Pathogenic Botryosphaeriaceae associated with *Mangifera indica* in the Kimberley Region of Western Australia

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Abstract

Members of the Botryosphaeriaceae, in particular *Lasiodiplodia theobromae*, *Neofusicoccum parvum, Neofusicoccum mangiferae* and *Botryosphaeria dothidea*, commonly cause stem cankers, dieback and stem end rot of mangoes worldwide. In the current study, eight taxa of Botryosphaeriaceae were identified as canker-associated fungi, pathogens, potential pathogens or endophytes of mangoes in the Kimberley, Australia. These include *Neoscytalidium novaehollandiae, Neoscytalidium dimidiatum, Pseudofusicoccum adansoniae, P. ardesiacum, P. kimberleyense, Lasiodiplodia* sp. 1, *Lasiodiplodia iraniensis* and *Lasiodiplodia pseudotheobromae.* The pathogenicity of a selection of these species toward fruit and branches was tested. All were pathogenic to mango in comparison to the control, with *Lasiodiplodia* spp. being the most pathogenic. It appears that either geographic isolation or the unique growing conditions in the Kimberley may have provided an effective barrier to the acquisition or establishment of known botryosphaeriaceous pathogens. Wounds caused by mechanical pruning may provide an entry point for infection, whilst severe pruning may increase plant stress.

Introduction

The horticultural industry in Australia is the second largest and fastest growing sector of agriculture. In 2008, the Australian mango industry contributed $100 million towards the $9 billion generated by the horticultural industry (www.mangoes.net.au; (DAFF 2008). Mango growing centres are concentrated in Queensland (QLD) but smaller centres have been established in northern New South Wales (NSW), Darwin and Katherine in the Northern Territory (NT), Kununurra in the Ord River Irrigation Area (ORIA), Carnarvon and Gingin in Western Australia (WA) (Australian horticultural fact sheet, 2008; www.mangoes.net.au).

Mangifera indica is endemic to north-eastern India and Myanmar, but is now grown in tropical climates world-wide (Kostermans and Bompard 1993).There is over 1000 cultivars of mango, while Australia grows only a few of these commercially. The most common cultivar grown in Australia is Kensington Pride. Mangoes typically produce flowers in the dry season and set fruit at the start of the wet season. These climatic factors in combination with rapidly ripening fruit provide conditions favourable to fungal disease development (Johnson 1993, 1997).

Mangoes are affected by a variety of pests and pathogens. About 5% of fruit is lost to post-harvest diseases, although this figure can rise to 100% if conditions favour disease development and proper management strategies are not in place (Johnson 2008). The major post-harvest diseases affecting mango are anthracnose, stem end rot (SER) and dendritic spot. Anthracnose is considered easy to control and is caused by *Colletotrichum gloeosporioides* and *Colletotrichum acutatum*, causing disease in young trees and fruit in orchards. SER is caused by a range of species of the Botryosphaeriaceae including *N. parvum*, *N. mangiferae*, *B. dothidea* and *L.*

theobromae (Slippers et al. 2005; Johnson 2008). These pathogens may establish asymptomatically in the field, with disease expression linked to stress or ripening fruit. These fungi express after the fruit have been harvested, causing a watery rot that starts from the stem end of the fruit then progresses throughout the rest of the fruit. Dendritic spot, which is characterised by irregular shaped superficial lesions on the surface of the fruit, is thought to be caused by *N. parvum* (Johnson 2008).

The same group of fungi that causes SER and dendritic spot have also been associated with mango decline. Mango dieback in the north (N) and NW of Australia has been associated with *N. dimidiatum and N. novaehollandiae* (Ray et al. 2010). Tip dieback has been reported in mango trees situated in Florida, associated with infection by *B. dothidea* (Ploetz et al. 1996) and *N. ribis* (Ramos et al. 1991; Ramos et al. 1997; Prakash and Eckert 2001). *Lasiodiplodia theobromae* also causes cankers, dieback and sap staining in other woody trees (Mohali 1993; Mohali et al. 2002; Slippers and Wingfield 2007; Punithalingam 1980). *Botryosphaeria dothidea* has also been reported to cause cankers and fruit rot in numerous woody plants such as apples (Brown-Rytlewski and McManus 2000), dieback in grapevines (Phillips 1998), and shoot and panicle blight in pistachios (Ahimera et al. 2003).

Species of Botryosphaeriaceae generally have the ability to colonise a wide range of woody hosts in various environments throughout the world (see Slippers and Wingfield 2007 for an extensive review). They occupy a range of niches, but are commonly studied because of their ability to switch between a quiescent and pathogenic lifestyle and cause disease. They can enter plant tissue through reproductive structures such as seeds or can enter the plant through wounds, stomata or lenticels and can infect the stems, twigs, roots and leaves (Brown and Hendrix 1981; Smith et al. 1996a; Smith et al. 1996b). These fungi have the ability to colonise hosts without any outward symptom development and they may remain quiescent inside the host for a length of time, where asymptomatic colonisation of hosts is characteristic of endophytes (Stone et al. 2004; Seiber 2007). This quiescent phase may be disrupted by some form of stress on the host; such as water stress, causing the pathogenic phase of the lifecycle to be triggered.

Historically, crop pathogens have been identified using morphological evidence (isolation and identification of potential pathogens from tissues that show disease symptoms by microscopic examination of spores). Morphological identification generally provides a quick and inexpensive means of identification. However, the telomorphs of many species of Botryosphaeriaceae are rarely encountered and the anamorphs often have overlapping morphological characteristics and share niches. This has led to confusion and misidentification of species, necessitating the use of molecular phylogenetic methods to confirm species level identification (Smith and Stanosz 2001; Burgess et al. 2005; Slippers et al. 2005; Burgess et al. 2006a; Crous et al. 2006).

