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Botryosphaeriaceae associated with baobab (*Adansonia digitata* L.) and marula (*Sclerocarya birrea* A. Rich.) in agroforestry systems in Kenya

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Indigenous fruit trees such as baobab and marula provide key nutrients and income for smallholders and enhance diversification of agroforestry systems in the drylands of Sub Saharan Africa. Cankers and diebacks are increasingly observed impacting baobab and marula in domestication trials and farms in Kenya, but little is known on disease occurrence and associated pathogens. Field disease incidence and severity was assessed. Fungal isolation and molecular identification was performed and pathogenicity of isolates was evaluated on baobab, marula and additional agroforestry trees. Nine taxa morphotypes belonging to genera *Lasiodiplodia*, *Neofusicoccum* and *Dothiorella* were identified co-occurring in both symptomatic and asymptomatic plant material. Seedlings inoculated with isolates of *L. pseudotheobromae*, *L. theobromae* and *N. parvum* showed similar symptoms with various degree of virulence. These findings suggest that species of Botryosphaeriaceae may occur as endophytes and also act as a disease complex, with the potential of infecting a wide range of trees in Eastern Kenya. Further investigation of ecology and impact of this potential threat to agroforestry systems in the African drylands, need to be performed in order to develop mitigation strategies.

Key words: *Adansonia digitata*, agroforestry, Botryosphaeriaceae, *Sclerocarya birrea*, tree cankers.

INTRODUCTION

Domestication of *Adansonia digitata* and *Sclerocarya birrea* within agroforestry systems in drylands of Kenya has contributed to nutritional security and source of livelihoods (Waldron et al., 2019). However, canker and dieback diseases associated with Botryosphaeriaceae fungi has greatly impacted trees healths in Africa, potentially frustrating the benefits of agroforestry for smallholder farmers (Graziosi et al., 2019).

Species of Botryosphaeriaceae have been reported to cause serious disease on woody plants worldwide (Jami et al., 2014). Reports of dying baobab in South Africa were associated with *Lasiodiplodia theobromae* and *Neofusicoccum parvum* (Roux, 2002). Health assessment of Australian baobab (*Adansonia gregorii*) trees revealed

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eleven Botryosphaeriaceous species including *Lasiodiplodia theobromae* (Sakalidis et al., 2011), *Lasiodiplodia crassispora*, *L. pseudotheobromae*, *Neofusicoccum ribis*, *Pseudofusicoccum adansoniae* and *Lucania parva*. *Graphium* species was also identified associated with baobab in South Africa (Cruywagen et al., 2010; Farr and Rossman, 2016)

Botryosphaeriaceae causing canker and dieback in Kenya has been reported on *Grevillea robusta* in Eastern Kenya (Njuguna et al., 2011), on Meliaceae (Muthama et al., 2017) and on Eucalyptus (Machua et al., 2016). However, no isolation attempts have been done on indigenous baobab and marula in Kenya. Severe cankers associated with Botryosphaeriaceae have been observed in Kenya on domestication trials of baobab and marula causing growth loss, fruit rot and tree mortality; additionally due to their domestication trials, the biotic interaction in agroforestry systems provide an intriguing situation to study. Hence, this is the first detailed study of Botryosphaeriaceae attacking *A. digitata* and *S. birrea* in East Africa. The role of these fungi in the ecology of the trees from which they were collected should be considered in future studies.

The objective of this study was to characterize the diversity of Botryosphaeriaceae associated with cankers of native trees *A. digitata* and *S. birrea* in Kenya and to assess their pathogenicity on these hosts.

MATERIALS AND METHODS

Field survey and sampling

Survey was conducted in Eastern Kenya in Makueni and Kitui County in 2018. Three sites were selected across the two Agro-ecological zones of Mukange, Tiva and Ikanga.

Makueni country is hot and dry receiving mean annual rainfall of 231 and 361 mm during long and short rains respectively. The mean maximum temperature of the area is 25°C and the mean minimum temperature is 13°C (Jaetzold et al., 2010). Kitui is hot and dry with high temperature throughout the year ranging from 16 to 34°C (Jaetzold et al., 2012).

Samples were collected from symptomatic and asymptomatic material from across five farms in Kitui and Makueni County. Symptomatic trees were sampled based on occurrence of various disease symptoms; such as dieback of shoots and branches, cankers on trunk with resin flow and diseased leaves, leaf spots or blights.

Fungal isolation, characterization and growth rate studies

A total of 102 symptomatic and 18 healthy trees were sampled. Pieces were cut from disease growing edge and also from healthy samples; surface were sterilized and blotted dry with sterile filter papers. Pieces were plated on petri dishes containing 2% malt extract agar (MEA) amended with streptomycin sulfate (100 mg/l) (Merck, Germany) and incubated at 25°C. The isolates were replicated three times. The cultures were monitored daily for two weeks and colonies resembling Botryosphaeriaceae were sub cultured to fresh 2% (MEA) plates until purification.

