**	**128**	**2**	**2**	**25**
Region	Mariepskop	15	12	7	***	***	5
	Letsetele Valley	59	54	52	1	***	1
	Constantia	48	15	15	***	***	***
	Hoedspruit	80	56	38	1	***	18
	Malelane	48	19	16	***	2	1
Cultivar	Sensation	65	54	45	1	***	8
	Tommy Atkins	92	77	60	1	***	16
	Kent	49	3	3	***	***	***
	Keitt	29	21	20	***	***	1
	Heidi	15	2	***	***	2	***
Plant part	Tree trunks	21	8	8	***	***	***
	Branches	74	55	49	***	2	3
	Leafs and leaf stems	14	1	1	1	***	***
	Blossoms	22	8	8	***	***	***
	Fruit	99	72	54	1	***	17
	Asymptomatic tissue	20	13	8	***	***	5

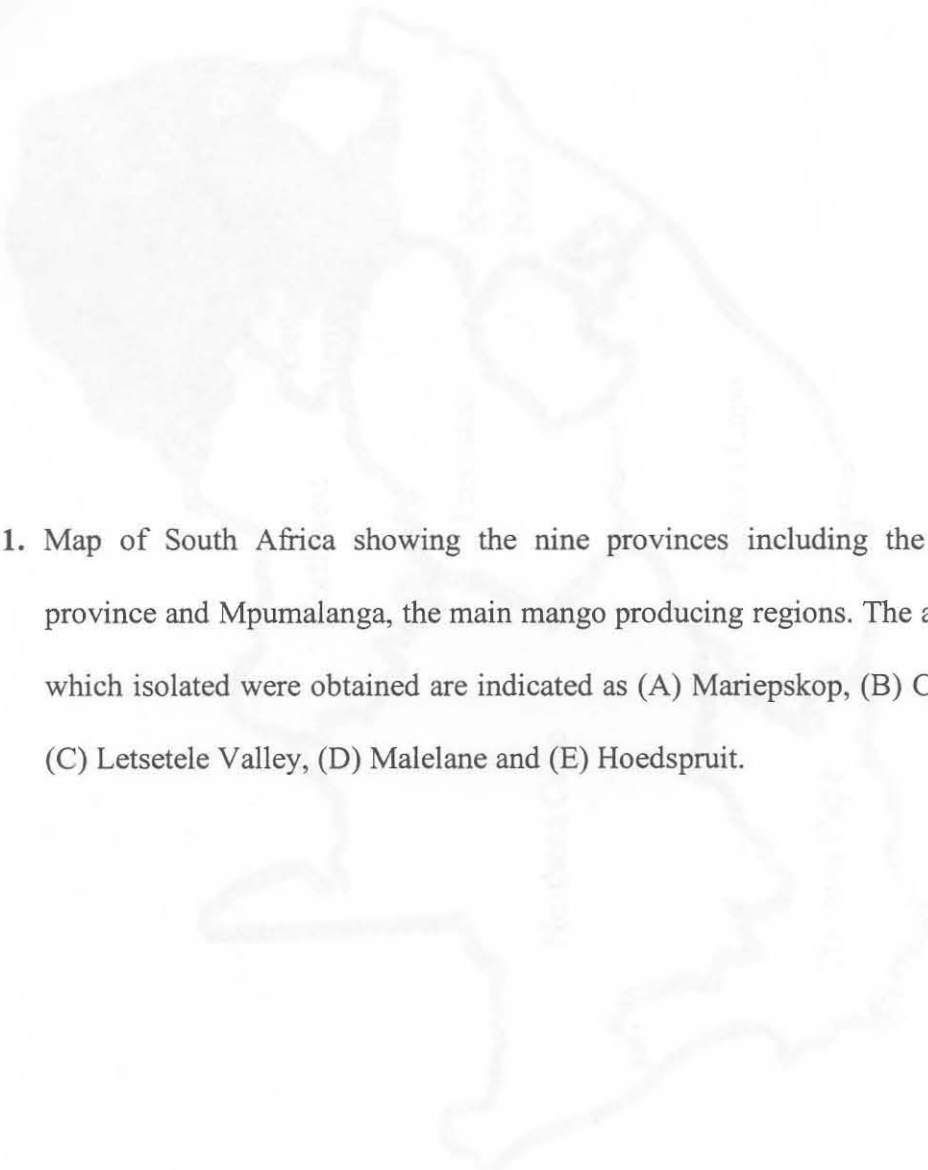
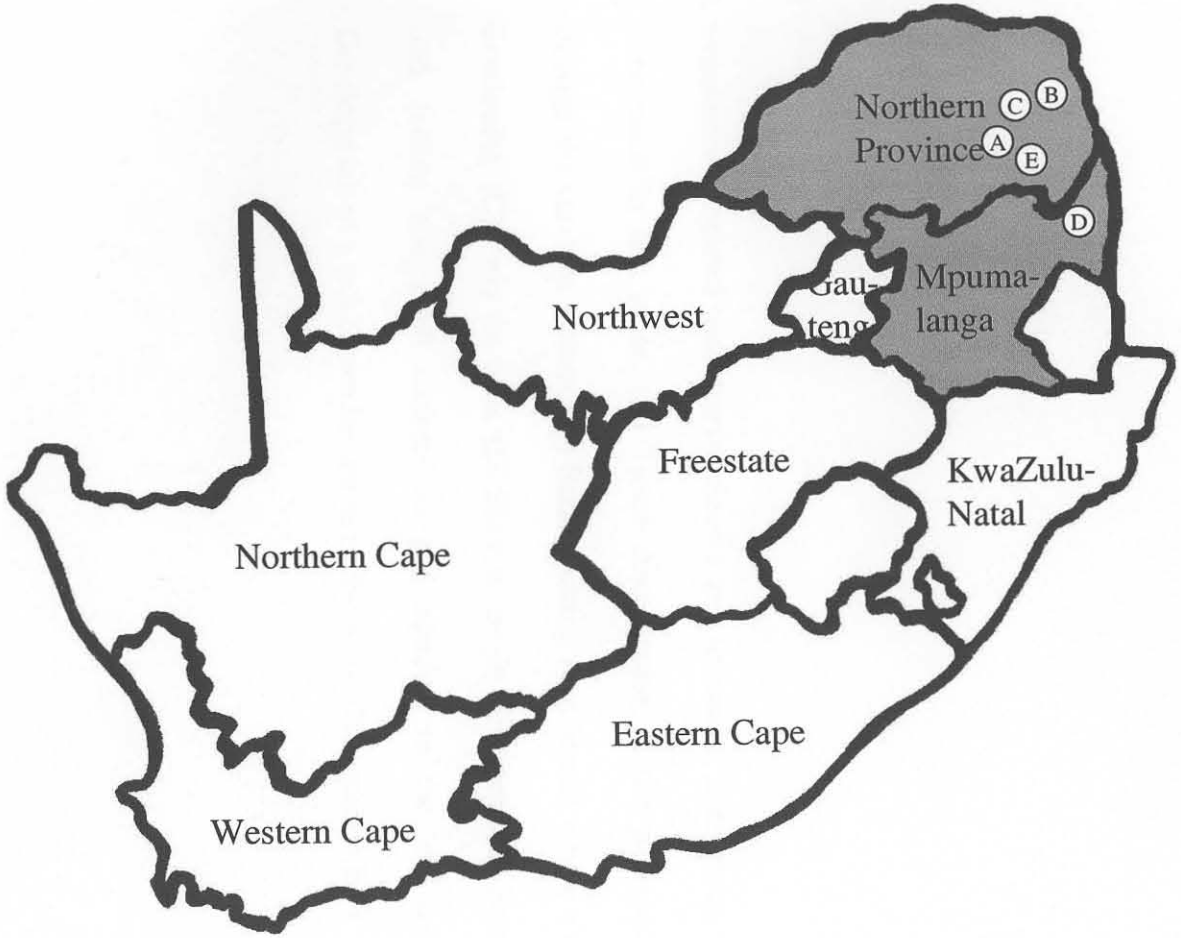


Figure 1. Map of South Africa showing the nine provinces including the Northern province and Mpumalanga, the main mango producing regions. The areas from which isolated were obtained are indicated as (A) Mariepskop, (B) Constantia, (C) Letsetele Valley, (D) Malelane and (E) Hoedspruit.