Recently, seven new species of Botryosphaeriaceae were described from native hosts in the Kimberley area of N Western Australia (Pavlic et al. 2008). Subsequent surveys in the Kimberley region have revealed dieback and cankers in mango orchards. In this study, we have isolated, identified and determined the pathogenicity of botryosphaeriaceous fungi associated with stem cankers and dieback of mango trees located in the Kimberley.

Materials and Methods

Isolation and data collection

Diseased stems and severe pruning wounds of *M. indica* were observed in orchards that utilised mechanical pruning (Fig. 3.1 A- D). Stem and twig samples were collected from *M. indica* exhibiting dieback and canker symptoms in Kununurra, Broome and Derby from orchards that both use mechanical pruning, or not (Fig. 3.1 E & F). One sample was also collected from *M. indica* in Tiwi Island, NT. Diseased stems and twigs were dipped in 70% ethanol and then flamed. Sterilised secateurs were used to remove ends off the wood, the ends were then discarded. The cut sections were plated out onto on half strength Potato Dextrose Agar (PDA) (19.5g BBL™ PDA, 7.5g agar and 1L distilled water) with Rifampicin added (SIGMA 95% HPLC added at 20mg/kg). Plates were incubated at room temperature (temperature 28 - 33°C), and after three to five days mycelia were subcultured onto half strength PDA plates. Cultures

were initially sorted into groups based on colony morphology. Cultures of all isolates were deposited at the WA culture collection, Agricultural Department of Western Australia (WAC) or Murdoch University (MUCC) (Table 3.1).

Figure 3.1 (A) Mechanical pruning of a mango orchard. (B- D) Pruning wounds on mango stems from mechanical pruning. (E) Dieback of branch in orchard where trees were pruned mechanically. (F) Lesion visible on darkened area of mango stem where trees were pruned mechanically. (G) Lesion caused by inoculation with *Lasiodiplodia* sp. 1 visible on surface and in wood. (H) Lesion caused by inoculation with *Neoscytalidium dimidiatum* visible on surface and in wood. (I) Stem inoculated with control agar plug. (J) Range of lesions produced by inoculation of mango fruit, black arrow indicates fruit inoculated with control agar plug.

Table 3.1 Isolates of Botryosphaeriaceae used in this study.

Code1	Identity	Source (if known)	Host	Location	Isolator	GenBank Accession^	
						ITS	EF-1α
MUCC738	*Lasiodiplodia hormozganensis**	stem	*Adansonia digitata*	Darwin, NT	ML Sakalidis	HM355855	GU199408
WAC13290	*L. iraniensis*	canker	*Mangifera indica*	Broome, WA	J Ray	GU172381	HM218824
WAC13297	*L. iraniensis*	stem	*M. indica*	Kununurra, WA	J Ray	GU172379	HM218823
WAC13281	*L. pseudotheobromae*#	stem	*M. indica*	Broome, WA	J Ray	GU172380	GU172412
WAC13280	*Lasiodiplodia* sp. 1*	canker	*M. indica*	Broome, WA	J Ray	GU172377	GU172408
WAC13300	*Lasiodiplodia* sp. 1	stem	*M. indica*	Broome, WA	J Ray	GU172378	GU172409
MUCC741	*Lasiodiplodia* sp. 2*	stem	*A. gregorii*	Darwin, NT	ML Sakalidis	GU199389	GU199411
WAC12693	*Neoscytalidium dimidiatum*	canker	*M. indica*	Kununurra, WA	J Ray	EF585538	EF585576
WAC13284	*N. dimidiatum*	stem	*M. indica*	Kununurra, WA	J Ray	GU172382	GU172414
WAC13285	*N. dimidiatum*	stem	*M. indica*	Kununurra, WA	J Ray	GU172383	GU172415
WAC13301	*N. dimidiatum*	stem	*M. indica*	Kununurra, WA	J Ray	GU172384	GU172416
WAC12690	*N. dimidiatum*	canker	*M. indica*	Kununurra, WA	J Ray	EF585537	EF585577
WAC13287	*N. dimidiatum*	stem (c)	*M. indica*	Kununurra, WA	J Ray	GU172385	GU172417
WAC13277	*N. dimidiatum*#	stem (c)	*M. indica*	Derby, WA	J Ray	GU172388	GU172420
WAC13276	*N. dimidiatum*#	stem (c)	*M. indica*	Broome, WA	J Ray	GU172389	GU172421
WAC13305	*N. dimidiatum*	stem	*M. indica*	Broome, WA	J Ray	GU172390	GU172422
WAC13274	*N. dimidiatum*#	stem	*M. indica*	Tiwi Island, NT	J Ray	GU172391	GU172423
WAC12691	*N. novaehollandiae*	canker	*M. indica*	Kununurra, WA	J Ray	EF585542	EF585574
WAC12688	*N. novaehollandiae*	canker	*M. indica*	Kununurra, WA	J Ray	EF585543	EF585575
WAC13296	*N. novaehollandiae*	canker	*M. indica*	Kununurra, WA	J Ray	GU172392	GU172424
WAC13286	*N. novaehollandiae*	canker	*M. indica*	Kununurra, WA	J Ray	GU172393	GU172425
WAC13288	*N. novaehollandiae*	stem	*M. indica*	Kununurra, WA	J Ray	GU172394	GU172426
WAC13289	*N. novaehollandiae*	stem	*M. indica*	Kununurra, WA	J Ray	GU172395	GU172427
WAC13291	*N. novaehollandiae*	stem	*M. indica*	Kununurra, WA	J Ray	GU172396	GU172428