After two weeks of incubation, nine morphotypes were distinguished by conidial characteristics aided by relevant keys, publications and books on Botryosphaeriaceae fungi (Burgess et al., 2019). Ten morphological groupings of Botryosphaeriaceae isolated from healthy and asymptomatic tissues of baobab and marula in the three sites were selected for molecular studies. Ten isolates of each Botryosphaeriaceae occurring in each site and tree species were selected for molecular studies.

Growth rates were assessed between 15 and 35°C at 5°C intervals in culture growth. Three replicates of each isolate were used. Mycelial plugs of 6 mm diameter were taken from actively growing edges of week-old single mycelia cultures and transferred to the center of MEA 90 mm diameter petri-dishes. Three perpendicular measurements were taken of the colony diameter daily until mycelium of the fastest growing isolates had covered the plates. Macromorphological changes in the growing colonies (upper and lower sides) were studied. The experiment was monitored for color changes using color charts of Rayner (1970), for two weeks. Pycnidia were mounted in 85% lactic acid on microscopic slides and examined using a microscope.

Ten isolates from each morphotype were chosen for DNA extraction. Genomic DNA was extracted using CTAB (3%) and phenol-chloroform DNA extraction method as described by Gardes and Bruns (1993) with modifications according to Ihrmark et al. (2002). Part of ITS r DNA region was amplified and sequenced using fungal specific primer ITS1F (5'-CTTGGTCATTTAGAGGAGTAA-3') (Gardes and Bruns, 1993) and ITS4 (5'TCCTCCGCTTATTGATATGC-3). The amplified PCR products were purified AMPURE PCR purification kit (Beckman Coulter, USA) following manufacturer's instructions. The samples were sequenced in both directions at least twice using the PCR Big Dye ® terminator cycle sequencing kit. PCR reaction mix was prepared as described by the manufacturer's instruction. The samples were sequenced in both directions twice using ITSF and ITS 4 primers.

Edited nucleotide sequences were submitted to NCBI database sequences and identified using BLASTN (Ying et al., 2015) www.ncbi.nlm,nih.gov/BLAST/blast. cgi Published sequences from GenBank were used to identify sequences obtained from this study.

Phylogenetic analyses were done for ITS sequenced data. The edited nucleotide data and those from GenBank were aligned using MUSCLE and phylogenetic analysis was done in MEGA 7. The evolutionary history was inferred using Neighbor-joining method (Saitou and Nei, 1987). The tree was drawn to scale. The evolutionary distances were computed using Maximum Composite Likelihood method of Kumar et al. (2016). All positions containing gaps and missing data were eliminated from dataset.

Pathogenicity trial

Three potential canker and dieback fungi obtained diseased and healthy tissues were selected for the pathogenicity trials: *N. parvum*, *L. theobromae* and *L. pseudotheobromae*. The species were selected on the basis that they were the species isolated most frequently from diseased and symptomless samples of *A. digitata* and *S. birrea* trees. Healthy 8-month-old seedlings of marula, baobab, *Acacia xanthophloea* and *Calodendrum capense* were chosen for the pathogenicity assay. The part to be inoculated was sterilized with 70% ethanol and a vertical incision of approximately I cm was made using sterile blade and bark carefully lifted up. Mycelial plug 5 mm^2 were excised from four day old cultures using cork borer and placed at the centre of the incision and covered with parafilm. After inoculation, the seedlings were assessed regularly for canker symptoms development for 6 months. All the seedlings were selected, slit longitudinally and the total length of the internal lesion was recorded.

To complete Koch's postulates, three inoculated stems per isolates were randomly selected for re-isolation of inoculated fungus.

