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Endophytes as potential pathogens of the baobab species *Adansonia gregorii*: a focus on the Botryosphaeriaceae

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Abstract

Adansonia gregorii (baobab) is an iconic tree species occurring in the NW of Australia. Dying baobabs, *A. digitata*, have been reported from southern Africa and as *A. gregorii* is closely related to *A. digitata*, surveys were conducted to assess the health of the Australian baobab. The endophytic microflora of *A. gregorii* and surrounding tree species was sampled and the ability of these endophytes to cause disease in *A. gregorii* was determined. Endophytes were isolated from asymptomatic baobabs across 24 sites in the Kimberley region, north-west Australia (NW Australia). Material was also taken from surrounding native tree species at three sites. Material was also collected from asymptomatic and dying *Adansonia* species in the George Brown Darwin Botanic Gardens and from a dying baobab in a nursery in Broome. Endophytic fungi isolated from these samples were identified using morphological and molecular methods. Eleven botryosphaeriaceous species were identified along with 18 other non-botryosphaeriaceous species; *L. theobromae*[1] was the most common species. The pathogenicity of the botryosphaeriaceous species to baobabs was determined by inoculating the taproot of seedlings and stems of young baobab trees. *Lasiodiplodia theobromae* was confirmed as a potentially significant pathogen of baobabs.

Introduction

Baobabs are iconic trees from the genus *Adansonia*, endemic to the deciduous forests of western and southern Madagascar, the savannah lands of Africa and the NW Australia. *Adansonia* belongs to a monophyletic group in the sub-family Bombacoideae. Baum (2003) used a combined molecular and morphological dataset to show that *Adansonia* started to diversify into its current divisions about 10 million years ago, forming three distinct evolutionary pathways, which eventually resolved to *A. gregorii*, *Adansonia digitata* and the six Malagasy baobab species. Using molecular, morphological and ecological data, *Adansonia* has been separated into three sections: *Brevitubae*, *Longitubae* and *Adansonia*. Section *Brevitubae* contains two species that are located in Madagascar both of which are pollinated only by mammals; *Adansonia suarezensis* is pollinated by fruit bats and *Adansonia grandidieri* is pollinated by a nocturnal lemur. Section *Longitubae* consists of five species all of which are pollinated by long-tongued hawkmoths; four in Madagascar (*Adansonia perrieri*, *Adansonia za*, *Adansonia rubrostipa* and *Adansonia madagascariensis*) and one in Australia (*A. gregorii*). *Adansonia digitata*, the only species in the section *Adansonia*, is endemic to southern Africa, and is mainly pollinated by bats (Baum 1995, 1995 1996). It is also the only baobab species that is an autotetraploid (2n ¼ 160), while other baobab species are diploid (2n ¼ 88) (Baum and Oginuma 1994). Recent work undertaken by J. Pettigrew (pers. comm.) has identified a second species of baobab in South Africa, *Adansonia kilima* prov. nom., which is diploid.

The current distribution of *Adansonia* has been the subject of much debate (Aubréville 1975; Baum et al. 1998; Wickens and Lowe 2008). Originally, the formation and break up of Gondwanaland were thought to account for the distribution of *Adansonia* sp. (Aubréville 1975). However, Gondwana separated into the continents of Australia, Africa, India, South America and Antarctica very early in the evolution of angiosperms, before *Adansonia* evolved, discounting the continental drift theory (Raven and Axelrod 1972) and (Wickens and Lowe 2008). Beard (1990) outlined the “floatation” hypothesis; seed pods of the “proto-baobab” floated across the Indian Ocean from Madagascar, landed on the NW coast of Australia and were then successful in germinating and establishing in this area. Leong Pock et al. (2009)

hypothesised that an ancestor of *Adansonia* migrated from the neotropics to West Africa, where seed pods germinated and that anthropogenic movement spread this species to Madagascar and Australia, indicating the centre of origin to be Africa, not Madagascar. Current work (J. Pettigrew, pers. comm.) supports the close relationship of *A. gregorii* to the African baobabs and indicates that *A. gregorii* split relatively recently from the African baobabs, supporting an anthropogenic (or possibly sea dispersal) mediated introduction of *Adansonia* into Australia. However, their data cast doubt on the neotropical origin of the ancestral *Adansonia*.

The majority of baobabs in Australia are located in the Kimberley region in the NW of Western Australia, specifically Dampierland, Central Kimberley, Northern Kimberley, Victoria Bonaparte Bioregion and the Ord-Victoria Plains Region (Wickens and Lowe 2008). Despite other areas in Australia offering a suitable climate, it is thought that the limited range of *A. gregorii* may be due to competition with other native tree species. Baobabs are able to outcompete other tree species in areas that have limited rainfall for nine months of the year (Lowe 1998). In Australia, baobabs serve ecological, commercial and cultural purposes. They provide food and shelter for bird species, reptiles, amphibians, invertebrates and can be a host to plant species such as mistletoe (Lowe 1998). They are grown commercially and the seedlings are harvested after a few months and used for food (the root is used in a similar manner to a starchy vegetable such as turnip and the leaves are used in salads) (Johnson et al. 2002). The trees are an iconic image of the Australian outback and serve as a major tourist attraction. The baobabs are also used by the Aboriginal people of the Kimberley as a source of food, fibre, water, shade and are integral to numerous Dreamtime stories (the Dreamtime describes the period in which the world was created) that include baobabs as their centre piece (Lowe 1998).

In southern Africa, dying baobabs have been reported (Anonymous 1991; Black 2004; Calvert 1989; de Meyer 2007; Moodie 2004; Piearce et al. 1994; Roux 2002) and surveys of these trees have indicated the presence of the fungal pathogen *L. theobromae* (Ascomycota: Botryosphaeriaceae) (Roux 2002). *Lasiodiplodia theobromae* is a cosmopolitan fungus, colonising a range of mainly woody hosts in the tropics and subtropics. It can cause canker, dieback, fruit and root rot in fruit and nut trees, vegetable crops and ornamental plants

(Punithalingam 1980) and is often isolated as an endophyte from healthy plants (Müllen et al. 1991). Species of the Botryosphaeriaceae are often described as opportunistic or latent pathogens for which there may be an extended period of latency (they are commonly isolated from apparently healthy material using endophyte isolation methods) before some trigger (e.g. host stress) causes them to become pathogenic often resulting in cankering of the host (Burgess et al. 2001; Burgess et al. 2005; Müllen et al. 1991; Smith et al. 1996; Taylor et al. 2005). Africa and Australia share a large amount of floral families, such as Myrtaceae and Proteaceae (Beadle 1981), and they also share the pathogens of many of these plants (Crous et al. 2000; Slippers et al. 2004; Slippers et al. 2005a). A survey of the Australian baobabs was deemed a prudent course of action to determine whether their susceptibility to infection from endophytic latent pathogens is similar to that in Africa. The main objectives of the present study were to: assess the health of these trees; isolate and identify the endophytic fungi of *A. gregorii* and surrounding tree species; and determine if these endophytes have the ability to cause disease in *A. gregorii*.

Baobabs were surveyed in 24 undisturbed sites in the Kimberley region and material was also taken from surrounding tree species at three sites. Endophytic fungi were isolated from these samples and identified using both molecular and morphological methodology. Additional material was taken from baobabs in disturbed urban sites: George Brown Darwin Botanic Gardens and from a nursery in Broome. The distribution and diversity of the various endophyte species and the pathogenicity of selected isolates towards *A. gregorii* were examined.