Code1	Identity	Source (if known)	Host	Location	Isolator	GenBank Accession^	
						ITS	EF-1α
WAC13273	*N. novaehollandiae**#	stem (c)	*M. indica*	Kununurra, WA	J Ray	GU172397	GU172429
WAC13303	*N. novaehollandiae*	stem	*M. indica*	Derby, WA	J Ray	GU172398	GU172430
WAC13304	*N. novaehollandiae*	stem	*M. indica*	Derby, WA	J Ray	GU172399	GU172431
WAC13275	*N. novaehollandiae**#	stem	*M. indica*	Derby, WA	J Ray	GU172400	GU172432
WAC12689	*Pseudofusicoccum adansoniae*	canker	*M. indica*	Kununurra, WA	J Ray	EF585534	EF585567
MUCC525	*P. adansoniae*	canker	*M. indica*	Kununurra, WA	TI Burgess	EF585527	EF585573
WAC13292	*P. adansoniae*	stem (c)	*M. indica*	Kununurra, WA	J Ray	GU172401	GU172433
WAC13278	*P. adansoniae*#	stem	*M. indica*	Kununurra, WA	J Ray	GU172402	GU172434
WAC13295	*P. adansoniae*	stem	*M. indica*	Derby, WA	J Ray	GU172403	GU172435
WAC13299	*P. adansoniae*	stem	*M. indica*	Derby, WA	J Ray	GU172404	GU172436
CMW26145	*P. adansoniae**	stem	*Acacia synchronica*	the Kimberley, WA	ML Sakalidis	*EF585525*	*EF585569*
CMW26146	*P. adansoniae*	stem	*Eucalyptus* sp.	the Kimberley, WA	ML Sakalidis	*EF585532*	*EF585570*
WAC13294	*P. ardesiacum*	stem	*M. indica*	Derby, WA	J Ray	GU172405	GU172437
WAC13279	*P. adansoniae/kimberleyense*#	stem	*M. indica*	Derby, WA	J Ray		
WAC13293	*P. kimberleyense*	stem	*M. indica*	Derby, WA	J Ray	GU172406	GU172438
WAC13298	*P. kimberleyense*	stem	*M. indica*	Derby, WA	J Ray	GU172407	GU172439
MUCC742	*P. kimberleyense**	stem	*A. rubrostipa*	Darwin, NT	ML Sakalidis	HM355856	GU199412
CMW26158	*P. kimberleyense*	stem	*A. gregorii*	the Kimberley, WA	MJ Wingfield	*EU144058*	*EU144073*
CMW26157	*P. kimberleyense*	stem	*Eucalyptus* sp.	the Kimberley, WA	ML Sakalidis	*EU144056*	*EU144071*
CMW26156	*P. kimberleyense*	stem	*A. synchronica*	the Kimberley, WA	ML Sakalidis	*EU144057*	*EU144072*
CMW26161	*P. kimberleyense*	stem	*F. opposita*	the Kimberley, WA	ML Sakalidis	*EU144059*	*EU144074*

*Indicates isolates used in the stem pathogenicity trial. #Indicates isolates used in the fruit pathogenicity trial. ^Sequence numbers in italics were obtained from GenBank. (c) Indicates isolates came from a tree also with a canker.

Molecular phylogenetic characterisation

DNA was extracted from freeze dried mycelia using a modified method from Graham et al. (1994) as described in Andjic et al (2007). A part of the internal transcribed spacer (ITS) region of the ribosomal DNA operon amplified using the primers ITS-1F (Gardes and Bruns 1993) and ITS4 (White et al. 1990) and part of the elongation factor 1-α (EF1-α) was amplified using EF1-728F & EF1-986R (Carbone et al. 1999) or EF1F & EF2R (Jacobs et al. 2004). The PCR reaction mixture and PCR conditions were as described previously (Burgess et al. 2005). PCR products were cleaned using sephadex G-50 (Sigma Aldrich) columns according to manufacturer's instructions. The purified filtrate was used in the sequencing reaction. Products were sequenced with the BigDye terminator cycle sequencing kit (PE Applied Biosystems) using the same primers that were used in the initial amplification. The products were also cleaned in sephadex G-50 columns and were separated by an ABI 3730 48 capillary sequencer (Applied Biosystems, Foster City, California).

In order to compare the isolates used in this study with related species, sequences of known vouchered isolates (where possible the type isolate was included) were obtained from GenBank (www.ncbi.nlm.nih.gov/Genbank). The isolate code, identity and accession numbers for these sequence data used given in TreeBASE (www.treebase.org access code: S10464). Parsimony analysis was performed on individual and combined datasets in PAUP (Phylogenetic Analysis Using Parsimony) version 4.0b10 (Swofford 2003). Non-informative characters were removed prior to analysis and characters were unweighted and unordered. The most parsimonious trees were obtained by using heuristic searches with random stepwise addition in 1000 replicates, with the tree bisection-reconnection branch-swapping option on and the steepest-descent option off. Maxtrees automatically increased by 100, branches of zero length were collapsed and all multiple equally parsimonious trees were saved. Estimated levels of homoplasy and phylogenetic signal (retention and consistency indices) were determined (Hillis and Huelsenbeck 1992). Branch and branch node supports were determined using 1000 bootstrap replicates (Felsenstein 1985).