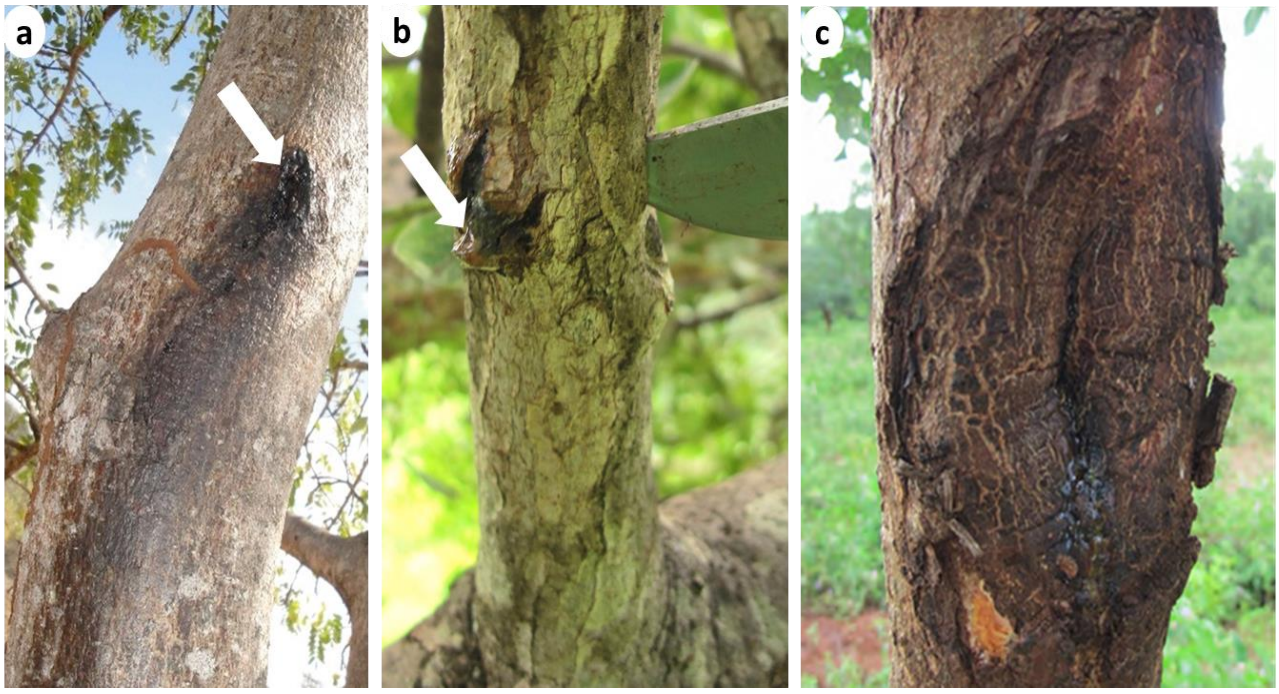


Figure 1. Disease symptoms observed in *Adansonia digitata* and *Sclerocarya birrea.*

Data analysis

GenStat Version 19.1 were used to analyze the data as needed and Minitab Version 15 was used to analyze field data collected. Pathogenicity trial was conducted using a randomized complete block design with four blocks comprising of 80 treatments including controls. Data was log transformed to satisfy the assumptions of ANOVA. One-way analysis of variance was used to assess difference in lesion lengths among fungal species with means separated using Turkey's test (p=0.05).

RESULTS

Field symptoms and abundance of fungal species on parts of *A. digitata* and *S. birrea* in Eastern Kenya

The main disease symptoms observed in the field were stem with resin flow (gummosis) (Figure 1). Cankers varied in size from small lesions to large open wounds.

The disease in the field was characterized by stem cankers with resin, dieback of branches and some leaf spots and blights. Disease incidence increased during dry season. About 87% of the diseased trees and 70% of healthy trees sampled yielded Botryosphaeriaceae fungi. Analysis of fungi occurring on different plant parts showed that most of the fungi were isolated from diseased stems (50.1%) followed by branches (33.5%), leaves (9.7%) and healthy plant parts (6.7%) (Table 1). *L. theobromae* and *L. pseudotheobromae* were the most frequently isolated species occurring on both symptomatic and asymptomatic tissues, with highest occurrence in dieback and canker symptoms.

Morphological and molecular characterization

All the isolates produced aerial mycelium that was initially white turning greyish white, dark green or blackish grey after two weeks. About 450 isolates were morphologically characterized and 38 molecularly identified. The morphotypes corresponded to three main genera; *Lasiodiplodia, Neofusicoccum and Dothiorella.*

In MEA culture *Lasiodiplodia* initially had dense whitish mycelium turning smokey grey, and olivaceous grey color on the reverse (Figure 2b, d, f and g).

Dothiorella colonies were initially white to smokey grey with woolly aerial mycelia, becoming pale olivaceous grey within 5 to 7 days (Figure 2c). In culture, aerial mycelia of *Neofusicoccum* were fluffy and white becoming grayish and pale olivaceous gray and bluish black on the reverse (Figure 2a).

Growth rate studies

The fungi differed in their growth rates at five temperatures (P<0.001) (Table 2). *N. parvum* was the fastest species and colonized the plate within 24 h, achieving maximum growth at 30 to 35°C. *Lasiodiplodia mahajangana* obtained maximum growth at 30°C. All fungi grew favorably between 25 and 35°C and poorly at 15°C.

Table 1. Significance of occurrence of Botryosphaeriaceae fungi species on healthy and diseased parts of *A. digitata* and *S. birrea* in Eastern Kenya.

