African Journal of Rural Development, Vol. 8 (2): April-June, 2023: pp. 92-106 ISSN 2415-2838

Pathogenicity of species in *Botryosphaeriaceae* associated with stem canker on *Eucalyptus* germplasms in Uganda

SYOFUNA, A.[1], NAKABONGE, G.[1], TUMWEBAZE, S.[1], NAMAYENGO, S.[1], TWEHEYO, M.[1], MUGABI, P.[1], NYEKO, P.[1], ZZIWA, A.[2], BALIKOOWA, K.[3] and BANANA, A.[1]

[1]Department of Forestry, Biodiversity and Tourism, School of Forestry, Environmental and Geographical Sciences, College of Agricultural and Environmental Sciences, Makerere University, P.O. Box 7062, Kampala Uganda

[2]Department of Agricultural and Biosystems Engineering, School of Food Technology, Nutrition and Bio-engineering, College of Agricultural and Environmental Sciences, Makerere University, P.O. Box 7062, Kampala Uganda

[3]Department of Environmental Management, School of Forestry, Environmental and Geographical Sciences, College of Agricultural and Environmental Sciences, Makerere University, P.O. Box 7062, Kampala Uganda

Corresponding author: gnakabonge@gmail.com

ABSTRACT

Botryosphaeria canker is threatening the successful establishment of commercial *Eucalyp*tus plantations in Uganda. This study investigated the pathogenicity of species of Botryosphaeriaceae associated with the canker disease and susceptibility of *Eucalyp*tus *grandis* W.Hill ex Maiden and its hybrid clones. Molecular characterization of the rRNA internal transcribed spacer (ITS) and β-tubulin gene regions revealed isolates of *Neofusicoccum parvum, Neofusicocum ribis, Neofusicoccum kwambonambiense, Pseudofusicoccum* sp. and *Lasiodiplodia sp.* Pathogenicity of *Botryosphaeriaceae* isolates was significant (P<0.05) and isolate AS-02 of *N. kwambonambiense* was the most aggressive and AS-6 *of Pseudofusicoccum spp*. the least aggressive. However, with all fungal isolates combined, hybrid clones (*Eucalyp*tus *grandis*× *Eucalyptus urophylla*) GU 7 and GU 8 exhibited the highest resistance to the disease and GC 796/2 and F1 (*E. grandis* from South Africa) was the most susceptible. The information generated in this study should be exploited for sustainable plantation forestry management in the region.

Keywords: Botryosphaeria canker, Lasiodiplodia, Neofusicoccum, plantation forestry, Pseudofusicoccum

RÉSUMÉ

Le chancre de Botryosphaeria menace l'établissement réussi des plantations commerciales d'Eucalyptus en Ouganda. Cette étude a investigué la pathogénicité des espèces de Botryosphaeriaceae associées à la maladie du chancre et la susceptibilité d'Eucalyptus grandis W. Hill ex Maiden et de ses clones hybrides. La caractérisation moléculaire des régions ITS (Internal Transcribed Spacer) de l'ARNr et du gène de la β-tubuline a révélé des isolats de Neofusicoccum parvum, Neofusicoccum ribis, Neofusicoccum kwambonambiense, Pseudofusicoccum sp. et Lasiodiplodia sp. La pathogénicité des isolats de Botryosphaeriaceae était significative (P<0,05), avec l'isolat AS-02 de N. kwambonambiense étant le plus agressif et l'isolat AS-6 de Pseudofusicoccum spp. étant le moins agressif. Cependant, avec tous les isolats fongiques combinés, les clones

Cite as: Syofuna, A., Nakabonge, G., Tumwebaze, S., Namayengo, S., Tweheyo, M., Mugabi, P., Nyeko, P., Zziwa, A., Balikoowa, K. and Banana, A. 2023. Pathogenicity of species in *Botryosphaeriaceae* associated with stem canker on *Eucalyptus* germplasms in Uganda. *African Journal of Rural Development* 8 (2):92-106.

hybrides (Eucalyptus grandis × Eucalyptus urophylla) GU 7 et GU 8 ont montré la plus grande résistance à la maladie, tandis que GC 796/2 et F1 (E. grandis d'Afrique du Sud) étaient les plus susceptibles. Les informations générées dans cette étude devraient être exploitées pour une gestion durable des plantations forestières dans la région.

Mots-clés: Chancre de Botryosphaeria, Lasiodiplodia, Neofusicoccum, Sylviculture de plantation, Pseudofusicoccum

Introduction

Eucalyptus species are among the most important plantation forestry tree species that have been introduced in many tropical and sub-tropical regions, Uganda inclusive (Varmola and Carle 2002; Sawlog Production Grant Scheme (SPGS), 2007). To relieve pressure on Uganda's Natural forests, *Eucalyptus* spp. were initially established to drain swamps around Kampala and drive steam engines for the railways (Stebbing, 1953). Currently, *Eucalyptus* trees are grown for utility poles, domestic and industrial fuel wood, building construction, aesthetic use in towns and as shelter belts on agricultural fields (Kaboggoza, 2011; Birara *et al.,* 2019). The *Eucalyptus* species commonly grown in Uganda is *Eucalyptus grandis*, its hybrid clones of *E. grandis*× *E. urophylla* (GUs; GU 7, GU 8, GU 21, GU 607 and GU 609), and hybrids of *E. grandis*× *E. camaldulensis* (GCs; GC 796, GC 784, GC 578, GC 540, GC 514, GC 550) (SPGS 2009; Turinawe *et al.,* 2014).

Despite the efforts put in the establishment of plantation forestry worldwide, diseases continue to pose a serious threat to this development initiative (Food and Agriculture Organization FAO, 2009). Disease surveys conducted in Uganda reported Botryosphaeria canker as the most widely spread disease occurring among *Eucalyptus* species and *Grevillea robusta* A. Cunn. ex R. Br. (Roux *et al*., 2001; Nyeko and Nakabonge 2008; Nakabonge *et al.,* 2019). Botryosphaeria canker is caused by pathogenic fungal species belonging to the family *Botryosphaeriaceae*, order *Botryosphaeriales* with 29 well characterized genera (Phillips *et al*., 2013). The commonly known genera include; *Botryosphaeria, Dothiorella, Fusicoccum, Pseudofusicoccum, Lasiodiplodia, Diplodia, Neoscytalidium,* and *Neofusicoccum* (Slippers and Wingfield, 2007; Bezerra *et al*., 2021; Garcia *et al*., 2021).

Symptoms that have been linked to Botryosphaeriaceae include twig, branch and main stem cankers, die-back of shoots or whole branches, and blue stain of sapwood (Slippers and Wingfield, 2007; Slippers *et al*., 2009; Carlucci *et al.,* 2015; Urbez-Torres *et al.,* 2016; Bezerra *et al*., 2021). Bark cracking, oozing, stem discoloration and malformation, as well as the occurrence of kino pockets in the xylem and occurrence of epicormic shoots are some of the other symptoms observed on trees infected with Botryosphaeria canker causing fungi (Gezahgne *et al*., 2004; Batista *et al.,* 2021). Severity and visible symptoms of infections were once used to identify the causal pathogens of Botryosphaeria canker (Niekerk *et al*., 2006; Ammad *et al.,* 2014). However, overlapping symptoms and morphological features has led to the use of molecular characterization for conclusive identification of the pathogens to species level (Crous *et al.,* 2009). The nuclear rRNA internal transcribed spacer region composed of ITS1/ITS2 intergenic sequences with well conserved 5.8 rRNA and the β-tubulin (tub) genes are frequently utilized in phylogenetic analysis of Botryosphaeriaceae (Li *et al.,* 2018).