Materials and methods

Fungal isolation and distribution

Undisturbed study sites

Stem and leaf material were collected from 24 native bushland sites in the Kimberley, Western Australia (Fig. 2.1). At all sites, material was taken from at least three *A. gregorii* trees. At sites

6, 18 and 22 material was intensively collected from both *A. gregorii* and other native flora. At these sites, six individual trees for each species found within a 100 m2 radius of *A. gregorii* were sampled. At site 6, flora sampled included *Acacia synchronicia*, a *Corymbia* sp., *Grevillea agrifolia*, *Lysiphyllum cunninghamii* and *Terminalia pterocarya*. Site 18 flora sampled included *A. synchronicia*, a *Bombax* sp., a *Calytrix* sp., *Crotalaria medicaginea*, *Eucalyptus camaldulensis*, a *Melaleuca* sp. and a *Terminalia* sp. At site 22 flora sampled included *A. synchronicia*, a *Eucalyptus* sp., *Ficus opposita* and *L. cunninghamii*.

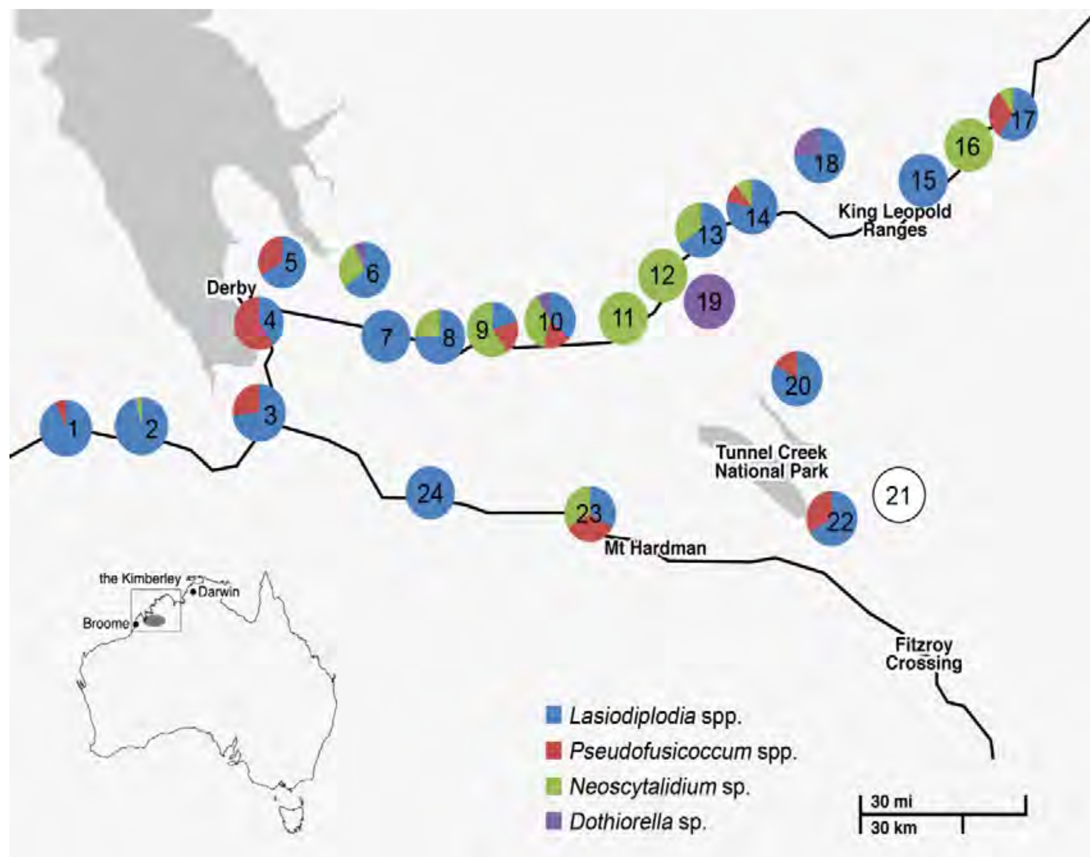

Figure 2.1 Map of sites where stem and twig samples of *Adansonia gregorii* were collected in Australia. The proportion of botryosphaeriaceous species from four genera (*Lasiodiplodia*, *Pseudofusicoccum*, *Neoscytalidium* and *Dothiorella*) is displayed in the pie charts; with the site number in the middle of the chart. At site 21 no botryosphaeriaceous species were isolated. The smaller Australia map depicts where the survey sites are located.

Disturbed urban study sites

Samples were also collected from seven species of *Adansonia* at the George Brown Darwin Botanic Gardens. These included *A. za*, *A. perrieri*, *A. rubrostipa*, *A. suarezensis*, *A. digitata*, *A. grandidieri* and *A. gregorii*. An additional sample from a dying *A. za* was also taken. Samples were also taken from a dying *A. gregorii* in a nursery in Broome, Western Australia.

Isolation

For endophytic isolations, stem samples were washed in several washes of water, bleach and ethanol as described by Taylor et al. (2009). Bark and wood were separated and sections were placed onto separate half-strength potato dextrose agar (PDA) (19 g PDA in 1 l distilled water, Difco™ PDA, Sparks, MD, USA, 7.5 g agar in 1 l distilled water). Direct isolations were also made from fruit bodies located on senescing branches of *A. gregorii*, as described by Pavlic et al. (2008). Cultures were allowed to grow for several weeks and as mycelium grew out of samples they were plated out until a pure culture was obtained. Cultures were initially sorted, by colony morphology, into two broad groups of Botryosphaeriaceae-like (fluffy white to grey-green cultures) and non-Botryosphaeriaceae (all others) (Slippers and Wingfield 2007). Within these two groups, isolates were further sorted into subgroups based on colony morphology and representative isolates from each morphological subgroup were selected for molecular identification. Cultures of all isolates belonging to the Botryosphaeriaceae are maintained on half-strength PDA slopes at the Murdoch University Culture Collection (MUCC) or in the culture collection of the Forestry and Agriculture Biotechnology Institute, University of Pretoria (CMW).

Molecular identification

Molecular identification of all species other than those newly described by Pavlic et al. (2008) was performed by growing the cultures on half-strength PDA plates for approximately 1 week at 20 °C. The mycelial mass was harvested and placed into 1.5 ml sterile Eppendorf tubes and freeze dried. A modified method from Graham et al. (1994) was used to extract DNA, as described in Andjic et al. (2007). A part of the internal transcribed spacer (ITS) region of the

ribosomal DNA operon was amplified for all isolates using the primers ITS-1F (Gardes and Bruns 1993) and ITS4 (White et al. 1990). Part of the elongation factor 1-α (EF1-α) was also amplified for all isolates using a combination of the following primers: EF1-728F and EF1-986R (Carbone et al. 1999) and EF1F and EF2R (Jacobs et al. 2004). PCR products were cleaned using Sephadex G-50 columns (Sigma Aldrich, Sweden). The columns were prepared as follows: 650 µl of Sephadex solution (3.33 g in 50 ml of distilled water) were added to clean Centri-Sep columns (Princeton Separations, Freehold, NJ). The columns were spun at 750 g for 2 min in a Microfuge18 bench centrifuge (Beckman Coulter, Germany) and the filtrate was discarded. The PCR product was added to the top of the column and was spun again at 750 g for 2 min. The filtrate was used in the sequencing reaction. Products were sequenced with the BigDye terminator cycle sequencing kit (PE Applied Biosystems, California, USA) using the same primers that were used in the initial amplification. The sequencing products were also cleaned in Sephadex G-50 columns and were separated by an ABI 3730 48 capillary sequencer (Applied Biosystems, California, USA). Identities of botryosphaeriaceous species were confirmed by phylogenetic analyses (statistically supported by 1 000 bootstrap replications) as described in Pavlic et al. (2008). A file of the combined and individual datasets is available in TreeBase (www.treebase.org, access code: S10433).