Tree species	Fungal species	Healthy			Diseased			% occurrence
		Leaves	Branch	Bark	Leaf spots and blight	dieback	Stem canker	
A. digitata	*L. theobromae*	–	++	+++	++	++++	+++++	18.2
	L. pseudotheobromae	–	–	+	–	+++	++++	15.2
	L. parva	–	+	+	–	++	+++	1.5
	N. parvum	+	++	++	++	++++	+++++	5.3
	L. crassispora	+	+	+	++	+++	+++	0.7
	D. longicollis	–	–	–	+	++	++	0.9
	D. sarmentorum	–	–	+	++	++	+++	0.8
	Lasiodiplodia sp.	–	++	++	++	++	+	1.3
S. birrea	*L. theobromae*	–	+	+	+	+++	++++	17.6
	L. pseudotheobromae	–	+	+	+	++	++	14.5
	L. parva	–	–	+	+	+	++	0.8
	L. crassispora	+	++	++	+	+++	+++	1.5
	L. mahajangana	–	+	–	+	++	++	0.1
	Lasiodiplodia sp.	–	–	+	++	+++	++++	9.5

- = Pathogen not detected in the tissue; **+ =** occurrence not significant; **++ =** 1-10% occurrence in the disease symptoms; **+++ =**10-20% occurrence; **++++ =** 20-30% occurrence; **+++++ = >**30%. Species with an occurrence of >++ were considered potentially important pathogen in the disease type.

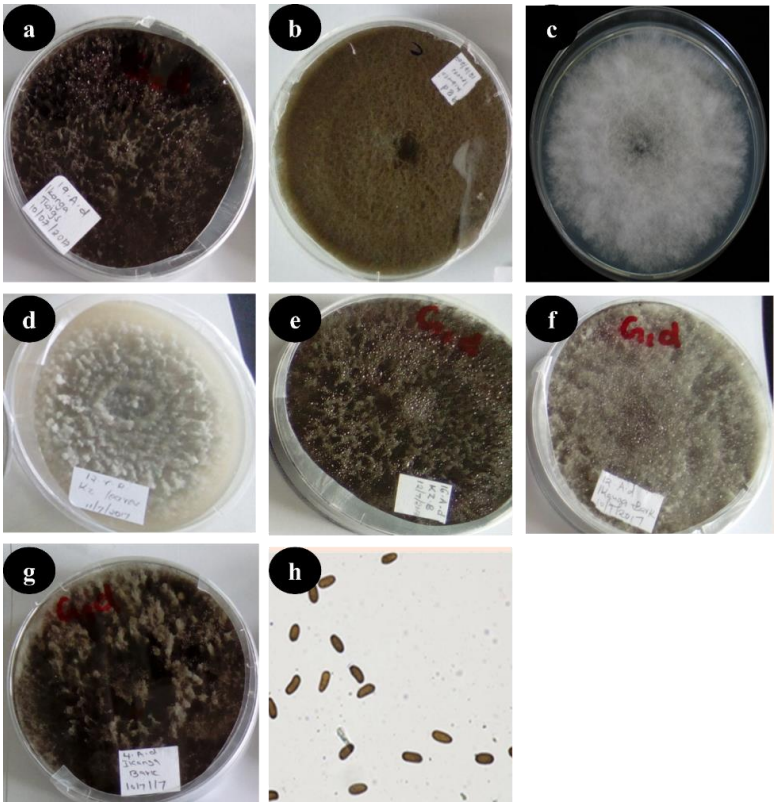


Figure 2. Macro and microscopic cultural and conidial characteristics of morphotypes. (a) *Neofusicoccum parvum*, (b) *Lasiodiplodia* sp., (c) *Dothiorella* sp., (d) *L. pseudotheobromae*, (e) *L. crassispora*, (f) *L. parva*, (g) *L. theobromae* and (h) spores of *Lasiodiplodia theobromae*.

Table 2. Growth rate of five Botryosphaeriaceae at five temperatures.

Temperature (°C)	Growth rate (mm/day)				
	Mean growth rates (mm day^{-1}) ± SE				
	N. parvum	*L. theobromae*	*L. pseudotheobromae*	*Lasiodiplodia sp.*	*L. mahajangana*
15	3.3±0.2^{aa*}	3.7±0.2^{aa}	4.0±0.2^{ad}	4.5±0.2^{ab}	6.3±0.3^{da}
20	8.8±0.3^{ab}	10.0±0.1^{ba}	5.2±0.6^{bd}	8.3±0.4^{aac}	10.0±0.4^{abe}
25	13.2±0.1^{ac}	14.9±0.1^{bb}	6.5±0.4^{cc}	14.8±0.2^{bb}	14.6±0.3^{bc}
30	15.0±0.0^{ad}	14.1±0.1^{ba}	9.6±1.9^{cd}	12.8±0.4^{abc}	15.3±0.2^{cd}
35	15.6±0.1^{ad}	10.8±0.3^{ab}	7.5±0.8^{ea}	7.9±0.3^{ad}	5.4±0.3^{ac}

*Mean colony diameter LSD at 95% confidence interval. *Letters followed by the same superscript across the column (different temperatures) were not significant at 95% confidence interval.