Studies on pathogenicity of Botryosphaeriaceae species have been conducted in many regions where the disease has been problematic (Mohali *et al.,* 2009; Nakabonge *et al.,* 2019). For instance, studies on susceptibility of selected Eucalyptus hybrid clones to Botryosphaeria canker have been conducted previously in Uganda (Nakabonge *et al*., 2019), however they focused on a

limited number of clones and species. Understanding the susceptibility of *E. grandis* and hybrid clones is essential in management of Botryosphaeria canker because the alternative mechanisms to control the disease such as the use of fungicides may not be sustainable (Old *et al.,* 2003; Nakabonge *et al.,* 2019). Therefore, the objectives of this study were to, (1) collect and identify Botryosphaericeae fungi associated with stem cankers of Eucalyptus trees in Uganda, (2) evaluate the pathogenicity of the identified Botryospheriaceae fungi on commonly grown Eucalyptus trees and (3) determine the level of susceptibility of *E. grandis* and its hybrid clones to Botryospheriaceae in Uganda. In contrast to prior studies that focused on one region, the current study followed a nationwide survey.

Materials and Methods

Sample collection and fungal isolations. A survey of Botryosphaeria canker disease was carried out in several districts of Uganda and samples were collected from Arua, Kabarole, Kyankwanzi, Luwero, Mayuge, Mbale, Mbarara, and Omoro districts (Figure 1).This was during 2019. Samples were collected from these areas based on previous reports of occurrence of Botryosphaeria canker disease (Nyeko and Nakabonge, 2008; A. Syofuna, unpulished data). Several symptomatic bark samples were randomly cut and collected from infected *Eucalyptus* trees countrywide. The samples were placed in individual paper bags, labelled and transported to Makerere University, School of Agricultural Sciences laboratory in Kampala, Uganda. The samples were then disinfected using 70 % ethanol and cut into approximately $3mm^2$ pieces that were transferred to Petri dishes containing Malt Extract Agar, (MEA: 20 g L^{-1}, 15 g L^{-1} agar, Biolab) and incubated at 25 ^{o} C to induce sporulation. Single hyphal tips were transferred to new MEA media. For each fungal isolate, four replicates were made by subculturing on 2 % MEA. From the four replicates, one was used for phylogenetic study while the other three were used for pathogenicity study.

DNA extraction, PCR amplification and Sequencing .For each isolate, 10-20g of growing mycelia were scraped off the surface of MEA plate and transferred to 1.5 ml Eppendorf tubes (Eppendorf, Germany). DNA was extracted using the Zymo Biomics DNA kit (Zymo Research Corporation, USA) following the instruction manual. The internal transcribed spacer (ITS) region of the ribosomal DNA (rDNA) was amplified using the primers ITS1 (5' TCCGTAGGTGAACCTGCGG) and ITS4 (5' TCCTCCGCTTATTGATATGC) (White *et al.* 1990).The Beta-tubulin gene fragment was amplified using the primers Bt2a5'GGTAACCAAATCGGTGCTGCTTC) andBt2b(5' ACCCTCAGTGTAGTGACCCTTGGC) (Glass and Donaldson, 1995). PCR amplification, reaction mixtures and conditions as well as visualization were conducted as described by Nakabonge *et al*. (2019). The PCR products were purified using the High Pure PCR Product Purification Kit (QIAGEN, GmbH, Hilden, Germany). The PCR products were sequenced in both directions (reverse and forward) using the Big Dye Cycle Sequencing kit with Amplitaq DNA Polymerase FS (Perkin-Elmer, Warrington, UK), according to the manufacturer's protocols on an ABI PRISM 3100 DNA Auto sequencer (Perkin-Elmer). The same primers used for the PCR amplification reactions were also used for sequencing of the ITS and the beta-tubulin gene regions. Amplicons were sequenced using the sanger sequencing method at Inqaba Biotechnical Industries (Pty) Ltd, Pretoria, South Africa.

Phylogenetic analysis. The forward and reverse sequences were combined using Cap 3 contig Assembly program (http://www.insilico.uni-duesseldorf.de/Cap3.html). Reference sequences of Botryosphaeriaceae fungi were derived from NCBI GenBank (https://www.ncbi.nlm.nih.gov/) for comparisons (Table 1). Multi-locus alignment of the assembled sequences and phylogenetic analysis was conducted using the software MEGA X through the Maximum-Likelihood method and

Tamura-Nei model (Tamura and Nei 1993; Kumar *et al.* 2018). The Tamura-Nei model was used because it corrects for multiple hits, taking into account the differences in substitution rates between nucleotides and the inequality of nucleotides frequencies. It also distinguishes transitional substitution rates between purines and traversional substitution rates between pyrimidines. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Joining and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. Bootstrap replicates (1000) were done on consensus parsimonious trees (Felsenstein, 1985). The tree was rooted with *Lecanosticta acicola* (von Thümen) Sydow isolate LNPV252.

Pathogenicity and Susceptibility tests. From the identified Ugandan isolates, AS-02 of *N. kwambonambiense,* AS-09 of *N. parvum*, AS-05 of *N. ribis*, AS -06 of *Pseudofusicoccum* sp., and AS-01 of *Lasiodiplodia* sp. were selected for pathogenicity tests. Eight Eucalyptus species and hybrid clones commonly grown in Uganda including six hybrid clones (GC540, GC550, GC796, GC796/2, GU7, GU8) and *E. grandis* species sourced from Australia (F2) and South Africa (F1) were selected for the experiment (Table 3). The trees came from a certified tree breeding centre where they were clearly labelled according to species and hybrids. The experimental design was a completely randomized block design with factorial treatment. The tree seedlings and plantlets were grown in one-liter buckets at the backyard of the School of Forestry at Makerere University for one year while the fungal isolates were grown on 2% malt extract agar (MEA) at 30ºC for two weeks before inoculation. Eight seedlings/plantlets of each Eucalyptus germplasms were inoculated on the stem (1.6 cm in diameter) approximately 8cm above the base with a $4mm^2$ mycelial plug of each fungal strain taken from the growing colony on MEA. A $4mm^2$ cork borer was used to remove the bark and expose the cambium. Agar plug of mycelium from each fungal culture was picked and placed under the bark of each of the Eucalyptus germplasms using a Scalpel blade with the mycelium facing the cambium. The inoculated areas were wrapped and sealed off with Parafilm to prevent desiccation and contamination (Chen *et al.,* 2011). As a control, three seedlings/plantlets of each germplasm were inoculated with sterile MEA plug. Each tree species/clone consisted of 40 trees including eight replicates (trees) for each of the five fungal isolates and control. Thus, the total number of seedlings/plantlets used in the experiment was 384 (8 germplasms X 8 replicates X 6 treatments). The inoculated plants were exposed to field environmental conditions, watered daily and observed for sixty days. The experiment was repeated once.

Two months after inoculation, the bark of the inoculated plants was removed and lesion lengths (cm) on the cambium were measured. Mean lesion length was compared for each fungal species and each Eucalyptus germplasms.. Stem lesion lengths were then analysed by One-way ANOVA performed with a General Linear Model to determine the pathogenicity of each of the fungal strains and susceptibility of the Eucalyptus germplasm. Factors considered were: fungal isolates (5) and *E. grandis* and its hybrid clones (8). Mean values were compared by Tukey's LSD test at 5 % probability level. Microsoft office excel was used to generate graphs for the comparison of all means.

Results

Isolation. A total of 34 samples were collected from symptomatic (Figure 2) Eucalyptus species and hybrid clones from eight districts of Uganda. Out of these, 15 fungal isolates were successfully obtained but only 9 single spore cultures that had attained full mycelial growth were used for DNA extraction.