Identities of non-botryosphaeriaceous isolates were determined by sequence similarity (Zhang et al. 2000) from Blast searches in GenBank (http://blast.ncbi.nlm.nih.gov/blast.cgi). Isolate identity was confirmed by sequence similarity to multiple matching sequences (ideally from different studies). Comparison of matching sequences and the queried sequence were compared through the neighbour joining tree generated by GenBank. Also, when available, references linked to matched sequences were checked. A conservative approach was used: if the isolates had a sequence similarity match of 97–99 %, and the matching sequences were linked to a reference and there were multiple matching sequences then isolates were named to species level. If there was a lower sequence similarity match, or matching sequence identities were different isolates were named to genus, family or class level.

Pathogenicity

Pathogenicity to baobab taproots

Baobab seedlings are characterised by a narrow leafy stem and a large swollen taproot which functions as a water storage organ in seedlings and young trees (Wickens and Lowe 2008). For this trial four-month-old baobab seedlings were harvested from a commercial baobab grower (Baobabs in The Kimberley, Kununurra), packed into cardboard boxes and transported to Perth in refrigerated transport within six days. The taproots were used for the pathogenicity trial (Fig. 2.2 A). Twenty-four isolates were selected for this test representing *L. theobromae*, *Lasiodiplodia margaritaceae*, *Lasiodiplodia crassispora*, *N. ribis*, *Pseudofusicoccum adansoniae*, *Pseudofusicoccum ardesiacum*, *Pseudofusicoccum kimberleyense*, *Neoscytalidium novaehollandiae* and *Fusicoccum ramosum* (Table 2.1). All isolates were grown on half-strength PDA agar plates for approximately 10 days at 20°C.

Wooden racks were built to house the inoculated taproots and these were all sprayed with 70% ethanol for surface sterilisation. The shoots were removed from the taproots, the taproots were then washed thoroughly in water, sprayed with 70% ethanol and then dipped in a bucket of sterile water. Using pruning shears sprayed with 70% ethanol, lateral roots and foliage were removed, in some cases taproots were trimmed if they were too long to fit inside plastic containers used for the trial. Both ends were immediately dipped in wax (to prevent desiccation) and left to dry. Prepared taproots were left in surface sterilised plastic containers overnight at room temperature.

The diameter and length of each taproot were measured and marked with an isolate and replicate number. Using a sterile scalpel blade a small lateral incision was made in the middle of the taproot, into which a 1cm^2 agar plug colonized with mycelium was inserted, with the mycelium orientated towards the outside of the taproot; this area was then lightly wrapped with parafilm. There were 10 replicates for each of the 24 isolates and 10 replicates for the control (agar without any mycelium).

Twenty-five groups (corresponding to 24 isolates and one control) were placed onto the lab bench; one taproot from each group was randomly collected and placed into a replicate group (1 of 10). Taproots from each replicate were placed in random order onto wooden racks (five taproots per rack), inside plastic crates (five racks in each crate and the bottom lined with paper towel soaked in sterilised water). The containers were then sealed with aluminium foil and tape and placed in a 25°C room.

After four days, all replicates were placed in the cold room at 4°C and taproot lesions were measured from one plastic crate at a time over a period of four days.

Table 2.1 Isolates collected in this study and used in pathogenicity trials.

Isolate	Fungal Species	Host	Location	GenBank Accession	
				ITS	EF
MUCC721	*Dothiorella longicollis#*	*Lysiphyllum cunninghami*	Site 22	GU199378	
CMW26267	*Fusicoccum ramosum#*	*Eucalyptus camaldulensis*	Site 18	EU144055	EU144070
MUCC707	*Lasiodiplodia theobromae**	*Grevillea agrifolia*	Site 6	GU199365	GU199391
MUCC708	*L. theobromae**	*Eucalyptus* sp.	Site 22	GU199366	GU199392
MUCC709	*L. theobromae#*	*Leceiceis* sp.	Site 22	GU199367	GU199393
MUCC710	*L. theobromae**	*Calytrix* sp.	Site 18	GU199368	
MUCC711	*L. theobromae#*	*Crotalaria medicaginea*	Site 18	GU199369	GU199394
MUCC712	*L. theobromae#*	*Adansonia gregorii*	Site 22	GU199370	
MUCC713	*L. theobromae**	*Ficus opposita*	Site 22	GU199371	GU199395
MUCC714	*L. theobromae#*	*Acacia synchronicia*	Site 6	GU199372	GU199396
MUCC715	*L. theobromae**	*L. cunninghami*	Site 6	GU199373	GU199397
MUCC716	*L. theobromae#*	*Corymbia* sp.	Site 6	GU199374	GU199398
MUCC717	*L. theobromae**	*A. gregorii*	Site 1	GU199375	GU199399
MUCC718	*L. theobromae#*	*Terminalia pterocamya*	Site 6	GU199376	GU199400
MUCC744	*L. theobromae*	*A. za* (dying)	Darwin		GU199404
MUCC735	*L. theobromae*	*A. za* (living)	Darwin	GU199385	GU199405
MUCC736	*L. theobromae*	*A. digitata*	Darwin	GU199386	GU199406
MUCC737	*L. theobromae*	*A. gregorii*	Darwin	GU199387	GU199407
MUCC720	*L. crassispora#*	*Corymbia* sp.	Site 6	GU199377	
CMW26162	*L. margaritaceae**	*A. gregorii*	Site 20	EU144050	EU144065
MUCC738	*L. parva*	*A. digitata*	Darwin		GU199408
MUCC739	*L. parva*	*A. za* (dying)	Darwin	GU199388	GU199409
MUCC740	*L. parva*	*A. gregorii*	Broome	GU199390	GU199410

Isolate	Fungal Species	Host	Location	GenBank Accession	
				ITS	EF
MUCC741	*L. parva*	*A. gregorii*	Darwin	GU199389	GU199411
MUCC743	*Neoscytalidium dimidiatum*	*A. perrieri*	Darwin		GU199413
MUCC537	*N. novaehollandiae**	*C. medicaginea*	Site 18	EF585540	EF585580
MUCC535	*N. novaehollandiae#*	*A. synchronicia*	Site 6	EF585536	EF585578
MUCC730	*Neofusicoccum ribis**	*E. camaldulensis*	Site 18	GU199384	
MUCC722	*Pseudofusicoccum adansoniae#*	*A. gregorii*	Site 20	GU199372	
MUCC723	*P. adansoniae#*	*G. agrifolia*	Site 6	GU199380	
MUCC724	*P. ardesiacum#*	*A. gregorii*	Site 3	GU199381	GU199402
MUCC725	*P. kimberleyense#*	*Eucalyptus* sp.	Site 22	GU199382	
MUCC726	*P. kimberleyense#*	*A. gregorii*	Site 22	GU199383	GU199403
MUCC742	*P. kimberleyense*	*A. rubrostipa*	Darwin		GU199412

*Isolates used in the taproot and in the young baobab tree trial #Isolates only used in the taproot trial

The parafilm was removed, the taproot was weighed, the lesion was scraped out and the taproot was reweighed immediately. The lesion length and width were also measured using callipers and a ruler. The presence of spores was recorded and specimens were mounted on slides for subsequent identification. Koch's postulates were tested by re-isolation from infected material.

Pathogenicity to young baobab trees

Fifty baobab trees (ca. 2m high and 2–3 years old) were purchased and shipped by the same company used for the taproot trial. They were planted within two weeks of harvest into 1m long PVC pipes in a potting medium consisting of 1/3 coarse river sand and 2/3 potting mix (2/5 coarse river sand, 2/5 composted pine bark fibres and 1/5 ground coco peat fibre) and were watered twice a day for 10 min by an automatic dripping system. The trees were planted along two rows on one side of an evaporative cooled (8–35 °C) glasshouse.