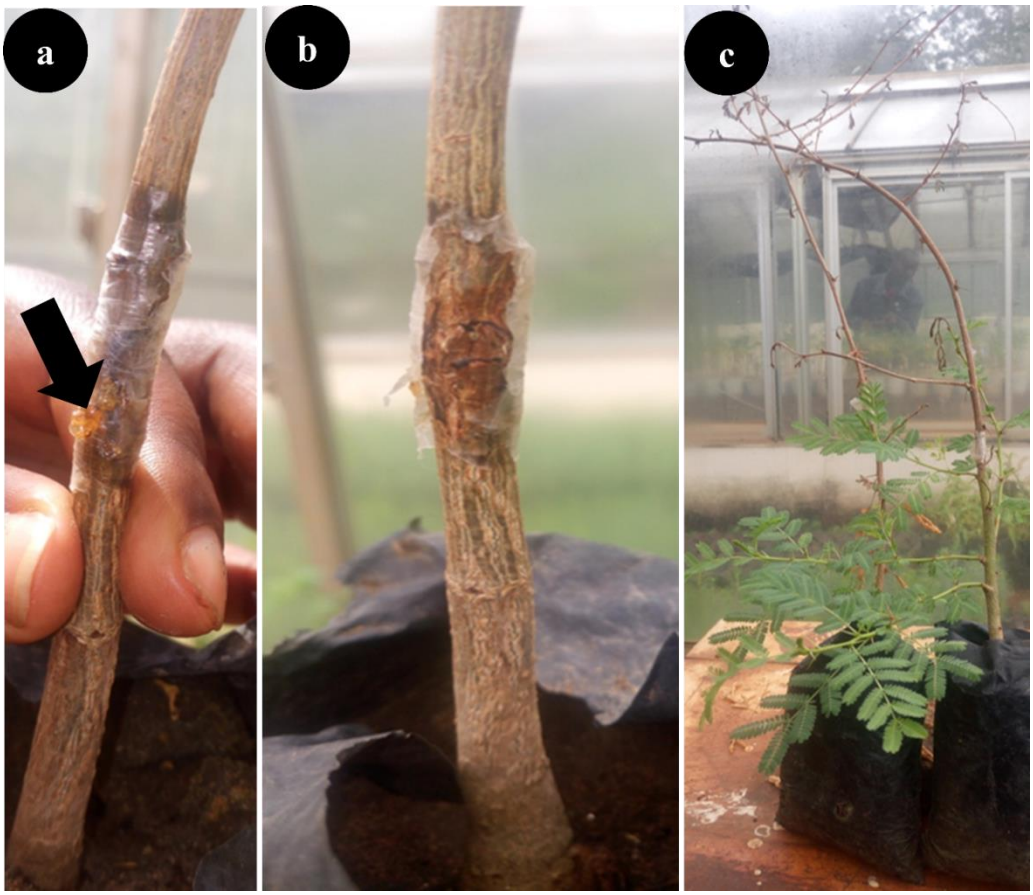


Figure 3. Symptoms developed on inoculated seedlings.