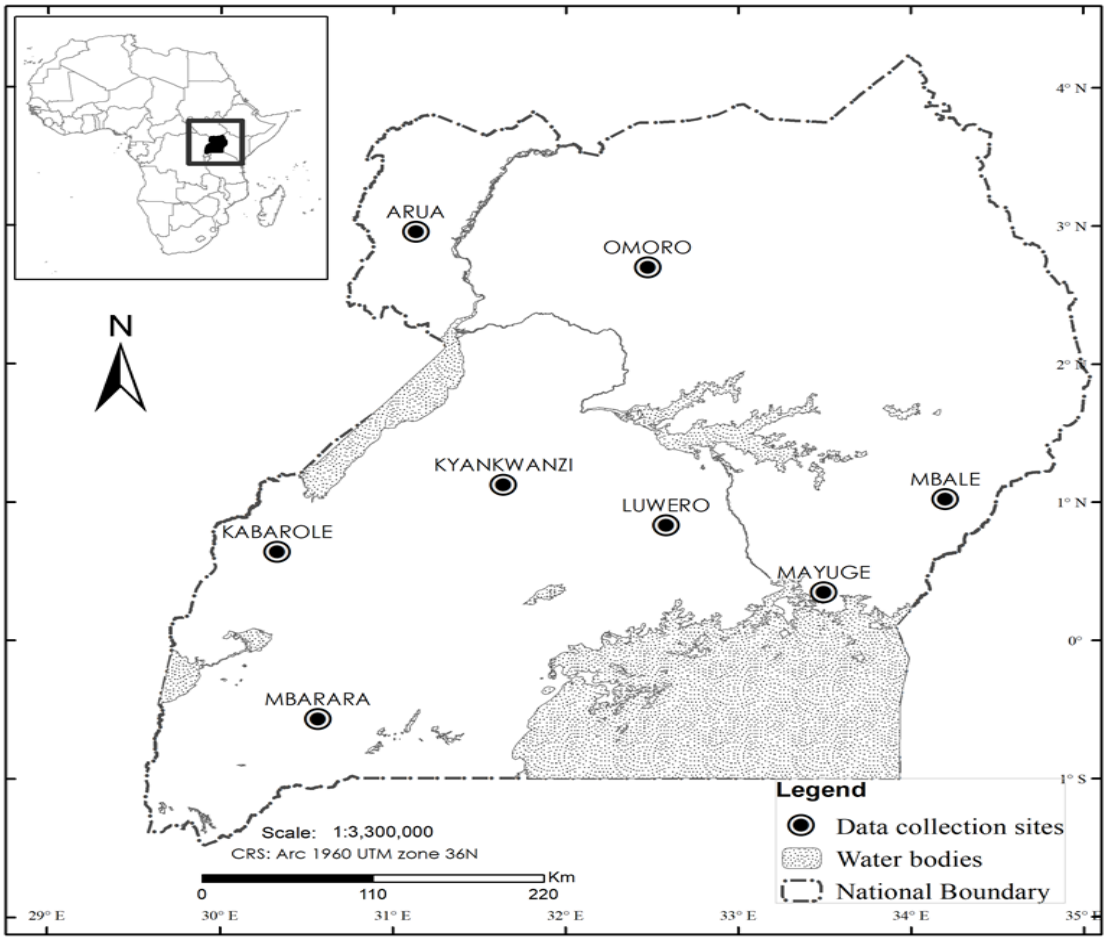

Figure 1. Map of Uganda showing districts where symptomatic bark samples were collected

Figure 2. Symptoms associated with Botryosphaeria canker in fields where samples were collected

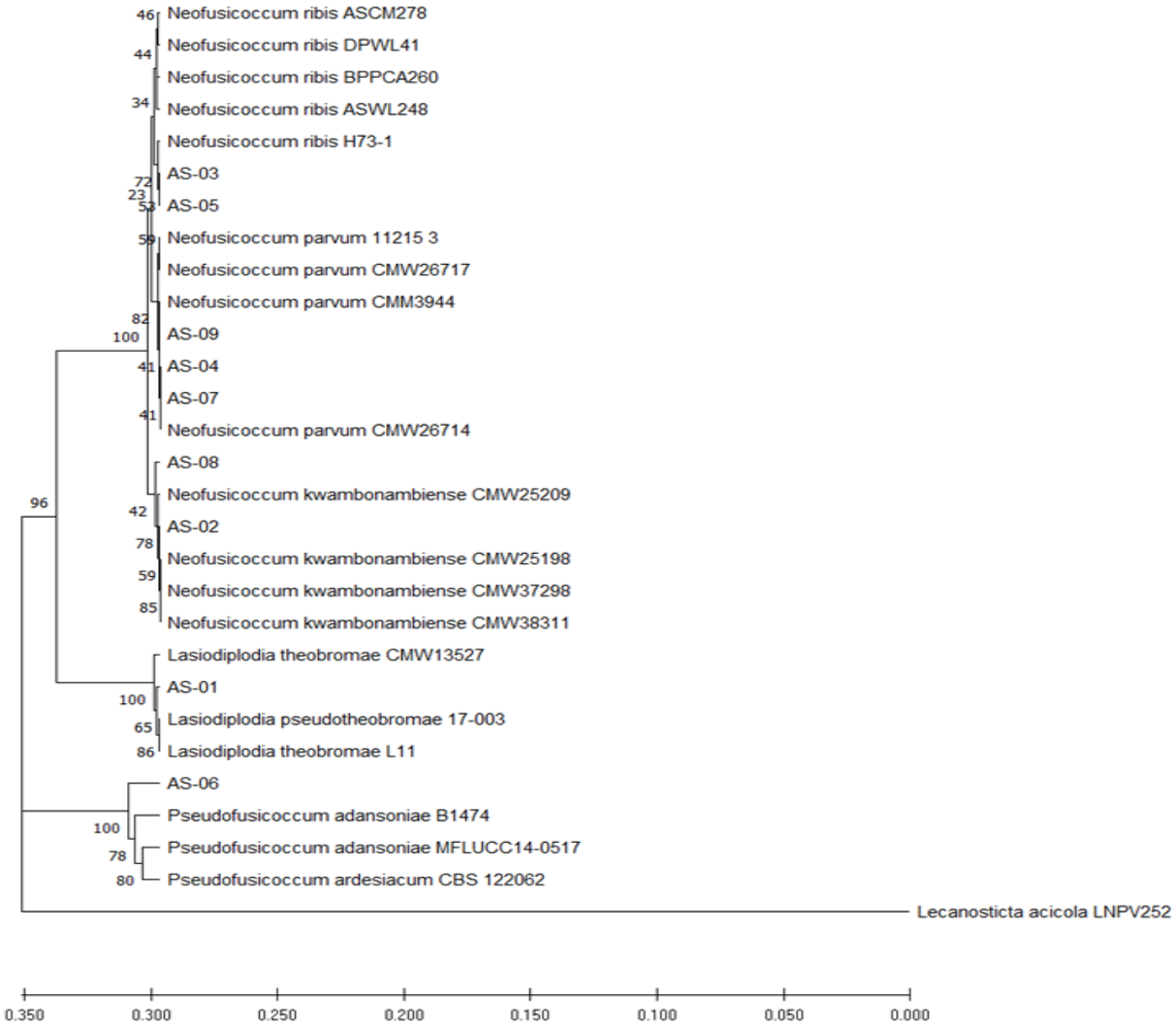

Figure 3. Phylogenetic analysis of the 2a sequences of *Botryosphae riecea* isolates

DNA sequencing and phylogenetic analysis. Phylogenetic analysis with the nine fungal isolates revealed five phylogenetic taxa. Blast searches against the GenBank nucleotide collection showed that the sequences of the fungal isolates were most similar to those shown in Table 1. Phylogenetic analysis of the aligned 29 sequences generated an optimal tree with highest log likelihood -1350.70 (Figure 3). Bootstrap values (1000 replicates) are indicated on the branches. The consensus phylogenetic tree generated for the combined sequences indicated that the Ugandan Botryosphaeriaceae isolates identified in the current study resided within five clades.; Isolate AS-01 resided in the *Lasiodiplodia* clade, AS-03 and AS-05 in the *N. ribis* clade, AS-02 and AS-08 in the *N. kwambonambiense* clade, isolates AS-04, AS-07, and AS -09 in the *N. parvum* clade, and isolate AS-06 in the

Pathogenicity of Botryosphaeriaceae to *E. grandis* and hybrid clones. Two months following inoculation, all fungus inoculated trees developed lesions. Brown discolorations stretching from the point of inoculation were observed with the bark peeled off (Figure 4). There was a significant difference in the length of lesion caused by the different fungal isolates ($F(5, 6) = 46.29$, $P=0.00$). The least significant difference test showed that isolates that grouped in *N. kwambonambiense, N. ribis* and *N. parvum* clades caused the longest lesion (Table 2). The lesions caused by isolates within the *Neofusicoccum* genus were significantly longer than for *Lasiodiplodia* and *Pseudofusicoccum* genera (Table 2). All fungal isolates produced significantly longer lesions than the control implying they were pathogenic.