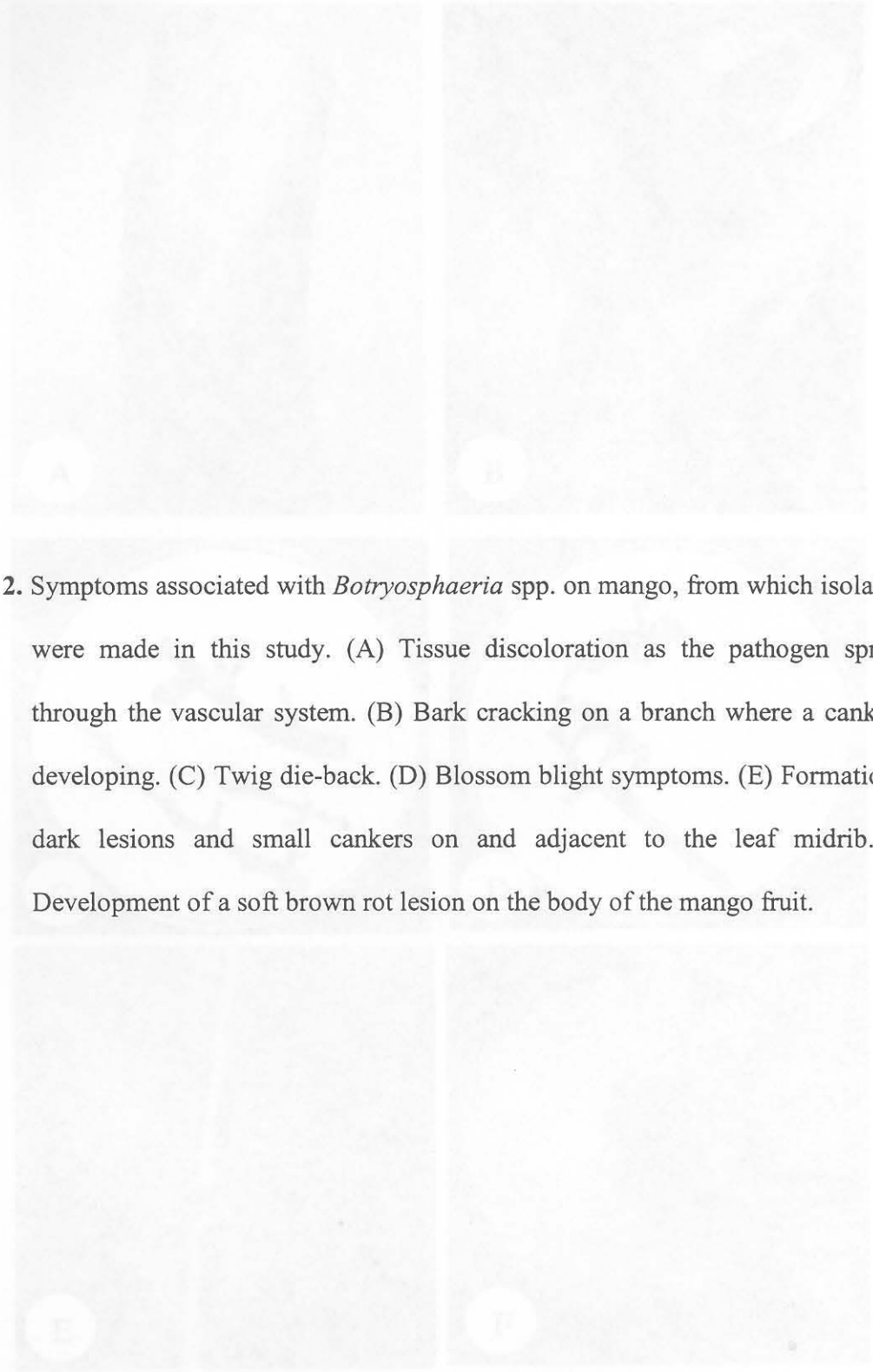


Figure 2. Symptoms associated with *Botryosphaeria* spp. on mango, from which isolations were made in this study. (A) Tissue discoloration as the pathogen spreads through the vascular system. (B) Bark cracking on a branch where a canker is developing. (C) Twig die-back. (D) Blossom blight symptoms. (E) Formation of dark lesions and small cankers on and adjacent to the leaf midrib. (F) Development of a soft brown rot lesion on the body of the mango fruit.