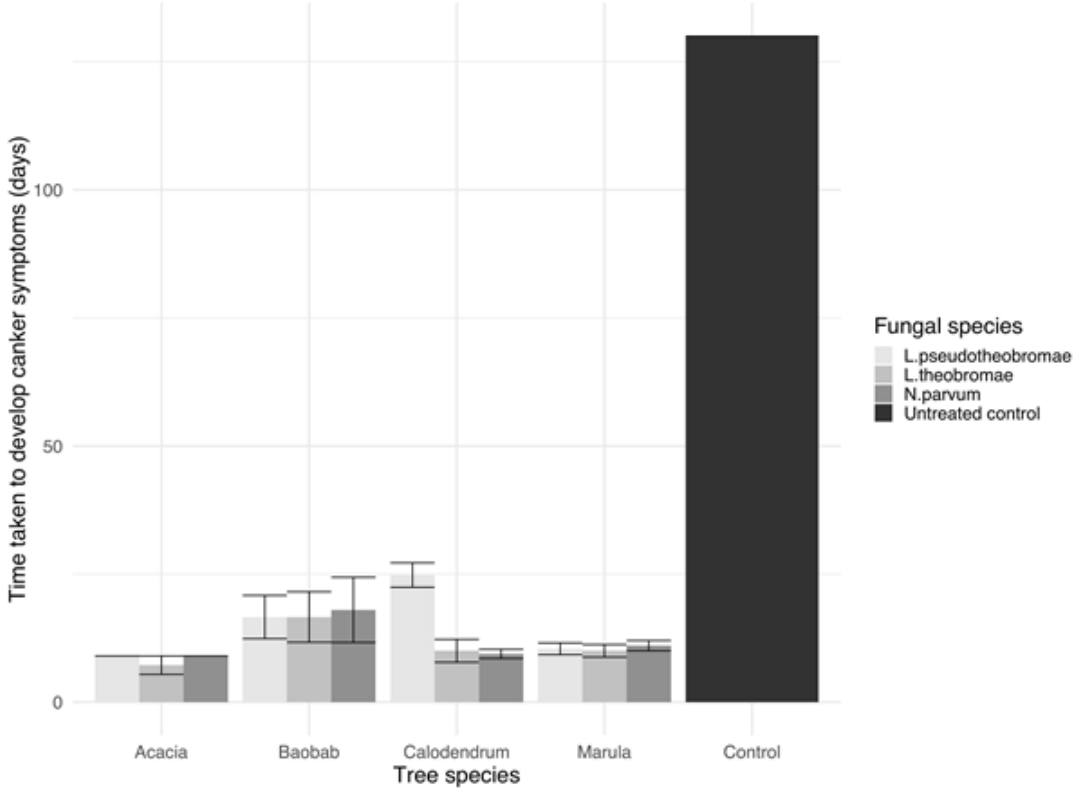
Pathogenicity of three Botryosphaeriaceae species

Seedlings of the entire four tree species inoculated with Botryosphaeriaceae showed canker and dieback disease symptoms as observed for these four tree species in the field. The main symptoms caused by the *L. pseudotheobromae*, *N. parvum* and *L. theobromae* on inoculated seedlings were cankers, dieback and wound healing (Figure 3). The earliest symptom observed on inoculated seedlings was resin production (gummosis), which occurred on approximately 90% of seedlings inoculated with Botryosphaeriaceae within 14 days. The seedlings developed canker symptoms characterized by necrosis of the inner bark and woody tissues, stem swelling and bending. Incidence of wound healing was highest on baobab and marula. Healed tissues were surrounded by layers of fleshy callous tissues around discoloured tissues where the fungus had been inoculated

Table 3. Summary of analysis of variance (ANOVA).

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Fungal pathogen	3	16.62418	5.541392	999.71	<.001
Tree species	4	0.732276	0.183069	33.03	<.001
Fungal pathogen. Tree sps	5	0.422677	0.084535	15.25	<.001
Residual	67	0.371383	0.005543		
Total	**79**	**18.15051**			

Variate: Number of days.



Figuure 4. Mean days to show early canker and dieback symptoms in *Adansonia digitata*, *Sclerocarrya birrea*, *Calodendrum capense* and *Acacia xanthophloea* seedlings inoculated with *L. pseudotheobromae*, *L. theobromae* and *N. parvum* under glass house conditions.

(Figure 3b). The symptoms caused by the three Botryosphaeriaceae species were generally indistinguishable among the four plant species.

There was significant difference in the mean number of days taken by each of the three inoculated fungal species to cause canker and dieback symptoms on the four tree species (p≤0. 001; Table 3). Seedlings inoculated with *L. pseudotheobromae* were the first to develop canker symptoms on *Acacia* followed by *Calodendrum* (Figure 4) *L. theobromae* came second in developing the symptoms and *N. parvum* was little slower. *Acacia* and *Calodendrum* were highly susceptible to Botryosphaeriaceae but *S. birrea* and *A. digitata* were less susceptible. Baobab and marula showed fastest wound healing after inoculation of Botryosphaeriaceae than other tree species inoculated with the same fungal species.

Internal lesions were identified by extensive discoloration of phloem and rotting of inner tissues (Figure 3). The size of the internal lesions in the four tree species were significantly different from the un-inoculated control seedlings and between fungal species (p<0.001) (Table 4). All Botryosphaeriaceae species caused the longest lesions in *A. xanthophloea* and *C. capense*, moderate lesion on Marula (*S. birrea*) and shortest lesion on Baobab (*A. digitata*) (Figure 5).

Table 4. Summary of analysis of variance (ANOVA) at 95% confidence interval.

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
fungal_sps	3	189.222	63.074	7.56	<.001
tree_species	4	584.554	146.138	17.52	<.001
fungal_sps. tree_species	5	393.818	78.764	9.44	<.001
Residual	67	558.988	8.343		
Total	**79**	**1726.582**			

Variate: lesion size (cm).