Based on the taproot pathogenicity trial (above), nine isolates of Botryosphaeriaceae were selected to assess their pathogenicity to young baobab trees: six isolates of *L. theobromae* from five different hosts were used (*A. gregorii*, *Eucalyptus* sp., *Calytrix* sp., *F. opposita* and *L. cunninghamii*); one isolate of *N. ribis* (host-*E. camaldulensis*); one isolate of *N. novaehollandiae* (host-*C. medicaginea*); and one isolate of *L. margaritaceae* (host-*A. gregorii*) (Table 2.1). These isolates were among the most pathogenic from the previous trial. All isolates were grown on half-strength PDA for ca. 14 days at 20 °C. The trees were divided into five replicate groups (each with 10 trees) and the isolates were randomly assigned to each tree within a replicate (randomised complete blocks). There were five replicates per isolate and four replicates for the control. A sterile scalpel blade was used to make a small lateral incision along the stem into which a 1cm^2 agar plug colonized with mycelium was inserted with mycelium facing the inner stem. This was then lightly wrapped with parafilm. The controls were inoculated with agar without mycelium.

After 6 months, the lesions were harvested by clipping the stems at least 15 cm away from the visible lesion margins. The width, length and depth of lesions were measured using callipers and a ruler. The presence of fruit bodies was recorded and slides were prepared. The stems were

cut in half at the centre of the initial mycelium plug insertion to determine the depth of lesion development. At the extreme margin of the lesions the wood was cut away using a knife to establish the extent of interior lesion development.

Data analysis

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 to determine the variability in lesion length exhibited between the isolates using SPSS for Windows version 17 (SPSS Inc., Chicago). Means were compared using Duncan's multiple range test. The relationship between lesion weight, stem diameter and stem width was determined by using a general linear model univariate analysis in SPSS.

Results

Fungal isolation and distribution of botryosphaeriaceous endophytes-undisturbed sites

A total of 383 fungal isolations were made from baobabs and other native trees in the Kimberley. All trees were asymptomatic, showing no visible signs of disease. The majority (248) of isolates belonged to the Botryosphaeriaceae (Table 2.2 and Table 2.3) and included seven new species described by Pavlic et al. (2008). In many cases, multiple isolations of different fungi were made from a single section of one stem. Also, multiple isolations of the same species were made from a single section of one stem (when these isolates grew out on the agar they formed vegetative compatibility barriers and were therefore considered different genotypes). Division of isolates into two groups of botryosphaeriaceous and non-botryosphaeriaceous characteristics was useful for this broad division. In total, 11 botryosphaeriaceous and 18 non-botryosphaeriaceous species were identified.

Fungi associated with A. gregorii

Eight botryosphaeriaceous species were isolated from *A. gregorii*: *L. theobromae*, *L. margaritaceae*, *Lasiodiplodia pseudotheobromae*, *P. adansoniae*, *P. ardesiacum*, *P. kimberleyense*, *N. novaehollandiae* and *Dothiorella longicollis* (Table 2.2). *Lasiodiplodia* was the genus most frequently isolated from *A. gregorii* (Fig. 2.1, Table 2.2). *Neoscytalidium novaehollandiae* and *Pseudofusicoccum* sp. were also commonly isolated. *Pseudofusicoccum* was always isolated in conjunction with *Lasiodiplodia* (Fig. 2.1). *Lasiodiplodia* species and *N. novaehollandiae* were sometimes found in the absence of other genera.

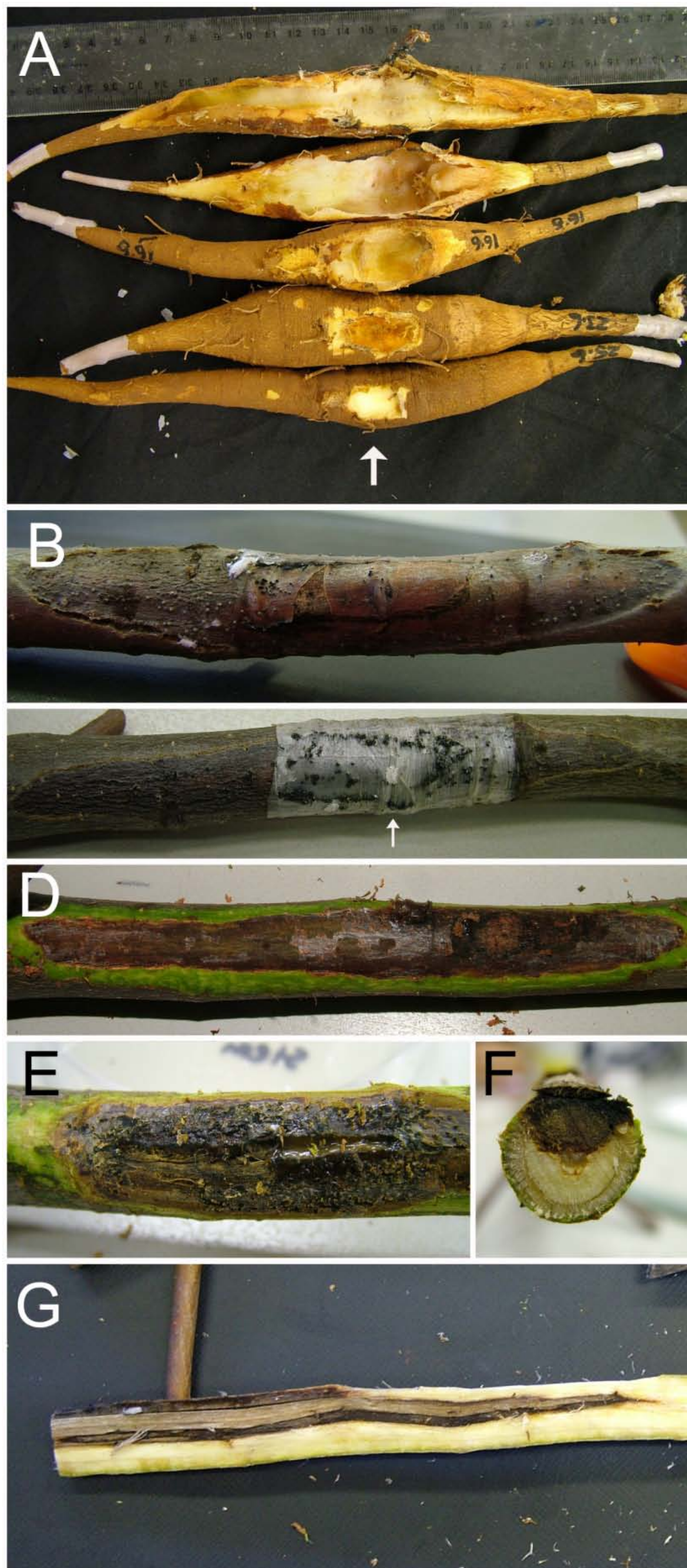

Figure 2.2 (A) Comparison of range of lesions in the taproot pathogenicity trial. The white arrow points to the control showing no lesion. (B) Lesion on the surface of the stem showing cracking of the stem (white arrow). (C) Lesion on the surface of the stem showing fruiting bodies emerging through parafilm (white arrow). (D) Lesion on the surface of the stem with epidermal layers scraped back. (E) Lesion on the surface of the stem with epidermal layers scraped back, showing cracking of the stem and sap exudates. (F) Stem cut in half at the site of inoculation showing lesion development into the pith. (G) Stem split lengthways showing lesion development below the surface. All stems and taproots are between 1.5 cm and 2 cm in diameter.

Botryosphaeriaceous species were isolated from 23 of the 24 sites. Out of these 23 sites, *Lasiodiplodia* species were isolated from all but four sites (11, 12, 16 and 19), *Pseudofusicoccum* species were isolated from 11 out of 23 sites and *Ne. novaehollandiae* from 13 out of 23 sites (Fig. 2.1, Table 2.2).