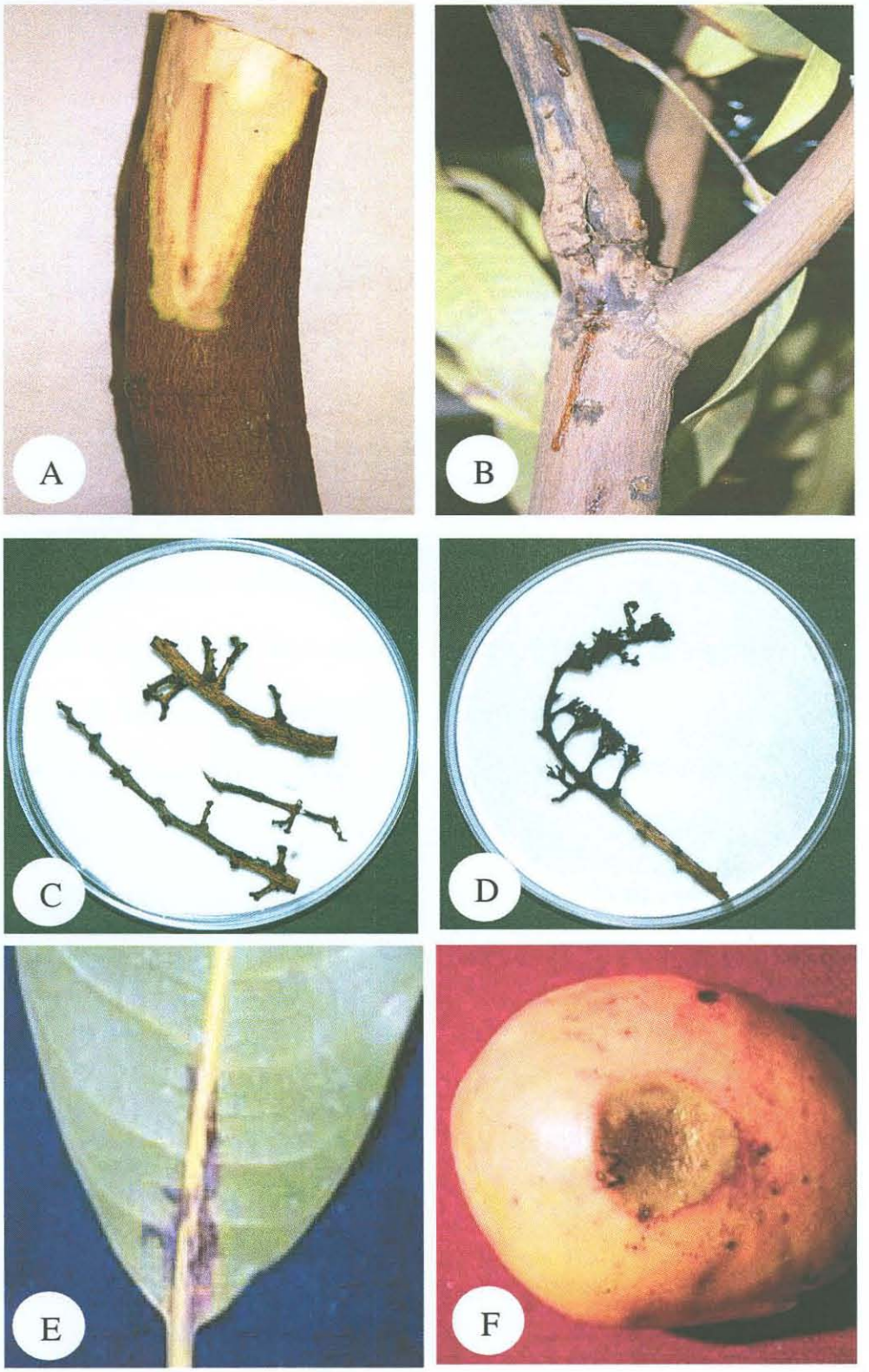
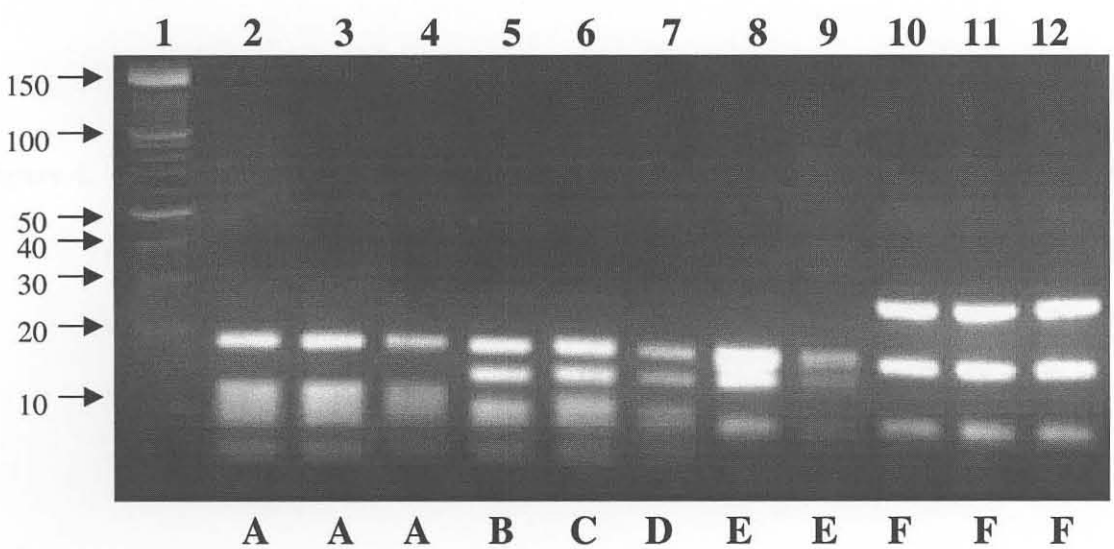