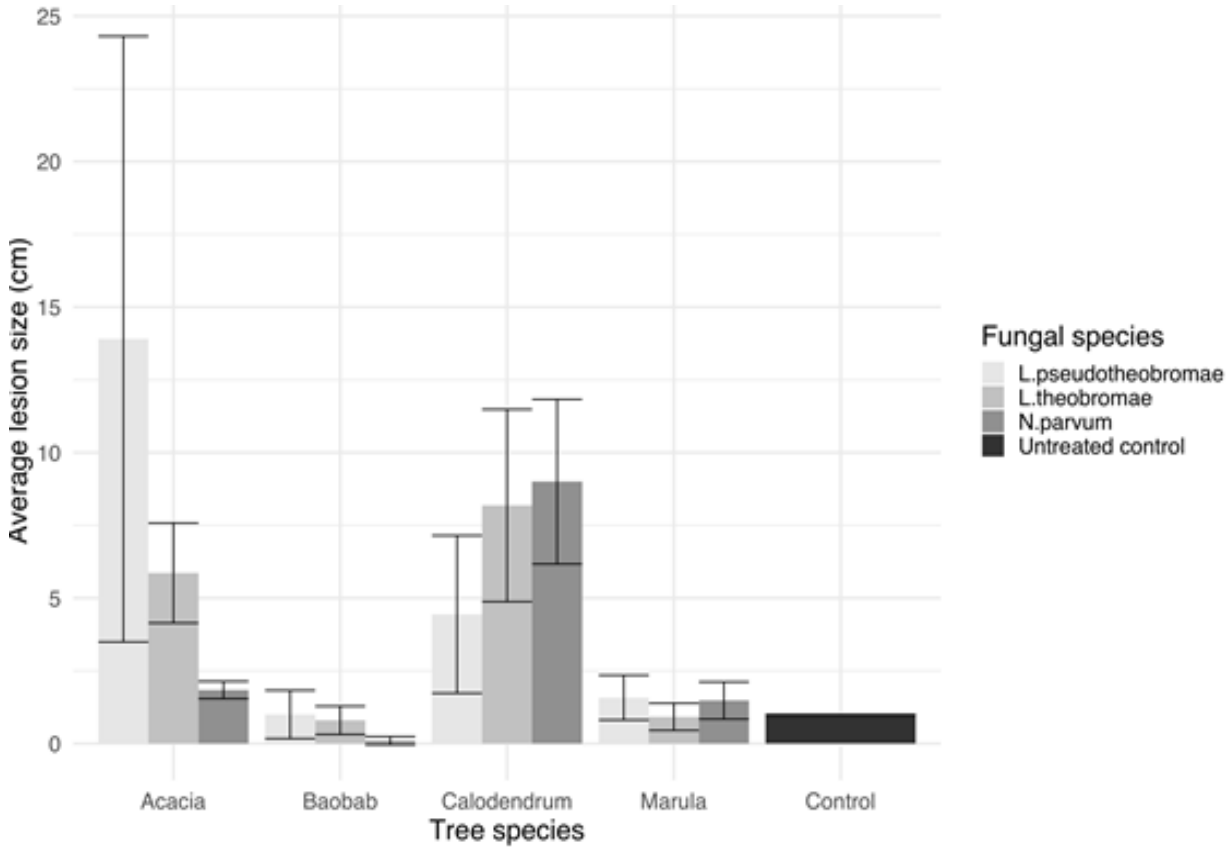


Figure 5. Mean internal lesion lengths (cm) after six months in *Adansonia digitata*, *Sclerocarrya birrea*, *Acacia xanthophloea* and *Calodendrum capense* seedlings inoculated with *Lasiodiplodia pseudotheobromae*, *L. theobromae* and *Neofusicoccum parvum* under glass house conditions in Kenya.

The longest lesion occurred on Acacia inoculated with *L. pseudotheobromae*, followed by *N. parvum* and *L. theobromae* showed slightly lower virulence.

Ranking of the scores obtained from the two variables (occurrence of early canker symptoms and size of internal lesions caused by each fungal species under glass house conditions) using Kruskal Wallis one-way analysis of variance showed the average rank for *L. pseudotheobromae*=50.67, *N. parvum*=49.67, *L. theobromae*=46.15 and Control=15.5 ($p<0.001$) as shown in Table 5.Therefore, *L. pseudotheobromae* was the most virulent species on all the tree species.

DISCUSSION

Canker and dieback disease was identified as a threat to cultivation of baobab and marula in Kenya and potential canker and dieback pathogens were also identified. This study described most comprehensive. Botryosphaeriaceae species associated with diseased and healthy samples of *A. digitata* and *S. birrea* in Eastern Kenya using

Table 5. One-way analysis of variance.