Host association of botryosphaeriaceous species

Fungi associated with other tree species

Ten botryosphaeriaceous species were isolated from native tree species other than *A. gregorii*: *L. theobromae*, *L. crassispora*, *L. margaritaceae*, *P. adansoniae*, *P. ardesiacum*, *P. kimberleyense*, *N. novaehollandiae*, *D. longicollis*, *Neofusicoccum ribis* and *F. ramosum* (Table 2.3). *Lasiodiplodia crassispora*, *N. ribis* and *F. ramosum* were never isolated from *A. gregorii.* The greatest number of species (five) was isolated from *G. agrifolia* on site 6 and a *Eucalyptus* sp. on site 22. Only one species was isolated from a *Melaleuca* sp. and a *Calytrix* sp. at site 18. No botryosphaeriaceous species were isolated from *Bombax* sp. (Table 2.3).

Site distribution of botryosphaeriaceous species

Across all sites and tree species

Lasiodiplodia was the most common genus (66 % of isolations), with four species (*L. theobromae*, *L. margaritaceae*, *L. crassispora*, and *L. pseudotheobromae*) isolated 163 times from 13 host species (Table 2.2 and Table 2.3). *Lasiodiplodia theobromae* was the most commonly isolated species from all hosts, except the *Eucalyptus* sp. and *F. opposita* (where *Pseudofusicoccum* sp. were most commonly isolated) and *E. camaldulensis* (a *Fusicoccum* and a *Neofusicoccum* species were isolated).

Table 2.2 Incidence and distribution of Botryosphaeriaceae on *Adansonia gregorii* at 24 sites (see Fig. 2.1) in the Kimberley, north-west Australia. Other native tree species were also sampled from sites in bold.

	Lasiodiplodia theobromae	*L. margaritaceae*	*L. pseudotheobromae*	*Pseudofusicoccum adansoniae*	*P. ardesiacum*	*P. kimberleyense*	*Neoscytalidium novaehollandiae*	*Dothiorella longicollis*	**Total**
Site 1	14					1			15
Site 2	27						1		28
Site 3	11				3	1			15
Site 4	2			3					5
Site 5	2				1				3
Site 6	**6**	**2**					**4**	**1**	**13**
Site 7	2								2
Site 8	3						1		4
Site 9	1			1			3		5
Site 10	4				1		4	1	10
Site 11							4		4
Site 12							2		2
Site 13	2						1		3
Site 14	7			1			1		9
Site 15	3						2		5
Site 16							2		2
Site 17	6			3			1		10
Site 18	**1**		**2**					**1**	**4**
Site 19								1	1
Site 20	2	3		1					6
Site 21									0
Site 22	**10**			**4**		**3**			**17**
Site 23			1	1			1		3
Site 24	2								2
Total	105	5	3	14	5	5	27	4	168

Lasiodiplodia pseudotheobromae was only isolated from baobabs at sites 18 and 23 and was not isolated from other species. *Lasiodiplodia crassispora* was only isolated once from a *Corymbia* sp. The only two species isolated from *E. camaldulensis* were *F. ramosum* and *N. ribis*, and they were not isolated from other hosts (Table 2.3). *Pseudofusicoccum* was the second most common genus (16 % of isolations) with three species (*P. adansoniae*, *P. ardesiacum* and *P. kimberleyense*) isolated 39 times from six host species (Table 2.2 and Table 2.3). *Neoscytalidium novaehollandiae* was the third most common genus (14 % of isolations) with 35 isolations from six host species (Table 2.2 and Table 2.3). *Lasiodiplodia* species were isolated from all hosts sampled except from a *Bombax* sp. and *E. camaldulensis* (Table 2.3).

Fungal isolation and distribution of non-botryosphaeriaceous endophytes-undisturbed sites

Identification

From 135 isolations, 18 different non-botryosphaeriaceous species were identified among the different hosts and locations (Table 2.4). Eleven isolate subgroups corresponded with a high sequence similarity to known species *Daldinia eschscholzii* (99 % match), *Gibberella moniliformis* (99 % match), *Neurospora cerealis* (99 % match), *Nigrospora sphaerica* (99 % match), *Aureobasidium pullulans* (98 % match), *Curvularia* sp. (99 % match), *Cytospora eucalypticola* (97 % match), *Geosmithia* sp. (99 % match), *Phoma* spp. (there were several *Phoma* species identified) (98–99 % match), *Sclerostagonospora* sp. (98 % match) and *Rhytidhysteron* sp. (97 % match). One isolate, an Amphisphaeriaceae species, only had a 94 % match with several different species, and it was identified to the family level of the closest matching species. Another two isolate subgroups – a Xylariaceae species and a Dothideomycete species – only had a 90 % match with several different species.

Table 2.3. Incidence and distribution of Botryosphaeriaceae on native tree species in close proximity of *Adansonia gregorii* at three sites in the Kimberley, north-west Australia. Six trees of each species were sampled at each site; numbers indicate number of isolations, whilst numbers in superscript indicate number of stems these isolates were obtained from.

	Lasiodiplodia theobromae	*L. crassispora*	*L. margaritaceae*	*L. pseudotheobromae*	*Pseudofusicoccum adansoniae*	*P. ardesiacum*	*P. kimberleyense*	*Neoscytalidium novaehollandiae*	*Dothiorella longicollis*	*Fusicoccum ramosum*	*Neofusicoccum ribis*	**Total**
					Site 6							
Acacia synchronicia	6^{2}							2^{1}				8
Corymbia grandiflora	6^{4}	1										7
Grevillia agrifolia	6^{4}		2^{2}		3^{3}	1		1				13
Lysiphyllum cunninghamii	10^{5}				1			2^{1}	1			14
Terminalia pterocarya	2^{2}		5^{2}									7
					Site 18							
A. synchronicia	1											1
Bombax sp.												0
Calytrix sp.	1											1
Crotalaria medicaginea	2^{2}							2^{1}				4
Eucalyptus camaldulensis										1	1	2
Terminalia sp.	2^{1}								1			3
Melaleuca sp.	1											1
					Site 22							
A. synchronicia					1		1					2
Eucalyptus sp.	1				2^{2}	1	2^{2}	1				7
Ficus opposita	2^{2}				2^{1}		1					5
L. cunninghamii	2^{1}								3^{2}			5
Total	42	1	7		9	2	4	8	5	1	1	80

Two isolate subgroups were identified as Sordariomycete species 1 and 2 (93 % and 97 % match respectively). Two isolate subgroups had no clear sequence homology to fungi listed on GenBank and have been called unknown 1–2 (Table 2.4).

Site distribution and host association

Eight of the subgroups were rare and were isolated only once from one site and one host (Table 2.4). The most widely distributed and most commonly isolated subgroup corresponded to unknown 1. This species was isolated from 11/24 sites and found on 7/13 tree species sampled. A *Rhytidhysteron* sp. and *N. cerealis* were also widely distributed (10/24 and 9/24 sites, respectively) but these had a much reduced host range, predominantly associated with *A. gregorii*. *Gibberella moniforms* and *C. eucalypticola* were both isolated from 8/13 trees species assessed in this study (including *A. gregorii*). Site 22 contained 9/18 non-botryosphaeriaceous species isolated, including two species unique to this site. Site 18 contained nine different species (three of these unique to the site), whilst six species were isolated from site 6, one unique to this site. Out of eighteen non-botryosphaeriaceous species, 12 were isolated from *A. gregorii* and four of these were unique to *A. gregorii* (Amphisphaeriaceae species, *Curvularia* sp., *Geosmithia* sp. and a *Xylariaceae* species).

Isolations from George Brown Darwin Botanic Gardens and a nursery in Broome

Isolations were made from seven different baobab species and one dying *A. za* in the George Brown Darwin Botanic Gardens. Botryosphaeriaceous species were obtained from five of the baobab species. All these isolates were sequenced and most were identified as *L. theobromae* (Table 2.1). One isolate was identified as *P. kimberleyense*, one as *N. dimidiatum* and three as *Lasiodiplodia parva* (Table 2.1). Isolations mad[1]e from the dying *A. za* were identified as *L. theobromae* and *L. parva*. Isolations made from a dying *A. gregorii* in Broome were identified as *L. parva* (Table 2.1).