Pathogenicity trial on excised stems

For this trial mango stems from unsprayed mature trees, Cv. Kensington Pride, were harvested from a commercial block in Skuthorpe, Broome. The cut ends were dipped in wax and transported via overnight air express to Murdoch University, Perth. Stems were halved and cut ends were dipped in wax. Nine isolates of the obtained isolates were selected for this test representing *Lasiodiplodia* sp. 1 (WAC13280), *L. hormozganensis* (MUCC738), *Lasiodiplodia* sp. 2 (MUCC741), *N. dimidiatum* (WAC13274 and WAC13277), *N. novaehollandiae* (WAC13275 and WAC13273), *P. adansoniae* (CMW26145) and *P. kimberleyense* (MUCC742) (Table 3.1). *Lasiodiplodia hormozganensis* and *Lasiodiplodia* sp. 2 were used as they were identified as part of the endophytic flora of Adansonia spp. in George Brown Darwin Botanic Gardens in the Northern Territory (Sakalidis et al. 2011). All isolates were grown on ½ strength PDA agar plates for approximately 5-10 days at 20˚C.

Using a sterile scalpel blade a small lateral incision was made halfway along the stem through the bark and cambium, avoiding the sapwood in the middle of the stem, into which a 0.5 cm^2 agar plug colonized with mycelium was inserted (with the mycelium orientated towards the inside of the stem). This area was lightly wrapped with parafilm. There were 9-11 replicates for each of the nine isolates as well as for the control (agar without any mycelium). Stems were randomly collected and sorted into replicate groups. Each replicate group was placed into zip-lock polythene bags (one at each end of the stems with tape wrapped loosely around the middle to hold together). These were then placed into a plastic container and incubated at approximately 22°C.

After 14 days, lesion development was measured. The parafilm was removed, and the lesion length was measured using callipers and a ruler. The length of the initial incision made to the stem was also recorded and this value was taken from the total lesion length. Koch's postulates were fulfilled by re-isolating the same fungal isolate from the lesions that were used for inoculation. Identity of each isolate was confirmed by culture and spore characteristics.

Pathogenicity trial on fruit

Based on the fungal species that were identified from symptomatic mango tissue nine isolates were selected for this test, namely *Lasiodiplodia* sp. 1 (WAC13280), *L. pseudotheobromae* (WAC13281), *N. dimidiatum* (WAC13274, WAC13276 and WAC13277), *N. novaehollandiae* (WAC13273 and WAC13275), *P. adansoniae* (WAC13278), and WAC13279 (which was later identified as a mixed culture of *P. kimberleyense* and *P. adansonia*e). The cultures were grown on half strength PDA, for five days at 28 - 33°C.

Unripe but mature Kensington Pride mangoes (unsprayed) were harvested from a commercial grower of mango trees in Humpty Do, NT. The fruit were transported back to the laboratory where they were washed in water, then submerged in 1.5% NaOH (Bleach) solution for two minutes, then rinsed in DI water. They were allowed to air dry and were stored overnight at 28 - 33°C in trays. The mangoes were sorted into nine replicates of 12 mangoes, and then randomly numbered 1-12 within the replicates and placed onto trays. Mangoes were wounded by pushing the tip of a sterile pipette just through the surface of the skin on the side of the mango. A colonised 7mm diameter agar plug was immediately aseptically placed mycelia side down into the wound (for the control, a non-colonised agar plug was used). The plug was cut from the inside edge of actively growing mycelium. Plastic bags were loosely placed over the trays and incubated at 28-33°C with diurnal light. After three days of incubation, the agar plugs were removed and each replicate of 12 mangos were placed into a large plastic bag with a moist paper towel, sealed and incubated as above. After another three days, the lesion diameter was measured in two directions on each mango, vertically toward the stem and horizontally.

For the fruit trial lesion length (l) and lesion width (w) data were combined to determine the lesion area using the equation “area of an oval = πlw”. For the fruit and stem trials normal distribution was assessed by plotting the raw data of lesion area on a p-plot. A one-way analysis of variance (ANOVA) was performed on raw and transformed data (unequal variances were determined using Levene’s test of homogeneity of variances). Means were compared using Duncan’s test for means using SPSS for Windows version 17 (Chicago, SPSS Inc.).

Results

Molecular phylogenetic characterisation

Recently, 8 new cryptic species have been described in the *L. theobromae* species complex without the designation of a type isolate for *L. theobromae sensu stricto.* For this reason we have refrained from naming any isolate *L. theobromae.* Eight taxa of Botryosphaeriaceae were identified from diseased mango stems or cankers. The most commonly encountered species included *N. novaehollandiae* (isolated 11 times), *N. dimidiatum* (isolated 10 times) and *P. adansoniae* (isolated six times). The remaining five taxa were isolated one to two times: *L. iraniensis* (twice), *Lasiodiplodia* sp. 1 (twice), *L. pseudotheobromae* (once), *P. ardesiacum* (once) and *P. kimberleyense* (once). Of the three growing areas in WA, four botryosphaeriaceous taxa were identified from mango in Broome, Derby contained five and Kununurra contained four. *Neoscytalidium dimidiatum* was found in all three areas. *L. iraniensis, N. novaehollandiae* and *P. adansoniae* were found in two areas. The remaining four taxa were each found in one area.

PCR products of approximately 570 bp were amplified for the ITSrDNA region, while PCR products of approximately 320bp were amplified for the EF1-α region. Phylogenies conducted on the combined datasets were used to identify isolates to species level. Of the 986 characters from 81 taxa, 418 were parsimony informative generating 228 trees of 1048 steps (CI= 0.699, RI= 0.965, GI= -0.294), characters were reweighted based on the maximum value of the consistency index and the analysis was rerun. This generated 56 trees of 735 steps (CI=0.791, RI= 0.978, G1= -0.280) (Fig 3.2). Twenty-five well supported clades were identified corresponding to twenty-three described species of the Botryosphaeriaceae and two potential new species (*Lasiodiplodia* sp. 1 and *Lasiodiplodia* sp. 2).