Figure 4. Developed lesions on Eucalyptus germplasm sixty days after inoculation with Botryosphaeriaceae fungi

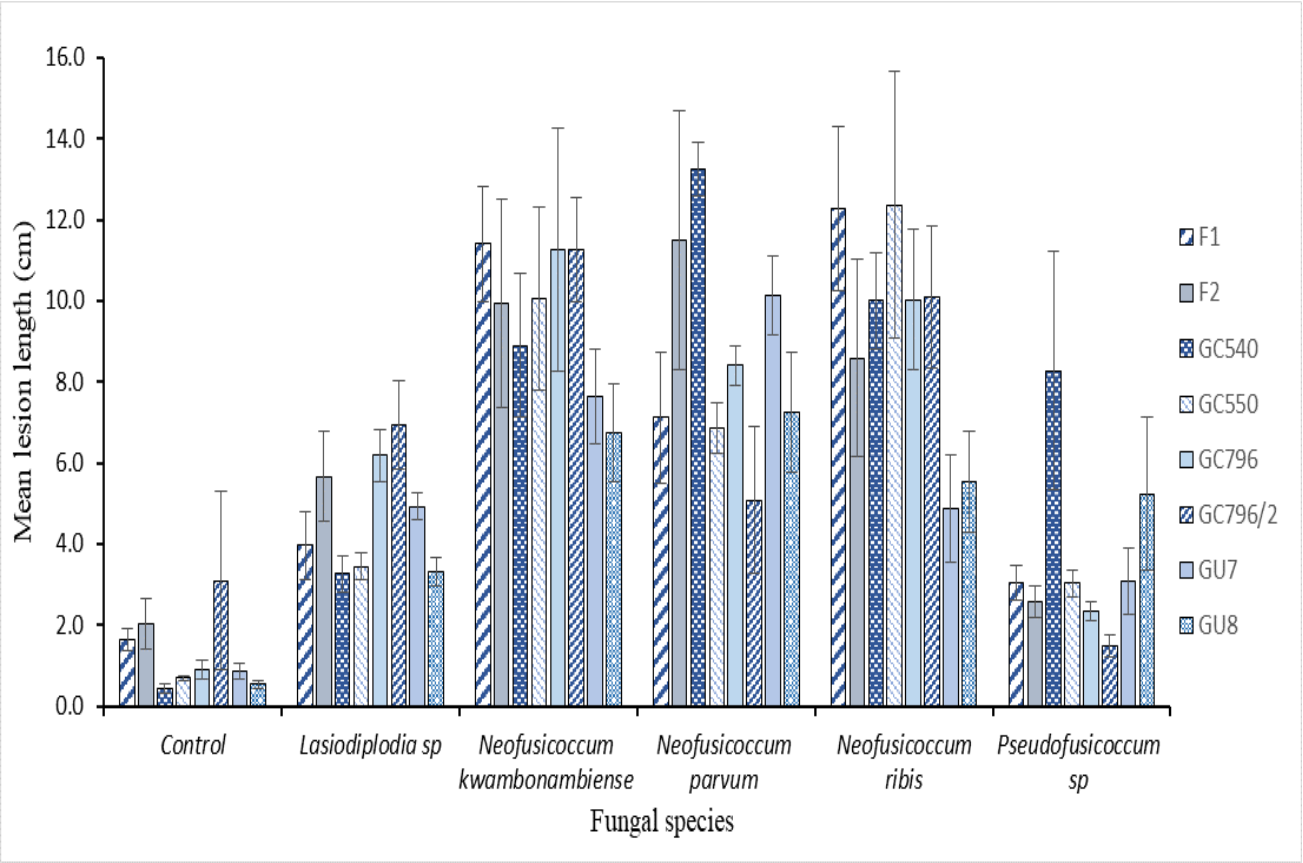

Figure 5. Lesion length measured 60 days after inoculation with different *Botryosphaeriaceae* isolates on *Eucalyptus* species and hybrid clones

Fungal isolates showed variation in virulence to the various Eucalyptus germplasms (Figure 5). *Lasiodiplodia* sp. was more virulent to GC796/2, GC796, F2, GU7, F1 than to GC540, GU8 and GC550. The isolate that resided in *N. kwambonambiense* was the most aggressive to F1, GC796, GC796/2, GC550, F2, GC540 than to GU7 and GU8. The isolate from the *N. parvum* group was more aggressive to GC540, F2, GU7, GC796, F1 than to GU8, GC550 and GC796/2. The N. ribis isolate was more aggressive to F1, GC550, GC796/2, GC796, GC540 than to F1, GU8, GU7. *Pseudofusicoccum* sp. was more aggressive to GC540 than to GC796/2.

Susceptibility of *E. grandis* and hybrid clones. There were significant differences in lesion length on *E. grandis* and hybrid clones when inoculated with *Botryosphaeriaceae* fungi $F_{(35, 36)} = 1.71$, P=0.014). This means all *Eucalyptus* trees in the study were susceptible to the pathogens. The levels of susceptibility varied in *E. grandis* and its clones. F2, GC 540, GU 7 and GC 796 were the most susceptible to isolate in the *N. parvum*, F1 and GC550 were the most susceptible to isolate in *N. ribis* group while GC796 and GC796/2 were susceptible to isolate in *N. kwabonambiense* (Figure 6). Comparisons of all means showed that GC 540, F1, GC 796/2 and F2 were the most susceptible to the canker pathogens followed by hybrid clone GC 796 and GC 550 while GU 7 and GU 8 were the most resistant with smallest lesions sizes formed after inoculation (Table 3).

Discussion

The phylogenetic analysis with the ITS rRNA and Beta tubulin sequence data of our fungal isolates revealed five groups within the Botryosphaeriaceae including; *N. ribis, N. kwambonambiense, N. parvum, Lasiodiplodia*sp.and *Pseudofusicoccum* sp. The isolation of *Botryosphaeriaceae* from samples collected during the current study confirms earlier reports of the occurrence of the fungal pathogens in Uganda and their association with Eucalyptus and its clonal hybrids (Nakabonge, 2002; Nakabonge *et al.,* 2019). Botryosphaeriaceae species are among the most important pathogenic fungi because of their ability to cause diseases on a wide range of valuable plant species (Slippers *et al*., 2004; Li *et al.,* 2015; Hilário *et al.,* 2020; Hlaiem *et al.,* 2020; Batista *et al.,* 2021).

The genus *Neofusicoccum* was first described by Crous *et al.* (2006) and includes many species that are important pathogens causing several plant diseases globally (Slippers *et al.* 2004; Thomidis *et al*., 2011; Ni *et al.,* 2012; Phillips *et al*., 2013). *Neofusicoccum parvum* (Pennycook and Samuels) Crous, Slippers and A.J.L.Phillips, was first described from Kiwifruit and *Populus* spp. in New Zealand. In this study, *N. parvum* isolates were recorded to be in Kabarole (western Uganda), Arua (northern Uganda) and Mayuge (eastern Uganda) districts. The wide distribution of the species poses a potential threat to plantation forestry development in Uganda (Jami *et al*., 2017; Nakabonge *et al*.,2019).