[1] **Table 2.4** (following page) Number of non-botryosphaeriaceous isolates (columns) on all hosts (rows) across all sites. GenBank accession numbers of isolates are presented in brackets after isolate name. Data in superscript indicates the number of sites the species was isolated from.

	Amphisphaeriaceae species (GU199414)	*Aureobasidium pullulans* (GU199415)	*Curvularia* sp. (GU199416)	*Cytospora eucalypticola* (GU199417)	*Daldinia eschscholzii* (DGU199418)	Sordariomycete species 1 (GU199419)	Dothideomycete species (GU199420)	*Geosmithia* sp. (GU199421)	*Gibberella moniliformis* (GU199422)	*Neurospora cerealis* (GU199423)	*Nigrospora sphaerica* (GU199424)	*Phoma* spp. (GU199425)	Sordariomycete species 2 (GU199426)	*Sclerostagonospora* sp. (GU199427)	*Rhytihysteron* sp. (GU199428)	Unknown 1 (GU199429)	Unknown 2 (GU199430)	Xylariaceae species (GU199431)	Total
Adansonia gregorii	1^{1}		1^{1}	1^{1}	2^{2}			1^{1}	2^{1}	13^{9}		3^{3}		1^{1}	13^{9}	16^{10}		1^{1}	55
Acacia synchronicia				8^{2}		1^{1}			2^{2}				3^{1}			5^{2}			19
Lysiphyllum cunninghami				1^{1}					1^{1}	2^{1}		1^{1}	6^{2}	1^{1}					12
Bombax sp.																1			1
Calytrix sp.		1^{1}		2^{1}												2^{1}			5
Corymbia sp.				4^{1}					2^{1}										6
Crotalaria medicaginea												2^{1}	1^{1}				1^{1}		4
Eucalyptus spp.				4^{1}					1^{2}	1^{1}	1^{1}		5^{2}		1^{1}	1^{1}			14
Ficus opposita					1^{1}				1^{1}							1^{1}			3
Grevillea agrifolia		5^{1}							1^{1}							1^{1}			7
Melaleuca sp.				2^{1}					2^{1}				1^{1}						5
Terminalia spp.				3^{1}			1^{1}												4
Total	1	6	1	25	3	1	1	1	12	16	1	6	16	2	14	27	1	1	135

Pathogenicity

Pathogenicity to baobab taproots

All lesions comprised soft, rotting material, and in some cases orangey, brown or black discolouration was observed on the top of the lesions, but generally there was no apparent discolouration within the lesion (Fig. 2.2 A). No lesions were observed on any of the 10 control taproots. Fungal fruit bodies were observed on the surface of some lesions and in each case the morphology of the spores produced was in accordance with the species used in the inoculation. Koch's postulates were proven with cultures of the expected fungal species being re-isolated from the margin of the infected material.

Levene's homogeneity of variance test returned a significant p-value ($p \leq 0.05$), indicating unequal variances. Data were transformed by a log function prior to ANOVA. There was a significant ($p = 0.002$) relationship between lesion width and taproot length, the longer the taproot the longer the lesion. Lesion weight was used to measure pathogenicity as it encompassed the actual volume rotted.

There was significant ($p \leq 0.05$) variation in pathogenicity between the isolates (Fig. 2.3). Mean lesion weight ranged from 0.07 g to 12.84 g. The most pathogenic isolates (MUCC707, MUCC708, MUCC710, and MUCC717) were *L. theobromae* and one isolate of *N. ribis* (MUCC730). *Lasiodiplodia theobromae* isolates were collected off three different hosts; *G. agrifolia*, *Eucalyptus* sp., *Calytrix* sp. and *A. gregorii*, respectively (Table 2.1). All isolates of *L. theobromae* caused lesions with the mean lesion weight ranging from 6.85 g to 12.84 g. The *N. ribis* isolate was collected from the bark of *E. camaldulensis* and produced a mean lesion weight of 9.12 g

Neoscytalidium novaehollandiae isolate MUCC537 (collected from *C. medicaginea*) produced a lesion mean of 3.32 g. Small lesions were formed by *P. adansoniae* (MUCC722 and MUCC723), *P. ardesiacum* (MUCC724) and *P. kimberleyense* (MUCC725 and MUCC 726), all collected from *A. gregorii* except for MUCC725 which was from a *Eucalyptus* sp., and MUCC723 which was collected from *G. agrifolia*. Other isolates representing *L. crassispora*

(MUCC720), *D. longicollis* (MUCC721) and *F. ramosum* (CMW26267) all formed small lesions.

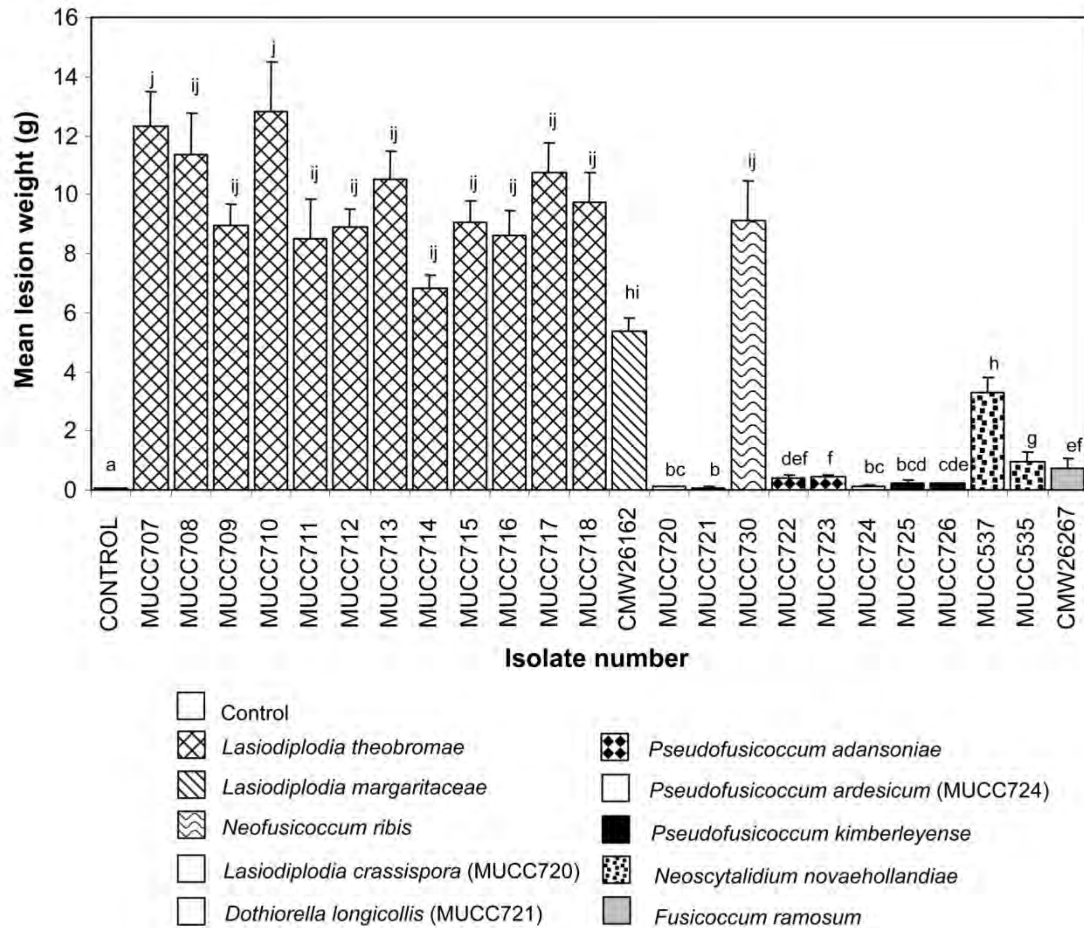

Figure 2.3 Mean lesion weight (g) from taproots in taproot pathogenicity trial (see Table 2.1 for details of each isolate). Bars represent the standard error of the mean. The same letter above the bar indicates means are not significantly (p >0.05) different using Duncan's mean separation test.