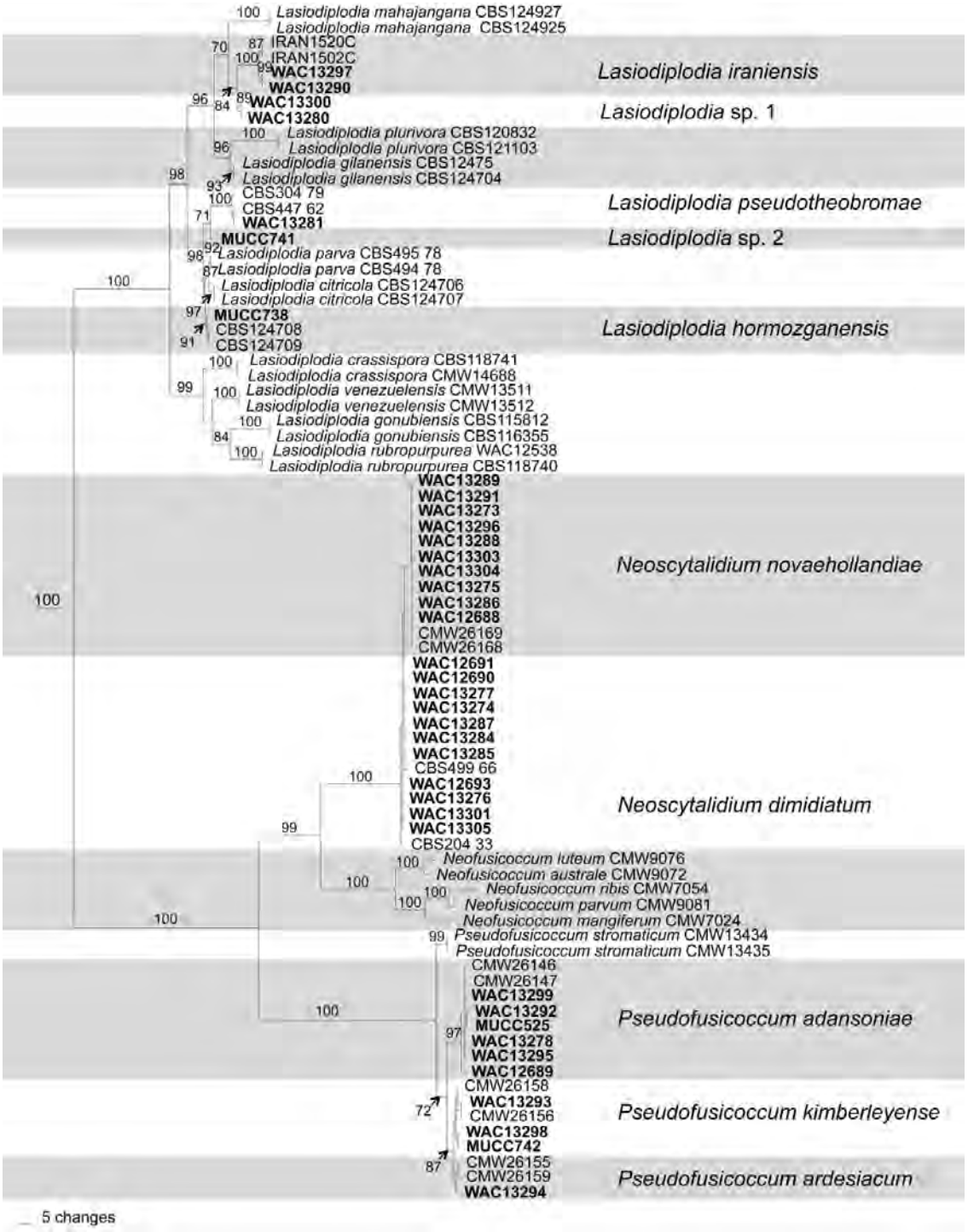

Figure 3.2 One of 56 most parsimonious trees of 735 steps resulting from the analysis of the combined ITS and EF1-α sequence. Those isolated in this study are in bold. The bootstrap values of the branch nodes are given in italics.

Two isolates (WAC13280 and WAC13300) sampled from mango in Broome were identified as *Lasiodiplodia* sp. 1. Two isolates one from a mango stem in Kununurra (WAC13297) and one from a mango canker in Broome (WAC13290) were identified as *L. iraniensis*. One isolate from a mango stem in Broome (WAC13281) was identified as *L. pseudotheobromae*.

Nine isolates (WAC12693, WAC13284, WAC13285, WAC13301, WAC12690, WAC13287, WAC13277, WAC13276 and WAC13305) obtained from mango stem or canker material collected in Kununurra, Broome and Derby were identified as *N. dimidiatum*. One isolate collected from a mango stem in Tiwi Island, NT was also identified as *N. dimidiatum*. Another eleven isolates (WAC12691, WAC12688, WAC13296, WAC13286, WAC13288, WAC13289, WAC13291, WAC13273, WAC13303, WAC13304, and WAC13275) obtained from mango stem or canker material in Kununurra and Derby were identified as *N. novaehollandiae*.

Six isolates (WAC12689, MUCC525, WAC13292, WAC13278, WAC13295 and WAC13299) from stem and canker material from mangoes in Kununurra and Derby were identified as *P. adansoniae*. One isolate (WAC13294) from the stem of a mango in Derby was identified as *P. ardesiacum*. Two isolates (WAC13293 and WAC13298) were identified as *P. kimberleyense*, from mango stems in Derby.

Pathogenicity trial on excised stems

Once inoculated, colonisation of tissues by the test isolates resulted in lesions that were visible as a slight darkening of the stem (Fig. 3.1 G & H). These became more pronounced as the epidermal layers were scraped away at harvest. In some cases, lesions extended along the phloem of the stem and were not evident on the surface. Data were transformed using a log function. Data were examined for outliers and these were removed prior to further analysis. All isolates produced lesions significantly ($p<0.05$) different from the control (Fig. 3.1 G, H & I). Symptom development following inoculation with the three *Lasiodiplodia* species produced the largest lesions; in particular, *L. hormozganensis* produced the largest lesions (mean=141mm).