Neofusicoccum kwambonambiense Pavlic. Slippers and M.J. Wingf, (Slippers, Crous and M.J. Wingf.) was first described from dying branches and pulp of ripe fruits of *Syzygium cordatum* and Eucalyptus species in Kwambonambi in South Africa (Pavlic *et al.,* 2007, 2009). The species was recorded on Eucalyptus germplasms from Mbarara (western) and Mayuge (eastern) districts in Uganda. Indeed *N. ribis* is a well-known pathogen of many woody species including Eucalyptus with a wide geographical distribution, thus its isolation from the same tree species in Uganda was not surprising. In this study, the fungus was found in Mbale, Kyankwanzi and Luwero districts in Uganda.

Lasiodiplodia species are important pathogens having been reported on a wide range of trees in temperate and tropical regions (Slippers and Wingfield, 2007; Njuguna, 2011; Li *et al*., 2019) and are also known to cause disease on Eucalyptus species (Li *et al*., 2015). The genus *Pseudofusicoccum* was established by Crous *et al.* (2006) for species resembling *Fusicoccum*, and are reported to be native to Australia (Pavlic *et al.,* 2008). *Pseudofusicoccum* spp. have

Table 1. Origin and number of samples collected per region

Region of Uganda	Origin/ District	Number of samples collected
Eastern Uganda	Mbale	3
	Mayuge	6
South western Uganda	Mbarara	4
Central Uganda	Kyankwanzi	5
	Luwero	4
Western Uganda	Kabarole	4
Northern Uganda	Omoro	3
	Arua	3

Table 2. *Botryosphaeria* isolates used in phylogenetic analysis including those currently identified from Uganda.

Isolate No	GenBank Identity	Origin of isolate	GenBank Accession No	
			ITS	Beta Tubulin
17-003	*L. pseudotheobromae*	South Korea	LC270865.1	LC314724.1
CMW13527	*L. theobromae*	Venezuela	KY473074.1	KY472965.1
L11	*L. theobromae*	China	KR260801.1	KR260830.1
AS-01*	*Lasiodiplodia sp*	Uganda	MW293875	MW303963
CMW25198	*N. kwambonambiense*	South Africa	KU997386.1	KU997562.1
CMW38311	*N. kwambonambiense*	Mozambique	KF432949.1	KF454704.1
CMW37298	*N. kwambonambiense*	Mozambique	KF432945.1	KF454700.1
CMW25209	*N. kwambonambiense*	South Africa	KU997390.1	KU997565.1
AS-02*	*N. kwambonambiense*	Uganda	MW293876	MW303964
AS-08*	*N. kwambonambiense*	Uganda	MW293882	MW303970
CMW26717	*N. parvum*	South Africa	FJ900611.1	FJ900638.1
CMM3944	*N. parvum*	Brazil	JX513636.1	KC794028.1
CMW26714	*N. parvum*	South Africa	FJ900610.1	FJ900637.1
11215_3	*N. parvum*	New Zealand	JX074743.1	JX398944.1
AS-04*	*N. parvum*	Uganda	MW293878	MW303966
AS-07*	*N. parvum*	Uganda	MW293881	MW303969
AS-09*	*N. parvum*	Uganda	MW293883	MW303971
ASCM278	*N. ribis*	Malaysia	MK557959.1	MK573986.1
DPWL41	*N. ribis*	Malaysia	MK557955.1	MK574002.1
BPPCA260	*N. ribis*	Malaysia	MK557958.1	MK573996.1
ASWL248	*N. ribis*	Malaysia	MK557957.1	MK573988.1
H73-1	*N. ribis*	Australia	HQ392733.1	HQ392754.1
AS-03*	*N. ribis*	Uganda	MW293877	MW303965
AS-05*	*N. ribis*	Uganda	MW293879	MW303967
MFLUCC14-0517	*P. adansoniae*	Thailand	KM396906.1	KM510364.1
B1474	*P. adansoniae*	Malaysia	KT968483.1	KX154810.1
CBS 122062	*P. ardesiacum*	Australia	KF766222.1	KX465069.1
AS-06*	*Pseudofusicoccum sp.*	Uganda	MW293880	MW303968

Isolates with * were collected in this study from Uganda

Table 3. Origin of Eucalyptus germplasm used for pathogenicity tests

***Eucalyptus* germplasm**	**Origin**
Eucalyptus grandis (F1)	South Africa
Eucalyptus grandis (F2)	Australia
GC 796/2	South Africa
GU 8	South Africa
GU 7	South Africa
GC 540	South Africa
GC 550	South Africa
GC 796	South Africa

Table 4. LSD groupings for mean length of lesions caused by different fungal species.

Fungal species	**Mean lesion length (cm)**	**Std error**	**Grouping**
N. kwambonambiense AS-2	9.59	0.673	A
N. ribis AS-5	8.94	0.770	A
N. parvum AS-9	8.35	0.687	A
Lasiodiplodia sp. AS-1	4.89	0.339	B
Pseudofusicoccum sp. AS-6	3.54	0.467	B
Control	1.27	0.202	C

Species with the same letters in grouping are not significantly different from each other at 5%.

Table 5. Comparison of mean lesion length of *Eucalyptus* germplasm after inoculation with Botryopsphaeriaceae pathogens

***Eucalyptus* species and hybrid clones**	**Mean lesion length (cm)**	**Std error**	**Grouping**
F1	6.803	0.884	A
F2	6.730	0.978	A
GC540	7.078	1.181	A
GC796/2	6.808	1.008	A
GC550	6.084	0.899	B
GC796	6.238	0.886	B
GU7	5.703	0.648	C
GU8	5.214	0.649	C
Control	1.27	0.476	D