Pathogenicity to young baobab trees

From initial surface observations, lesions were evident as a slight darkening of the stem (Fig. 2.2 B & C). These became more pronounced as the epidermal layers were scrapped off (Fig. 2.2 D & E). In some cases, lesions extended along the pith (Fig. 2.2 F & G) of the stem and were not evident on the surface (Fig. 2.2 G). In numerous cases there was a cracking of the stem along the lesion, and fruit bodies (and in some cases mycelium) formed along the cracks and sap exudates (Fig. 2.2 B–E). In some cases, lesions did not extend beyond the length of the initial

incision; in these cases fruit bodies were often present underneath the parafilm. All isolates produced fruit bodies on at least two out of five potential lesions. No fruit bodies were observed on trees inoculated with control plugs. Fruit bodies were examined microscopically to confirm the morphological similarity between the inoculated and recovered fungi.

Levene's homogeneity of variance test returned a significant p-value ($p \leq 0.033$) indicating unequal variances. Lesion length, width and depth data were transformed by a square root function and all analyses were rerun. Lesion width and lesion depth followed the same patterns as results from lesion length data. There was no significant relationship between lesion length and tree height. There was a significant ($p = 0.018$) correlation between lesion length and stem diam, the smaller the diameter the longer the lesion.

Lesion length was significantly ($p \leq 0.001$) different between isolates. *Lasiodiplodia theobromae* again produced larger lesions than the other species. The lesions produced after inoculation of *A. gregorii* stems by *L. theobromae* resulted in the lesion lengths ranging from 3 to 25 cm (mean = 10.68 cm). The largest lesions were produced by a *L. theobromae* (MUCC708) isolate that had been collected from a *Eucalyptus* sp. *Lasiodiplodia theobromae* isolates collected from *A. gregorii* (MUCC717), *G. agrifolia* (MUCC707) and a *Calytrix* sp. (MUCC710) all produced similar large lesion sizes. *Lasiodiplodia theobromae* isolates collected from *L. cunninghamii* (MUCC715) and *F. opposita* (MUCC713) produced moderate lesions sizes. Four of the six *L. theobromae* isolates produced lesions greater than those of *N. ribis* (MUCC730), *L. margaritaceae* (CMW26162) and *N. novaehollandiae* (MUCC537) which exhibited reduced lesion severity (means = 3.46 cm, 2.94 cm and 3.54 cm, respectively) (Fig. 2.4).

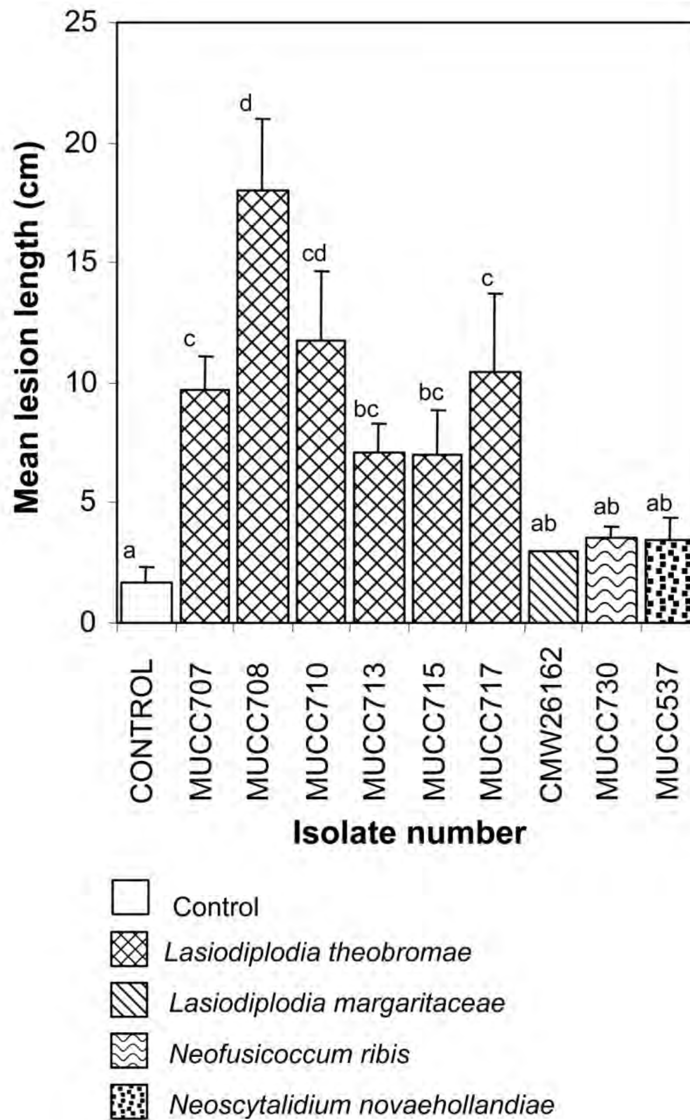

Figure 2.4 Mean lesion length (cm) in 2-3-year-old *Adansonia gregorii* trees in the young tree pathogenicity trial (see Table 2.1 for details of each isolate). Bars represent the standard error of the mean. The same letter above the bar indicates means are not significantly (p>0.05) different using Duncan's mean separation test.

Discussion

From undisturbed sites in the Kimberley, 29 fungal species were isolated as stem endophytes from asymptomatic trees, including 11 botryosphaeriaceous species (*L. theobromae*, *L. margaritaceae*, *L. crassispora*, *L. pseudotheobromae*, *P. adansoniae*, *P. ardesiacum*, *P. kimberleyense*, *N. novaehollandiae*, *D. longicollis*, *N. ribis* and *F. ramosum*). These species occurred on *A. gregorii* and surrounding hosts (*L. crassispora*, *N. ribis* and *F. ramosum* were not isolated from *A. gregorii* and *L. pseudotheobromae* was only isolated from *A. gregorii*). *Lasiodiplodia* spp., *Pseudofusicoccum* spp. and *N. novaehollandiae* were the most common genera, with *L. theobromae* as the dominant endophyte, sampled from most sites and most tree species (except for *Bombax* sp. and *Eucalyptus* sp.).

From the Darwin Botanical Gardens and a nursery in Broome, four botryosphaeriaceous species were isolated (*L. theobromae*, *L. parva*, *P. kimberleyense* and *N. dimidiatum*). Interestingly, two species (*N. dimidiatum* and *L. parva*) were collected at these two sites but were not isolated in the survey of the more remote undisturbed sites. Also, two of the trees (one in the Botanical Gardens and the only baobab in the nursery) were dying and these trees were associated with *L. theobromae* (only the Botanical Gardens) and *L. parva* (both sites).

Lasiodiplodia parva was recently described from cassava field soil in Colombia and *Theobroma cacao* in Sri Lanka (Alves et al. 2008). Previously, this species has been misidentified as *L. theobromae* as ITS sequence and spore morphology are similar. GenBank Blast searches of the ITS and EF-1α sequence confirm this misidentification. Within Australia (data obtained in this study), it has only been found in highly disturbed environments (a dying baobab in a nursery in Broome and one in the Botanical Gardens in Darwin), which suggests a human-mediated introduction.