The two isolates of *N. dimidiatum* produced significantly ($p<0.05$) different mean lesion lengths from each other (Fig. 3.3).

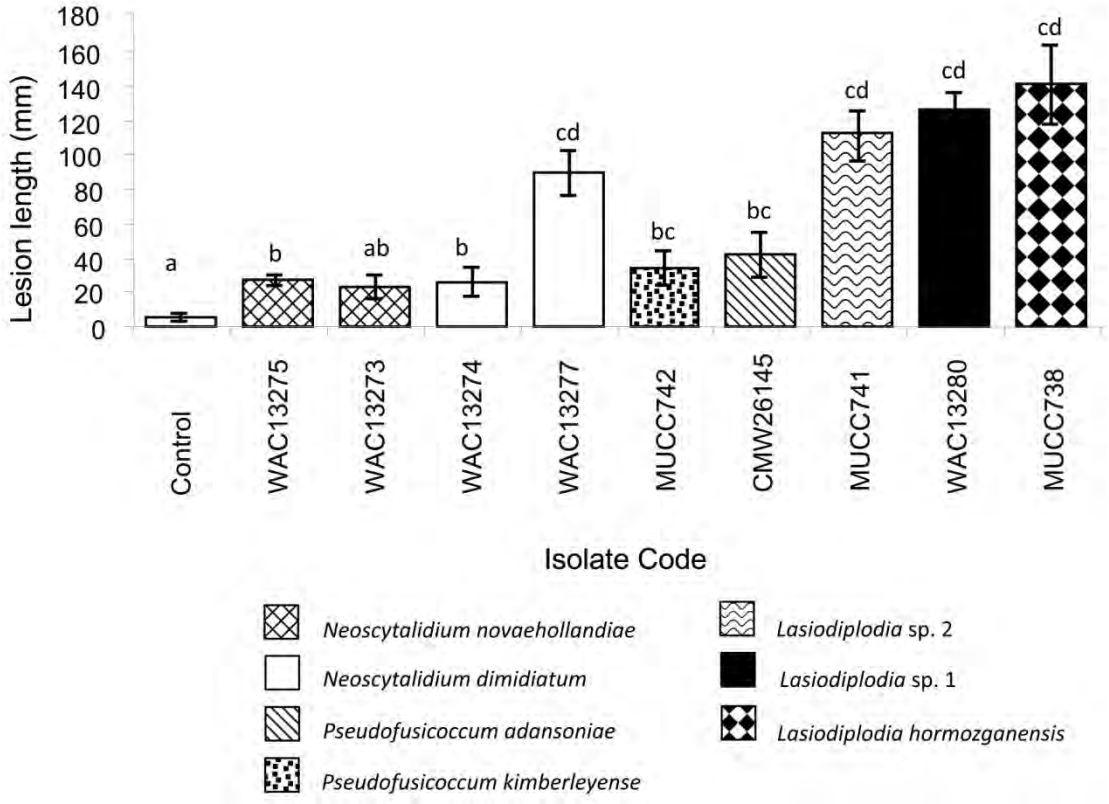

Figure 3.3 Mean length (mm) of lesions in Kensington Pride mango stems. The bars represent the standard error of the mean, lesion lengths which are significantly different ($p<0.05$) will have a different letter above the error bar.

Pathogenicity trial on fruit

Inoculations resulted in irregularly shaped, roughly circular black brown lesions on the surface of the fruit (Fig. 3.1 J). Data from the lesion area were transformed using a log function. All isolates produced lesions significantly ($p<0.05$) different from the control except for the mixed culture of *P. kimberleyense/adansoniae* (WAC13279) (Fig. 3.4). There was a great degree of intra- and inter-specific variation (Fig. 3.4). *Lasiodiplodia* sp. 1 (WAC13280) produced the largest lesions, with *L. pseudotheobromae* (WAC13281) and *N. dimidiatum* (WAC13274) also producing large lesions (Fig. 3.4).