Sample	Size	Mean rank
L. pseudotheobromae	20	50.67
N. parvum	20	46.15
L. theobromae	20	49.67
Control (pure agar inoculation)	20	15.5
P value (Chi-square probability)	**< 0.001**	

morphological and molecular data on ITS region. Three fungal genera were identified by means of phenotypic characters and DNA sequence analyses. Majority of the isolates represented *L. pseudotheobromae*, *L. theobromae* and *N. parvum* forming more than 50% of the Botryosphaeriaceae associated with both *A. digitata* and *S. birrea*. The three fungal species showed a strong endophytic association with asymptomatic tissues of baobab and marula (Begoude et al., 2010). The endophytic nature of Botryosphaeriaceae could be triggered to pathogenic phase by unfavourable climatic conditions (Osoro et al., 2017). The abundance and isolation frequency of *L. theobromae*, *L. pseudotheobromae* and *N. parvum* on diseased plant parts indicated they could play a role in the disease. *L. theobromae, L. pseudotheobromae and N. parvum* are serious pathogens of woody tree species in Africa (Slippers et al., 2017). *L. theobromae* has been referred to as a widely distributed fungi in tropical and sub-tropical regions and is reported to infect more than 500 plant species, the fungi has been associated with shoot blight, dieback and stem cankers in a diverse group of hosts (Adesemoye et al., 2014). The wide range of temperatures in which the species of Botryosphaeriaceae described here can grow (with optimum ranging from 25 to 30°C) make it hypothesized that high temperatures favors the pathogenic phase of this pathogens. *L. mahajangana*, *L. theobromae*, *L. pseudotheobromae*, *L. iraniensis* and *L. crassispora* had previously been isolated from *S. birrea* (Mehl et al., 2017), while *L. crassispora*, *L. pseudotheobromae*, *L. parva* and *L. mahajangana* have been associated with *A. digitata* (Cruywagen *et al*., 2017). Reports of dying Baobab in South Africa were associated to *L. theobromae* and *N. parvum. L. Pseudotheobromae* appears to have a wide host range and geographic distribution (Rodrıguez-Galvez et al., 2017). It has been associated with cankers, dieback and stem rot in mangoes (Ismail et al., 2012), trunk canker in *Acacia* (Castro Medina et al., 2013). *L. pseudotheobromae* is the most isolated species from baobabs in Africa with isolations from both asymptomatic and symptomatic tissues. It caused lesion on Australian *A. gregorii* but few isolates were observed in Kenya Baobab.

The ability of these pathogens, *L. pseudotheobromae*, *L. theobromae* and *N. parvum* to cause disease on four tree species were tested. The focus species were *A. digitata* and *S. birrea*, the other two species, *C*. *capense* and *A. xanthophloea* were included because they are agroforestry trees and the disease seemed to be widespread on several hosts on farms. Early canker development gave a rapid indication of virulence on all tree species and ranking analysis showed *L. pseudotheobromae* to be the most virulent on all the four tree species. The three fungal species isolated from baobab and marula could cause the disease not only on same species but also on *C. capense* and *A. xanthophloea.*

Therefore, the susceptibility of the four tree species to attack by all the fungi tested indicated the plurivorous nature of Botryosphaeriaceae (Jeff-ego and Akinsanmi, 2018). Emergence of pathogens with a wide host range pose serious health risk to other crops within Agroforestry and reduce productivity of the system. Trees adapted to dry areas should be restricted to their climatic conditions; emphasis on site specificity for trees should be encouraged as a disease management strategy to reduce destruction by pathogens.

Wound healing characterized by formation of callous tissues around infected parts was an indication of a host response to limit the spread of infection from the point of inoculation. No wound healing occurred in *Acacia* and *Calodendrum*, which indicated that high relative susceptibility to infection was connected to low wound healing. Baobab and marula were the least susceptible to infection among the four tree species tested, which are indigenous to semi-arid areas of Kenya and seemed to be better adapted to semi-arid conditions. The three fungi produce indistinguishable symptoms and it was not possible to isolate the primary cause of the disease supporting previous observation by Njuguna et al. (2011). It is therefore concluded that canker and dieback disease was a disease complex. Co-occurrence of Botryosphaeriacae fungi as observed may increase the ability of the pathogens to overcome the host's resistance especially under unfavorable environmental conditions, although its benefits are not yet clear. *Acacia xanthophloea* and *Calodendrum capense* were highly susceptible to the disease whereas *A. digitata* and *S. birrea*, the native species showed least susceptibility making it suitable for agroforestry systems in semi-arid areas. This study concluded that unspecific plurivorous nature of pathogens with stressful environment in

agroforestry system will increase the virulence of the fungi and susceptibility of the host.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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