GenBank searches indicated that most of the non-botryosphaeriaceous genera matched cultures that were collected as part of other published and unpublished endophytic studies. The genera identified were predominantly from the Dothideomycete and Sordariomycete, in which many endophytes are found. The most commonly isolated species “unknown 1” closely matched an

unidentified endophyte previously collected from *Platycladus orientalis* (conifer) in North Carolina, USA (Hoffman and Arnold 2008), and occurred in seven hosts including *A. gregorii* in this study. *Neurospora cerealis* is a widely distributed endophyte (García et al. 2004) and was found on three hosts including *A. gregorii* in the present study. Interestingly, this isolate matched a culture obtained from Koala faeces (Peterson et al. 2009). *Gibberella moniliformis*, found on eight hosts including *A. gregorii*, is a known latent pathogen causing maize ear rot (Pamphile and Azevedo 2002). *Cytospora eucalypticola* was found on eight hosts including *A. gregorii* in this study and is considered to be a wide-spread latent pathogen (Keane et al. 2000). *Rhytidhysteron* sp. was widely distributed on *A. gregorii* and the genus is known as a common tropical saprotroph and as a parasite of woody plants (Murillo et al. 2009). *Daldinia eschscholzii* is a common wood decaying saprotroph (Whalley 1996). *Phoma* species are wide-spread saprotrophs, endophytes and latent pathogens (Aveskamp et al. 2008; Qi et al. 2009). *Aureobasidium pullulans* was found in two hosts and is common in moist environments on plant leaves (Crous et al. 2004) (Robert et al.). Eight isolates occurred as singletons: *N. sphaerica* a wide-spread saprotroph (Crous et al. 2004) (Robert et al.); *Geosmithia* sp. (commonly associated with bark beetles) (Kolařík et al. 2008); *Cuvularia* sp. (Redman et al. 2002) and (Phongpaichit et al. 2006); Sordariomycete species 1; a Xylariaceae species; an Amphisphaeriaceae species; Dothideomycete, and an Ascomycete species (unknown 2).

The host range of most of the newly described botryosphaeriaceous endophytes was extended from the original description of these species (Pavlic et al. 2008). *Fusicoccum ramosum* has the most restricted host range occurring only in *E. camaldulensis*. *Pseudofusicoccum ardesiacum* was associated with three tree species, *A. gregorii*, *Eucalyptus* sp. and *G. agrifolia* (new record in the present study). In addition to *A. gregorii*, *L. margaritaceae* also occurred in *T. pterocarya* and *G. agrifolia* (both new records). The host range of *D. longicollis* was extended from *L. cunninghamii* and *T. pterocarya* to include *A. gregorii*. *Pseudofusicoccum kimberleyense* and *P. adansoniae* were previously identified from *A. gregorii*, *A. synchronicia*, a *Eucalyptus* sp. and *F. opposita* (Pavlic et al. 2008), but in the present study *P. adansoniae* was also isolated from *L. cunninghamii* and *G. agrifolia*. *Neoscytalidium novaehollandiae* had a wide host range,

occurring on six hosts; *A. gregorii*, *A. synchronicia*, *C. medicaginea*, *G. agrifolia*, *L. cunninghamii* (new record) and a *Eucalyptus* sp. (new record).

The pathogenicity trials, in particular the young tree pathogenicity trial, demonstrated that *L. theobromae*, and in the taproot trial, *N. ribis*, were significantly more pathogenic than the other species considered in the study. In both trials, there was variation in the pathogenicity of *L. theobromae* isolates and in some cases, mean lesion size and weight of *L. theobromae* were similar to lesions produced by *N. ribis*. *Neofusicoccum ribis* was not collected from *A. gregorii* in this study, but it is present in the environment and could potentially colonise *A. gregorii* trees. *Neoscytalidium novaehollandiae* and *L. margaritaceae* produced moderate lesions, while isolates of *P. adansoniae*, *P. ardesiacum*, *P. kimberleyense*, *L. crassispora*, *D. longicollis* and *F. ramosum* produced minor lesions. Both trials produced similar results, demonstrating that either could be confidently used to assess potential pathogenicity of endophytes of *A. gregorii*. However, *N. ribis* was shown to be highly pathogenic to the taproots of *A. gregorii* but only produced moderate lesions in the young trees. The time taken to set up, run (two weeks) and process the results of the taproot trial was much less then the young tree trial (six months required for lesion development). The resources and space required were much less than for the young tree trial. Baobab taproots (from three-month-old seedlings) are a cost-effective and less intensive in situ method, useful for a quick assessment of pathogenicity of fungal endophytes to *A. gregorii* and potentially other *Adansonia* species. However, subsequent screening in young trees is required to definitively test the potential pathogenicity of fungal endophytes.

The ability of *L. theobromae* to behave both as an endophyte (evident from isolation from healthy plant tissue) and as a pathogen (evident from lesion development in baobab taproot and tree) reinforces its position as a latent pathogen. Endophytes are defined by their ability to asymptomatically live in host tissue (Saikkonen 2007; Seiber 2007; Stone et al. 2004). Endophytes are likely to have evolved from pathogens (Carroll 1988; Saikkonen 2007; Seiber 2007). Latent pathogens are characteristically asymptomatic for part of their lifecycle (endophytic stage). If the host becomes stressed by some means such as water stress, this may trigger the latent pathogen to behave aggressively and attack the host. Subsequent disease

symptoms may enable the dissemination of fungal propagules into the environment and new hosts.

The Botryosphaeriaceae are a well researched group of latent pathogens (Smith et al. 1996) (Flowers et al. 2003; Slippers and Wingfield 2007). They have a cosmopolitan host range and wide geographical distribution (see Slippers & Wingfield 2007 for an extensive review). Species with a wide host range often also behave as latent pathogens (Slippers et al. 2005a; Slippers et al. 2005b) and (Slippers et al. 2009) with the ability to cause disease symptoms on hosts, as shown in pathogenicity trials (Fraser and Davison 1985; Shearer et al. 1987) (Mohali et al. 2009; Smith et al. 1994) and as disease records in the field (Barnard et al. 1987; Fraser and Davison 1985; Shearer 1994; Úrbez-Torres et al. 2008). Some of the Botryosphaeriaceae exhibit a very restricted host range such as *D. santalui* restricted to *S. acuminatum*, and *D. moneti* restricted to *A. rostellifera* and *A. cochlearis* (Taylor et al. 2009). These species appear to be non-pathogenic to their hosts.

This study took place in the NW Australia, a tropical climate with a mean annual temperature of 28 °C. Current climate models indicate more extreme weather conditions in this area (2007) but the impact on flora is as yet unknown. This may lead to the potential range of the pathogens (and in some cases known hosts) expanding. A rise in temperature, longer dry periods and increase in storm severity during the wet season, would lead to an increase in stress and also increasing storm related wounds on plants. This may provide triggers to allow latent pathogens to switch to the pathogenic phase of their lifecycle. *Lasiodiplodia theobromae*, the dominant endophyte and latent pathogen encountered in the current study, has an extremely large host range (Punithalingam 1980); however, it does seem to be limited to tropical and sub-tropical environments. Increasing temperatures may result in a major range expansion by this pathogen placing more known and unknown hosts at risk.

The severity of disease caused by latent pathogens should not be underestimated. Of particular importance to forestry and horticultural industries is the fact that endophytes are generally not scrutinized as part of quarantine measures (Slippers and Wingfield 2007). They have been

spread undetected around the world in germplasm (Burgess et al. 2004).Their ability to colonise a wide range of hosts (Seiber 2007; Stone et al. 2004)and their ability to cause disease on known and new hosts (Slippers et al. 2005b) emphasize their importance towards tree health and perhaps call for the need for routine molecular based screening of plant material for latent pathogens.

This trial indicates the potential threat that *L. theobromae* presents to the iconic baobab trees; however, the threat of *L. parva* remains to be verified. The dying baobab in Broome was reported with similar disease symptoms to those of dying baobabs in South Africa. It is clear that *L. theobromae* normally occurs as an endophyte of *Adansonia* species. As shown in the present trial, endophytes of *A. gregorii* may also behave as pathogens. The risk posed by latent pathogens is currently underestimated. Further work to understand the triggers that cause the pathogenic phase of these endophytes are imperative as well as mapping the current distribution of known latent pathogens and to highlight the risk to native and non-native flora.

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