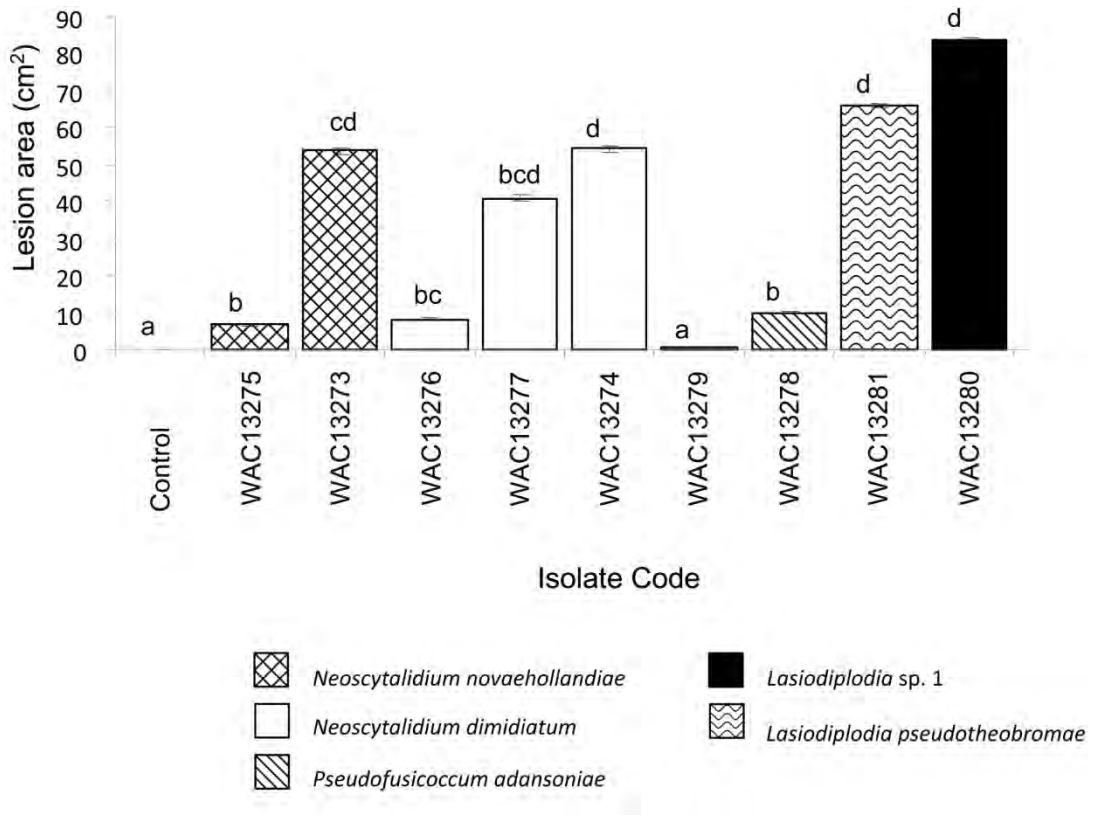

Figure 3.4 Mean area (mm^2) of lesions in Kensington Pride mango fruit. The bars represent the standard error of the mean, lesion lengths which are significantly different ($p<0.05$) will have a different letter above the error bar.

Discussion

Eight species of Botryosphaeriaceae from three genera were isolated from cankers and tip dieback of mango trees in the Kimberley (Broome, Derby and Kununurra). The main taxa of Botryosphaeriaceae pathogenic to mangoes normally associated with diseased mango trees in elsewhere in the world (*N. parvum*, *N. mangiferae* and *B. dothidea*) were not recorded in the Kimberley. Instead, a different suite of pathogenic species were associated with mango diseases in the Kimberley. This unique suite of fungi were isolated from mango trees showing dieback and canker symptoms and produced lesions during pathogenicity trials using mango fruit and excised stems. This difference in fungal population of mango may be due to a number of reasons such as (i) extreme climatic conditions (high temperatures, violent storms, flooding and drought conditions) (ii) the effectiveness of quarantine in preventing the movement of the

common pathogens into the region or (iii) the proximity to endophytes that dominate the native vegetation that are better suited to the environment.

The eight taxa of Botryosphaeriaceae obtained from diseased mango material in this study (*L. iraniensis*, *Lasiodiplodia* sp. 1, *L. pseudotheobromae*, *N. novaehollandiae*, *N. dimidiatum*, *P. adansoniae*, *P. kimberleyense* and *P. ardesiacum*) have been reported from native vegetation in the Kimberley Region with the exception of *Lasiodiplodia* sp. 1 and *N. dimidiatum* (Pavlic et al. 2008; Sakalidis et al. 2011). Three species; *L. pseudotheobromae*, *P. adansoniae* and *P. ardesiacum* have not been reported previously from mangoes. Species of Botryosphaeriaceae inked with decline of mangoes in eastern Australia by Slippers et al (2005) have been identified as *N. parvum*, *N. maniferae*, *B. dothidea* and *P. kimberleyense* (identified in the study as "*Dothierella*-long"). The disparity between pathogens of mango in eastern Australia and Western Australia is intriguing. Despite the observed ability of species of Botryosphaeriaceae to colonise known hosts, they do not always do so, even if the fungal species are known to be present in the area assessed. Regional specificity within members of this group has been observed (Slippers and Wingfield 2007). For example, *N. australe* is a common associate of eucalypts and other hosts in Western Australia (Burgess et al. 2006b; Taylor et al. 2009) but rare elsewhere in Australia (Cunnington et al. 2007).

Ray et al. (2010) reported the occurrence of *N. novaehollandiae* and *N. dimidiatum* in association with dieback of mango trees in the NW and north-central (NC) parts of Australia (Kununurra, Derby, Broome and the NT). *Neoscytalidium dimidiatum* was isolated from mango, but never on native tree species. Conversely, *L. margaritaceae*, *D.longicollis*, and *F. ramosum* have been isolated from native trees (Pavlic et al. 2008; Sakalidis et al. 2011) but not from mango trees. This may be a consequence of the small number of trees sampled in the current study, or may reflect the colonisation ability of the fungal species.

The spectrum of taxa of Botryosphaeriaceae colonising the mango trees and fruit may reflect what was introduced on the germplasm and what is present the local environment (Johnson 1997). Introduced fungal species may be less well adapted climatically or less readily spread in

soil and pest borne inoculum. Mangoes that have been introduced to exotic habitats may not have the complete suite of fungal microflora that occurs within their natural environment (Johnson 1997). The depauperate array of endophytes introduced with the mango may have disrupted the balanced microecology of the host (Johnson 1997), this may then reduce the plant defence system, resulting in increased colonisation of locally adapted endophytes and increasing the potential for a pathogenic lifestyle of latent pathogens (Redman et al. 2001; Kogel et al. 2006) both naturally occurring within the host and acquired from the surrounding environment. In this study, it would seem that there has been a horizontal movement of fungi endemic to the Kimberley environment into mango trees.