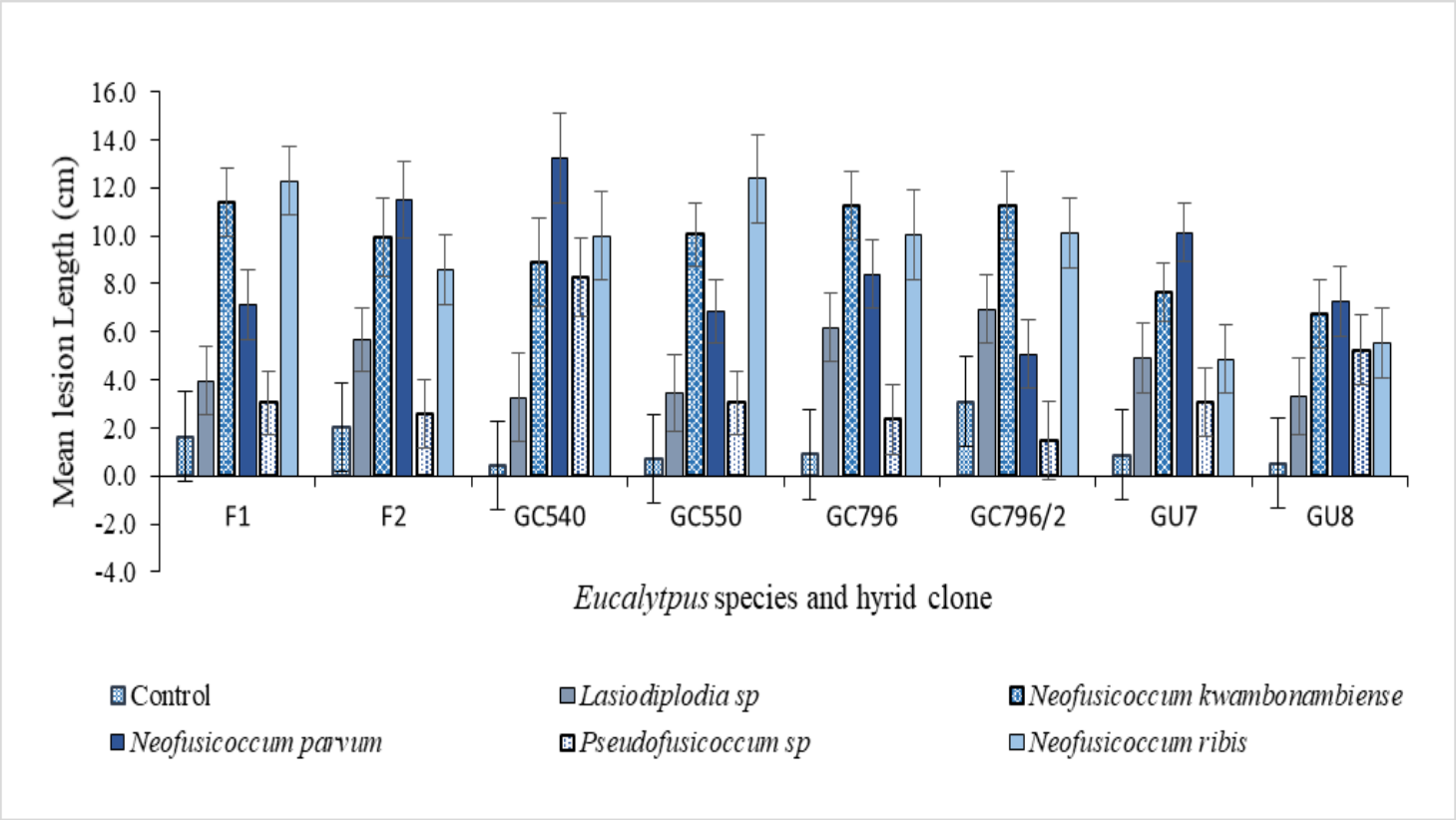

Figure 6. Mean lesion length (cm) following inoculation of *E. grandis* (F1, F2) and hybrid clones (GC540, GC550, GC796, GC796/2, GU7, GU8) with *Botryosphaeriaceae* species.

have also been isolated from *Jatropha podagrica*, in India (Sharma *et al*., 2013), from *Tinospora cordifolia* Miers (Mishra *et al*., 2019) and Spondias species in Brazil (Gonçalves *et al.,* 2016). This study represents the first report of occurrence of *Pseudofusicoccum* sp. on Eucalyptus species in Uganda, particularly in Kyankwanzi district (central Uganda).

All Botryosphaeriaceae species used in the study were pathogenic to inoculated *E. grandis* and hybrid clones tested. The resulting lesions appeared sunken, indicating cell necrosis characteristic of Botryosphaeria canker (Phillips *et al.,* 2013; Bezerra *et al*., 2021). However, for some three trees inoculated with sterile media, inoculation wounds were callused over. This is in contrast with other studies for example Heerden *et al.* (2005) and Nakabonge *et al.* (2019), where no lesions developed on controls. However, small lesions can be developed even on control trees due to defensive reactions of trees to inoculation wounds other than disease (Pavlic *et al.,* 2009).

Differences in aggressiveness among the Botryosphaeriaceae species were also observed. Isolate AS-02 in *N. kwambonambiense* clade was the most aggressive to *E. grandis* and hybrid clones as it produced the longest lesions. This finding was consistence with the findings by Barradas *et al*. (2016, 2019) who reported that *N. kwambonambiense* was the most pathogenic among the fungal species tested on some Eucalyptus trees. On the other hand, Gezahgne *et al.* (2004), Mohali *et al*. (2009) and Nakabonge *et al.* (2019) reported *N. parvum* as the most aggressive Botryosphaeria species to Eucalyptus hybrid clones in Ethiopia, Venezuela and Uganda respectively. Other studies have noted that the aggressiveness of some members of this family depends on different types of hosts (Batista *et al*., 2021).

Neofusicoccum isolates were more aggressive compared to other Botryosphaeriaceae species in this study. Among Neofusicoccum species, isolates AS-09 in *N. parvum* and AS-05 in *N. ribis* were less aggressive than that in *N. kwambonambiense. N. parvum* was recently reported to be

pathogenic on Eucalyptus clones by Nakabonge *et al.* (2019). Additionally, studies on blueberry indicated significant lesion lengths caused by *N. ribis* on both wounded and non-wounded shoots confirming its high pathogenicity level (Tennakoon *et al*., 2017). The other members in the genus are also known to show high pathogenicity on other species such as Almond trees (Olmo *et al.,* 2016).

Previous studies (Chen *et al.,* 2011; Li *et al*., 2015) have reported Lasiodiplodia species as being pathogenic to Eucalyptus species and hybrid clones whereas Mohali *et al.* (2009) found that *L. theobromae* was not pathogenic to Eucalyptus hybrid clones. In this study, the isolate AS-01 in the *Lasiodiplodia* clade was less aggressive than *Neofusicoccum* species but was still pathogenic compared to control. Because some members in the genus have been found to be greatly pathogenic to other hosts such as grapes (Rodríguez-Gálvez *et al*., 2015), it is likely that the pathogenicity of Lasiodiplodia species depends on the interaction of host and fungal species (Garcia *et al*., 2021).

The isolate AS-06 in the *Pseudofusicoccum* sp. clade was the least virulent. Although pathogenicity tests of *Pseudofusicoccum* sp. on Eucalyptus trees have not been widely studied, on *Mangifera indica* and Spondias species, it was the least pathogenic compared to *Lasiodiplodia sp* and *N. parvum* (Gonçalves *et al.,* 2016). The previous study indicated *Pseudofusicoccum* sp. as a weak pathogen but it may still pose a threat when it is new in the region and capable of adapting to cause severe disease to its hosts (Santini and Ghelardini 2015).

One of the justifications put forward for growing Eucalyptus hybrid clones in Uganda was their ability to tolerate diseases (SPGS 2008) compared to *E. grandis.* This has been confirmed in this study since some clones were more resistant to Botryosphaeria canker disease compared to *E. grandis.* Lesions that developed on inoculated Eucalyptus trees and hybrid clones indicated variation in susceptibility. In general, hybrid clones GU7 and GU8 exhibited higher resistance than GC550, GC796/2, F1, F2 while clones GC 796 and GC 540 had moderate resistance to Botryosphaeriaceae fungal inoculations. This study confirms earlier reports by Nakabonge *et al.* (2019) where clone GU 7 showed high tolerance to Botryosphaeria canker. The variation in susceptibility of Eucalyptus clones as was also reported by Heerden *et al.* (2005) against *Chrysoporthe cubensis*.

The difference in susceptibility could be exploited in management of the disease by planting tolerant clones and species in high disease pressure areas, since this strategy is the most effective in management of canker diseases (Nakabonge *et al*., 2019). Additionally, species in the Botyrosphaeriaceae family are weak pathogens that infect physiologically stressed trees, therefore, silviculture practices that minimise stress to grown trees could help in managing damage by Botryosphaeria canker diseases.

Based on the findings of this study it is concluded that that:

1. The isolates identified in this study were positioned in five phylogenetic clades representing *N. kwambonambiense, N. ribis, N. parvum, Lasiodiplodia* sp. and *Pseudofusicoccum* sp.

2. Amongst the identified isolates, AS-02 isolate of N. *kwambonambiense* was the most aggressive to Eucalyptus germplasm tested followed by isolate AS-05 of *N. ribis,* AS-09 of *N. parvum,* AS-01 of *Lasiodiplodia* sp. and AS-06 of *Pseudofusicoccum* sp.

3. All *E. grandis* and hybrid clones tested in the study were susceptible to the Botryosphaeriaceae species isolated in the study when compared with the control.

4. The level of susceptibility varied depending on the fungal isolates interacting with the Eucalyptus germplasms. Generally, hybrid clones; GU 8 and GU 7 were the least susceptible followed by GC550 and GC796. The most susceptible were F1, F2 and hybrid clones GC540 and GC796/2.

5. Furthermore, *E. grandis* (F2-Australia) was less susceptible to Botryosphaeria canker disease than (F1-South Africa).