Figure 3. A 3% agarose gel indicating polymorphic banding patterns after digestion of the PCR amplicon with the restriction enzyme *Cfo*I. Lane 1 represents the 100bp marker. Lanes 2-12 represents (A) *Fusicoccum* parvum, (B) *F. indigoticum*, (C) *F. mangiferum,* (D) *F. aesculi*, (E) *F. bacilliforme* and (F) *B. rhodina*.


1 2 3 4 5 6 7 8 9 10 11 12
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A A A B C D E E F F F

Figure 4. A 3% agarose gel indicating polymorphic banding patterns after digestion of the PCR amplicon with the restriction enzyme *Alu*I. Lane 1 represents the 100bp marker and lanes 2-4 represent (A) *F. indigoticum*, (B) *F. aesculi* and (C) *F. mangiferum*.

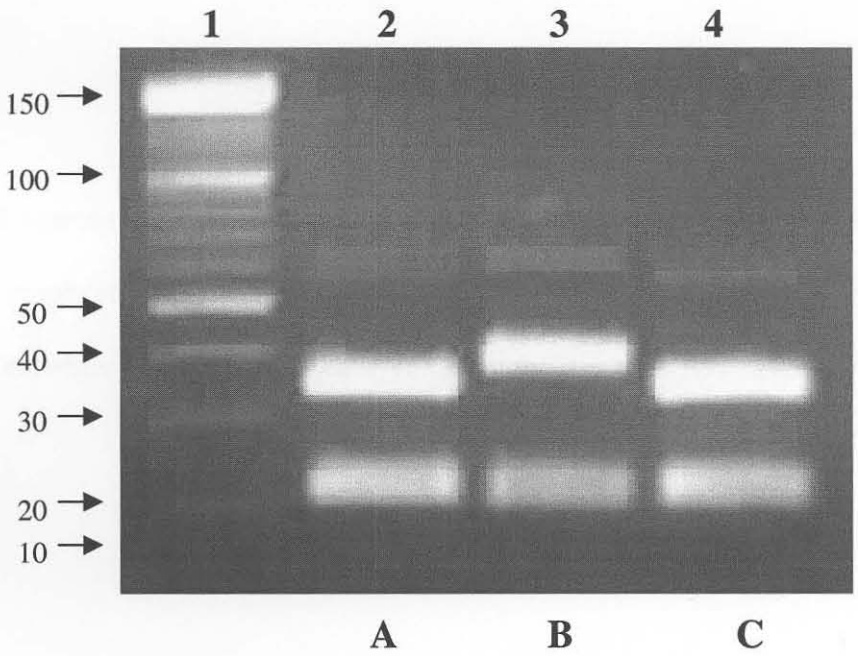
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Figure 5. A 3% agarose gel indicating polymorphic banding patterns after digestion of the PCR amplicon with the restriction enzyme *Bst*71I. Lane 1 represents the 100bp marker, while lanes 2-3 represent (A) *F. indigoticum* and (B) *F. mangiferum.*