It is important to establish the infection pathway of pathogenic fungi as an understanding of the infection pathway may lead to management practices that can reduce inoculum and infection of hosts. Species of Botryosphaeriaceae can infect plants via endophytic colonisation, wound infection, and seed to seedling colonisation, as well as by soil infection of fallen fruit and seed (Brown and Hendrix 1981; Smith et al. 1996a; Smith et al. 1996b; Johnson et al. 1997; Slippers and Wingfield 2007; Johnson 2008). Insects may also have a role in transmission of species (Slippers and Wingfield 2007; Adair et al. 2009). Johnson (2008) noted that spores of pathogenic Botryosphaeriaceae could persist in the soil and leaf litter in mango orchards and suggested that these fungi are likely to invade plants particularly during establishment (seed and soil transmission) and after pruning.

Pruning wounds provide a major entry point for potential pathogens (Cooke et al. 1998; Johnson 2008) and they also contribute to tree stress. In many commercial orchards in Kununurra and elsewhere, mango trees are mechanically pruned. Mechanical pruning does not always make a clean cut and often the branches are shattered. Lateral branches grow from below the pruning wound, but then often die. It appears that the shattered branches may provide an excellent entry point for pathogenic Botryosphaeriaceae.

The relative success of colonisation is also influenced by temperature. For example, species in the *L. theobromae* complex and *P. kimberleyense* are consistently found in warm tropical-sub-

tropical conditions whilst *N. parvum* is generally found in temperate conditions (Burgess et al. 2006a; Pavlic et al. 2007; Sakalidis et al. 2011). Previous cross inoculation studies have shown that the temperature that fruit are kept at influences the dominance of one pathogen over another. When fruit that were co-infected with *L. theobromae* and *N. parvum* were incubated at various temperatures, *L. theobromae* produced large lesions at 30°C, *N. parvum* dominated at 13°C and there was mixed expression at 18-25°C (Johnson 1993; Johnson 2008). In the present study, the fruit were kept at temperatures of 28-33°C that may have favoured the development of lesions of *L. theobromae*.

In the present study, mango fruit and stems were used to prove and compare pathogenicity between isolates that were collected from mango stems with dieback or a canker. All pathogens that were isolated from cankered mango stems produced lesions. On the mango fruit, *Lasiodiplodia* sp. 1 and *L. pseudotheobromae* produced the largest lesions followed by *N. dimidiatum* and *N. novaehollandiae*. *Neoscytalidium dimidiatum* and *N. novaehollandiae* were commonly isolated from mango branches with dieback or canker (Ray et al. 2010) but they have not been isolated from mango fruit in the field, or associated with SER previously, yet they are capable of causing lesions when artificially inoculated into fruit. The mechanics of infection has not been established, these species may behave as latent pathogens colonising mango trees prior to harvest and expressing once fruit is harvested or infecting through the skin of the fruit directly causing disease.

On the mango stems *L. hormozganensis*, *Lasiodiplodia* sp. 1, *Lasiodiplodia* sp. 2 and one of the *N. dimidiatum* isolates produced the largest lesions. The results of the excised stem pathogenicity test indicate that *Lasiodiplodia* spp. may be more pathogenic than *N. dimidiatum* and *N. novaehollandiae*. However, previous work on mango dieback in the N, NW of Australia indicated that *Neoscytalidium* spp. were most frequently isolated from symptomatic tissue (Ray et al. 2010). *Lasiodiplodia hormozganensis* was recently described from *M. indica* and *Olea* sp in Iran (Abdollahzadeh et al. 2010) and from non-native environments in Australia (originally identified as *L. parva*). *Lasiodiplodia hormozganensis* was isolated from *A. digitata* and a dying

A. za in the NT and from a dying *A. gregorii* in Broome (Sakalidis et al. 2011). *Lasiodiplodia* sp. 1 and 2 fall within the *L. theobromae* species complex, and represent additional cryptic species to the eight described *Lasiodiplodia* species within the complex (Damm et al. 2007; Alves et al. 2008; Abdollahzadeh et al. 2010; Begoude et al. 2010).

Latent pathogens are characterised by asymptomatic infection of the host and disease expression is linked to host stress (Stone et al. 2004; Seiber 2007; Dakin et al. 2009). Plant material (including seeds and cuttings) may harbour potential pathogens asymptomatically and "healthy" material can be spread throughout different geographical areas. In many cases this may pose no significant risk to the host as long as the hosts are well maintained. It has been shown that even if potential pathogens are detected in host plants, if the hosts are well managed and not stressed, then post-harvest disease is reduced (Willingham et al. 2001; Willingham et al. 2004). The Kimberley region has an extreme climate (mean average temperatures from 20°C to 36°C) (www.bom.gov.au). Additionally, farmers often induce water stress as this has been shown to improve fruiting (Bally et al. 2000). Variable climate extremes are likely to contribute to host stress levels especially of a non-native host such as mango.

Good orchard management and postharvest handling and marketing are the key to prevention and reduction of diseases (Johnson 2008). Efforts to reduce inoculum spread via improved orchard hygiene, pruning wounds and stress management would be of benefit (Johnson 2008). The establishment of orchards in regions with a shorter rain season may assist in reducing disease development (Johnson 2008). More work is required to clarify the relationship between the species of Botryosphaeriaceae that are associated with mango dieback in N and NW of Australia, to confirm the direction of gene flow, are the fungi moving from the native vegetation into the crop or vice versa, the role that *Neoscytalidium* spp. and how environmental factors contribute to disease expression.

This study has shown that species associated with cankers and stem dieback of mango trees can produce lesions in mango fruit. We have demonstrated that endophytes present in the native vegetation are also present in mango and are able to cause disease in mango stems and fruit.

Further sampling of horticultural crops and native hosts in any isolated area of Australia may reveal additional previously undescribed endophytes. These fungi may pose an economic risk to the mango industry.

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