We hope that the information provided in this study will support the planting and managing of Eucalyptus plantation forests in Uganda.

Declaration of conflict of interest

The authors declare no conflict of interest

Acknowledgement

The authors thank The German Academic Exchange Service, DAAD and Africa Development Bank for funding of the study.

References

Ammad, F., Benchabane, M., Toumi, M., Belkacem, N., Guesmi, A., Ameur, C. and Merah, O. 2014. Occurrence of *Botryosphaeriaceae* species associated with grapevine dieback in Algeria. *Turk. J. Agri and For.* 38:865-876.

Barradas, C., Phillips, A.J.L., Correia, A., Diogo, E., Bragança, H. and Alves A. 2016. Diversity and potential impact of *Botryosphaeriaceae* species associated with *Eucalyptus globulus* plantations in Portugal. *Eur J Plant Pathol*. 146:245-257.

Barradas, C., Pinto, G., Correia, B., Jesus, C. and Alves, A. 2019. Impact of *Botryosphaeria, Diplodia* and *Neofusicoccum* species on two Eucalyptus species and a hybrid: From pathogenicity to physiological performance. *For Pathol*. 49:1-11.

Batista, E., Lopes, A. and Alves, A. 2021. What do we know about *Botryosphaeriaceae*? An overview of a worldwide cured dataset. *Forests* 12:1-18.

Bezerra, J.D.P., Crous, P.W., Aiello, D., Gullino, M.L., Polizzi, G. and Guarnaccia, V. 2021. Genetic diversity and pathogenicity of *Botryosphaeriaceae* species associated with symptomatic citrus plants in Europe. *Plants* 10:1-31.

Birara, A., Abate, T. and Mekie, T. 2019. *Eucalyptus*: The popular exotic tree crop in Ethiopia. *Acta Sci. Agric.* 3:50-56.

Bose, T., Roux, J., Burgess, T.I., Shaw, C. and Wingfield, M.J. 2019. Susceptibility of *Eucalyptus grandis* and *Acacia mearnsii* seedlings to five *Phytophthora* species common in South African plantations. *For. Pathol*. 2018:1-8.

Bovolini M.P., Lazarott, M., Gonzatto, M.P., Junior, N.B. and Filho, R.L. 2018. Susceptibility of clonal seedlings of *Eucalyptus* spp. to powdery mildew disease. *Floresta e Ambiente*. 25:1-9.

Carlucci, A., Cibelli, F., Lops, F. and Raimondo, M.L. 2015. Characterization of *Botryosphaeriaceae* species as causal agents of Trunk diseases on Grapevines. *Plant Dis*. 99: 1678-1688.

Chen, S.F., Pavlic, D., Roux, J., Slippers, B., Xie, Y.J., Wingfield, M. J. and Zhou, X. D. 2011. Characterization of *Botryosphaeriaceae* from plantation-grown Eucalyptus species in South China. *Plant Pathol*. 60: 739-751.

Crous, P.W., Groenewald, J.Z., Summerell, B.A., Wingfield B.D. and Wingfield M.J. 2009. Co-occurring species of *Teratosphaeria* on *Eucalyptus*. *Persoonia* 22:38-48.

Crous, P.W., Wingfield, M.J., Mansilla, J.P., Alfenas, A.C. and Groenewald, Z. 2006. Phylogenetic reassessment of *Mycosphaerella* spp. and their anamorphs occurring on Eucalyptus. II. *Stud. Mycol*. 55:99-131.

Food and Agriculture Organization (FAO). 2009. Global review of forest pests and diseases: A thematic study prepared in the framework of the Global forest resources assessment 2005. Food and Agriculture Organisation of the United Nations, Rome.

Felsenstein, J. 1985. Confidence limits on Phylogenies: an approach using bootstrap. *Evolution* 39:783-791.

Garcia, J.F., Daniel, P., Lawrence, D.P., Morales-Cruz, A., Travadon, R., Minio, A., Hernandez-Martinez, R., Rolshausen, P.E., Baumgartner, K. and Dario, C.D. 2021. Phylogenomics of Plant-Associated *Botryosphaeriaceae* Species. *Front Microbiol.* 12:589.

Gezahgne, A., Roux, J., Slippers, B. and Wingfield, M.J. 2004. Identification of the causal agent of *Botryosphaeria* stem canker in Ethiopian Eucalyptus plantations. *S. Afr. J. Bot*. 70:241-248.

Glass, N.L. and Donaldson, G.C. 1995. Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous ascomycetes. *Appl. Environ. Microbiol.* 61:1323-1330.

Gonçalves, F.J.T., Freire, F.D.C.O., Lima, J.S., Melo, J.G.M. and Câmara, M.P.S. 2016. Patogenicidade de espécies de *Botryosphaeriaceae* endofíticas de plantas da Caatinga do estado do Ceará em manga e umbu-cajá. *Phytopathologica* 42: 43-52.

Heerden, S.W., Amerson, H.V., Preisig, O., Wingfield, B.D. and Wingfield, M.J. 2005. Relative pathogenicity of *Cryphonectria cubensis* on *Eucalyptus* clones differing in their resistance to *C. cubensis*. *Plant Dis.* 89:659-662.

Hilário, S., Lopes, A., Santos, L. and Alves A. 2020. *Botryosphaeriaceae* species associated with blueberry stem blight and dieback in the Centre Region of Portugal. *Eur. J. Plant Pathol* 156:31-44.

Hlaiem, S., Zouaoui, B.M., Yangui, I. and Ben, J.M.L. 2020. Identification and pathogenicity of *Diplodia seriata* and *Diplodia africana* related to lentisk dieback in Tunisia. *Arch. Phytopathol*. 53:99-111.

Iturritxa, E., Slippers, B., Mesanza, N. and Wingfield, M.J. 2011. First report of *Neofusicoccum parvum* causing canker and die-back of *Eucalyptus* in Spain. *Australas Plant Dis Notes.* 6:57-59.

Jami F., Slippers, B., Wingfield, M.J. and Gryzenhout, M. 2014. *Botryosphaeriaceae* species overlap on four unrelated native South African hosts. *Fungal Biol*. 118: 168-179.

Jami, F., Wingfield, M.J., Gryzenhout, M. and Bernard, S.B. 2017. Diversity of tree-infecting *Botryosphaeriales* on native and non-native trees in South Africa and Namibia. *Australas Plant Pathol*. 6:529-545.

Kaboggoza, J. 2011. Forest Plantations and Woodlots in Uganda. A platform for Stakeholders in African Foresrty. *African Forest Forum*. 17:1 -60.

Kumar, S., Stecher, G., Li, M., Knyaz, C. and Tamura, K. 2018. MEGA X: Molecular evolutionary genetics analysis across computing platforms. *Mol. Biol. Evol.* 35:1547 -1549.

Li, G.Q., Arnold, R.J., Liu, F.F., Li, J.Q. and Chen, S.F. 2015. Identification and Pathogenicity of *Lasiodiplodia* species from *Eucalyptus urophylla* × *grandis*, *Polyscias balfouriana* and *Bougainvillea spectabilis* in Southern China. *J. Phytopathol*. 163: 956-967.

Li, G., Slippers, B., Wingfield, M.J. and Chen, S. 2020. Variation in *Botryosphaeriaceae* from Eucalyptus plantations in YunNan Province in southwestern China across a climatic gradient. *IMA Fungus* 11:1-49.

Li, H.L., Jayawardena, R.S., Xu, W., Hu, M., Li, X.H., Liu, J.H., Hyde, K.D. and Yan J. 2019. *Lasiodiplodia theobromae* and *L. pseudotheobromae* causing leaf necrosis on *Camellia sinensis* in Fujian Province, China. *Can J Plant Pathol.* 41: 277-284.

Lopes, A., Barradas, C., Phillips, A.J.L. and Alves, A. 2016. Diversity and phylogeny of *Neofusicoccum* species occurring in forest and urban environments in Portugal. *Mycosphere* 7: 906-920.