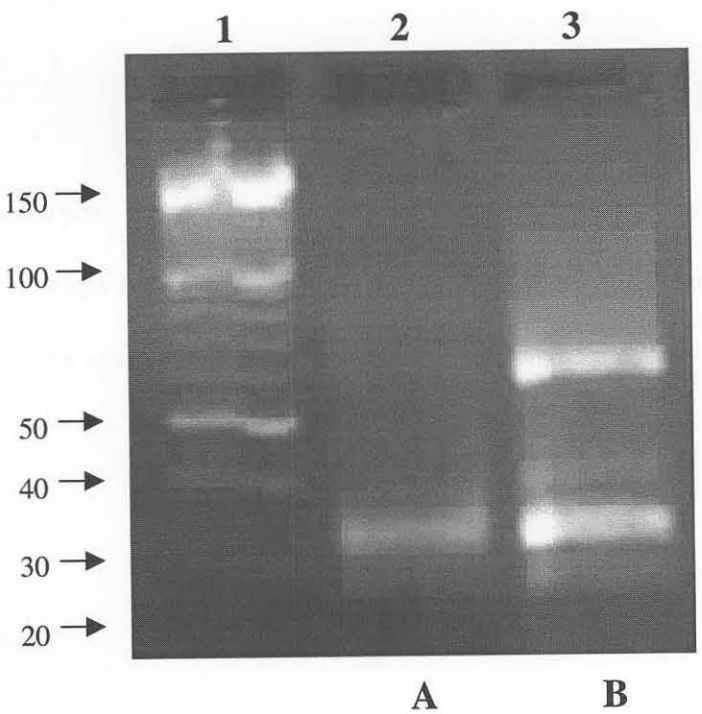
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Figure 6. Restriction enzyme maps generated to identify the *Botryosphaeria* spp. from mango. The maps indicate product sizes generated after digestion of the ITS PCR amplicons (indicating ITS1, 5.8S and ITS4) with (A) *Cfo*I, (B) *Alu*I and (C) *Bst*71I.

A. CfoI

Botryosphaeria parva

121 ↓ 22 ↓ 182 ↓ 90 ↓ 64 ↓ 72 (550)

F. indigoticum

143 ↓ 182 ↓ 90 ↓ 64 ↓ 71 (550)

F. bacilliforme

147 ↓ 181 ↓ 89 ↓ 138 (555)

F. mangiferum

145 ↓ 182 ↓ 90 ↓ 64 ↓ 74 (555)

F. aesculi

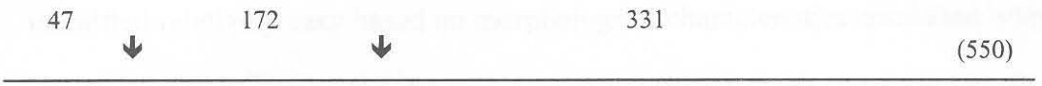
146 ↓ 180 ↓ 88 ↓ 70 ↓ 72 (556)

B. rhodina

284 92 ↓ 138 ↓ (514)

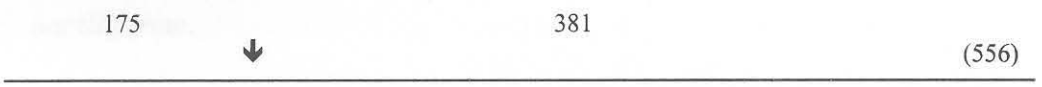
B. *Alu* I

F. indigoticum



F. mangiferum

F. aesculi



C. *Bst* 71I

F. indigoticum

F. mangiferum