Mishra, A., Gond, S.K., Sharma, V.K., Verma, S.K., Kumar, J., Singh, D.K. and Kharwar, R.N. 2019. Characterization of *Pseudofusicoccum adansoniae*, an Endophytic fungus residing in photosynthetic root of *Tinospora cordifolia*, a Medicinal Plant. Proceedings of the National Academy of Sciences India Section B. *Biol Sci.* 89:1319-1326.

Mohali, S. R, Slippers, B. and Wingfield, M. J. 2009. Pathogenicity of seven species of the *Botryosphaeriaceae* on *Eucalyptus* clones in Venezuela. *Australas Plant Pathol.* 38:135-140.

Nakabonge, G. 2002. Diseases Associated with Plantation Forestry in Uganda. Master's Thesis, Univesity of Pretoria.

Nakabonge, G., Namukasa, E., Tumwebaze, S.B. 2019. Susceptibility of *Eucalyptus* hybrid clones to *Botryosphaeria* canker in Uganda. *J Sustain Forest.* 39:407-416.

Ni, H.F., Yang, H.R., Chen, R.S., Liou, R.F. and Hung, T.H. 2012. New *Botryosphaeriaceae* fruit rot of mango in Taiwan: Identification and pathogenicity. *Bot. Stud.* 53:467-478.

Niekerk, J.M., Fourie, P.H., Halleen, F. and Crous, P.W. 2006. *Botryosphaeria* species as grapevine trunk disease pathogens. *Phytopathol. Mediterr.* 45: 43-53.

Njuguna, J.W. 2011. Stem Canker and Dieback Disease on *Grevillea robusta Cunn ex R . Br.:* Distribution, causes and implications in the Agroforestry system in Kenya. Doctoral thesis, Swedish University of Agricultural Sciences.

Ngobisa, N.I.C., Zainal-Abidin, M.A., Wong, M.Y. and Wan-Noordin, M.W.D. 2013. *Neofusicoccum ribis* associated with leaf blight on rubber (*Hevea brasiliensis*) in Peninsular Malaysia. *Plant Pathol.* 29:10-16.

Nyeko, P. and Nakabonge, G. 2008. Occurrence of pests and diseases in tree nurseries and plantations in Uganda. Study Commissioned by the Sawlog Production Grant

Old, K.M., Wingfield, M.J. and Qing, Y.Z. 2003. A Manual of Diseases of Eucalypts in South-East Asia. *Cifor.*

Olmo, D., Vegetal, L.D.S. and Agr, S.D.M. 2016. Characterization and Pathogenicity of *Botryosphaeriaceae* species isolated from Almond Trees on the Island of Mallorca (Spain). *Plant Dis.* 100:2483-2491.

Pavlic, D., Coutinho, T.A. and Wingfield, M.J. 2009. Molecular and phenotypic characterization of three phylogenetic species discovered within the *Neofusicoccum parvum /N . ribis* complex. *Mycologia* 101:636-647.

Pavlic, D., Slippers, B., Coutinho, T.A. and Wingfield, M.J. 2007. *Botryosphaeriaceae* occurring on native *Syzygium cordatum* in South Africa and their potential threat to *Eucalyptus*. *Plant Pathol.* 56:624-636.

Pavlic, D., Wingfield, M.J., Barber, P., Slippers, B., Hardy, G.E.S.J. and Burgess, T.I. 2008. Seven new species of the *Botryosphaeriaceae* from baobab and other native trees in Western Australia. *Mycologia.* 100:851-866.

Phillips, A.J.L., Alves, A., Abdollahzadeh, J., Slippers, B., Wingfield, M.J., Groenewald, J.Z. and Crous P.W. 2013. The *Botryoshaeriaceae*: genera and species known from culture. *Stud. Mycol.* 76:51-167.

Rodas, C.A., Slippers, B., Gryzenhout, M. and Wingfield, M.J. 2009. *Botryosphaeriaceae* associated with Eucalyptus canker diseases in Colombia. *For Pathol*. 39:110-123.

Rodríguez-Gálvez, E., Maldonado, E. and Alves, A. 2015. Identification and pathogenicity of *Lasiodiplodia theobromae* causing dieback of table grapes in Peru. *Eur J Plant Pathol*. 141: 477-489.

Roux, J., Coutinho, T. A., Byabashaijja, D. and Wingfield, M. 2001. Diseases of plantation *Eucalyptus* in Uganda. *S. Afr. J. Sci.* 97: 16-18.

Santini, A. and Ghelardini, L. 2015. Plant pathogen evolution and climate change. *CAB Rev*. 10:35. 10.1079/ PAVSNNR201510035.

Sawlog Production Grant Scheme (SPGS). 2007. Tree species for commercial Timber production and where they grow best in Uganda. Plantation Guidelines. No: 5 & 6.

Sawlog Production Grant Scheme (SPGS). 2008. News of Uganda's commercial tree planting fund for the private sector. Plantation Guidelines No: 21.

Sawlog Production Grant Scheme (SPGS). 2009. Growing Eucalypts for Timber , Poles & Fuelwood: SPGS Plantation Guidelines. No: 9 and10.

Sharma, R., Kulkarni, G. and Shouche, Y. S. 2013. *Pseudofusicoccum adansoniae i*solated as an endophyte from *Jatropha podagrica*: New record for India. *Mycotaxon* 123: 39-45.

Slippers ,B., Burgess, T., Pavlic, D., Ahumada, R., Maleme, H., Mohali, S. and Wingfield, M. J. 2009. A diverse assemblage of *Botryosphaeriaceae* infect *Eucalyptus* in native and non-native environments. Southern Forests: *J. For. Sci*. 71: 101-110.

Slippers, B., Fourie, G., Crous, P. W., Coutinho, T. A., Wingfield, B. D, Carnegie, A. J. and Wingfield, M. J. 2004. Speciation and distribution of *Botryosphaeria* spp. on native and introduced *Eucalyptus* trees in Australia and South Africa. *Stud. Mycol.* 50: 343-358.

Slippers, B. and Wingfield, M. J. 2007. *Botryosphaeriaceae* as endophytes and latent pathogens of woody plants: diversity, ecology and impact. *Fungal Biol. Rev*. 21: 90-106.

Stebbing, E.P. 1953. Uganda Forest Deparment: Annual Report for 1951. *Nature.* 171: 1060 -1061.

Tamura, K. and Nei, M. 1993. Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. *Mol. Biol. Evol*. 10: 512-526.

Tennakoon, K.M., Ridgway, H., Jaspers, M. and Jones, E. 2017. Production of *Neofusicoccum* species conidia and their pathogenicity on wounded and non-wounded blueberry shoots. *N.Z. Plant Prot*. 70: 209-214.

Thomidis, T., Michailides, T. J. and Exadaktylou, E. 2011. *Neofusicoccum parvum* associated with fruit rot and shoot blight of peaches in Greece. *Eur J Plant Pathol*. 131:661-668.

Turinawe, H., Mugabi, P. and Tweheyo, M. 2014. Density, Calorific value and Cleavage strength of selected hybrid Eucalypts grown in Uganda, *Maderas-Cienc Tecno* 16:13-24.

Urbez-Torres, J., Medina, F. C. and Mohali, S. 2016. *Botryosphaeriaceae* species associated with cankers and dieback symptoms of *Acacia mangium and Pinus caribaea var. hondurensis* in Venezuela. *Plant Dis.* 100:2455-2464.

Varmola, M. I. and Carle, J. B. 2002. The importance of hardwood plantations in the tropics and sub- tropics. *Int. For. Rev*. 4: 1-20.

White, T.J., Bruns, T. D., Lee, S. B. and Taylor, J. W. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. pp.315-322. In: Innis, M. A, Gelfand, D. H., Sninsky, J.J. and White, J.W. Eds. A Guide to Molecular Methods and Applications. Academic press, New York.