Stem Canker and Dieback Disease on *Grevillea robusta* Cunn ex R. Br.

Distribution, Causes and Implications in Agroforestry Systems in Kenya

Jane Wangu Njuguna
Faculty of Natural Resources and Agricultural Sciences
Department of Forest Mycology and Pathology

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Cover: Top: Severe shoot and branch dieback, Down: Damaged growth rings and resin ooze from infected heartwood (photo: Njuguna Jane 2005)

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Abstract

A widespread stem canker and dieback disease threatened the cultivation of *Grevillea robusta* on farms in Kenya. Disease index increased from 18% in the humid zone to 67% in the semi-arid zones and strongly correlated with altitude and length of drought period in the zones.

Analysis of morphological characteristics and molecular data of the internal transcribed spacer (ITS) rDNA revealed that forty fungal species were associated with *G. robusta*. Seven species of the *Botryosphaeriaceae* comprised 42% of the total isolations. Five fungal species known to cause canker and dieback symptoms on woody tree species, *Neofusicoccum parvum*, *Lasiodiplodia theobromae, Diplodia seriata, Botryosphaeria* sp. and a *Phomopsis* sp. were selected for pathogenicity tests on four agroforestry tree species: *Grevillea robusta, Senna siamea*, *Azadirachta indica* and *Melia volkensii*. *Neofusicoccum parvum* was the most virulent pathogen, *L. theobromae, Botryosphaeria* sp, and *D. seriata* were moderate and *Phomopsis* sp. was the least virulent. Under hot conditions, *G. robusta*, *S. siamea* and *A. indica* were most susceptible to the Botryosphaeriaceae pathogens and the only native species *M. volkensii* was least susceptible.

The symptoms caused by the Botryosphaeriaceae species in the field and laboratory conditions were indistinguishable leading to the suggestion that they formed a disease complex. Phylogenetic analyses showed that the fungal species associated with *G. robusta* also occurred in other plant species. The plurivorous nature of these pathogens threatens trees as well as crops in agroforestry systems. The study recommended that species site matching should be emphasized in agroforestry systems with a focus on native tree species such as *M. volkensii*.

Keywords: Agroforestry, Botryosphaeriaceae, canker and dieback disease *Grevillea robusta*, *Senna siamea, Azadirachta indica, Melia volkensii*.

Author's address: Jane Wangu Njuguna, SLU, Department of Forest Mycology and Pathology, P.O. Box 7026, SE-750 07 Uppsala, Sweden
E-mail: jane.njuguna@slu.se

Dedication

To my son Phil Karinga Njuguna, for growing up in the tender ages without my care to be strong always telling me that, "mum I can help you do the homework so you finish and come home".

To my son Ian Makumi Njuguna, you showed extra inner strength. Together we put up brave faces but we knew we were weak. You performed well in your examinations. Continue to work hard.

To my daughter Angela Nduta Njuguna, you grew up too quickly to take care of your brothers and also to become my greatest source of strength. You always told me "mum is everything fine at home". I wish you well in your endeavor to be a lawyer.

To my husband Dr. Joseph Njuguna Makumi, for your love, bravely and encouragement throughout this journey. Your exceptional ability to be father and mother to our three children together with looking after the entire family since the passing on of our parents is admired by many. "*Mwendwa wakwa, Ngai witu arokwongerera matuku maingi*".

To my parents Josphat Karinga Njagi and Mary Njoki Karinga, your love, prayers and sacrifice to us created the foundation of togetherness in us. Your constant calls made me feel that I was never alone. Dad, mum you are my heroes.

To my sisters, brothers, nieces and nephews, for never making my children feel alone by visiting them at school and home. Muthoni, Wawira, Njagi, Kananu, Wanjiku, Wairimu, Ndwiga, Mwaniki, Kinuthia na Njue thank you for looking after my children. God bless you all.

Contents

List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I Njuguna J. W., Barklund, P., Ihrmark, K., and Stenlid, J. (2011). A canker and dieback disease is threatening the cultivation of *Grevillea robusta* on small-scale farms in Kenya. *African Journal of Agricultural Research* In press.

II Njuguna J. W., Barklund, P., Ihrmark, K., and Stenlid, J. Fungal diversity associated with canker and dieback disease in *Grevillea robusta* in five agro-ecological zones in Kenya. (Submitted manuscript).

III Njuguna J. W., Barklund, P., Ihrmark, K., and Stenlid, J. Botryosphaeriaceae associated with *Grevillea robusta* in Kenya. (manuscript)

IV Njuguna J. W., Barklund, P., and Stenlid, J. Investigation into the pathogenicity of four Botryosphaeriaceae species and *Phomopsis* sp. to *Grevillea robusta, Senna siamea*, *Azadirachta indica* and *Melia volkensii* in Kenya (Submitted manuscript).

Abbreviations

AEZ	Agro-ecological zone
ASAL	Arid and semi-arid areas
CTAB	Cetyltrimethyl ammonium bromide
DNA	Deoxyribonucleic acid
FABI	Forestry and Agriculture Biotechnology Institute
ITS	Internal transcribed spacer
KEFRI	Kenya Forestry Research Institute
MEGA4	Molecular Evolutionary Genetics Analysis software
PCR	Polymerase Chain Reaction
rDNA	ribosomal Deoxyribonucleic acid
SLU	Swedish university of Agricultural Sciences
Tef 1-alpha	Translation elongation factor
ASALs	Arid and semi-arid lands

1 Background

1.1 The role of the forestry sector in Kenya

Kenya's natural forest cover has been declining at an alarming rate and currently stands at less than 2% of the total land area due to increased demand for forest products from the ever increasing population. According to the Kenya Forest Service reports, Kenya is estimated to have lost about 10% of its forest cover since 1968. About 80% of Kenya's rural population depends on fuel-wood for cooking, lighting and heating. The little forest cover is important as water catchment areas which supply water for domestic and industrial uses particularly generation of electricity which heavily relies on water. Furthermore the country experiences acute electricity shortages due to decreasing water levels forcing the energy sector to search for alternative sources of energy.

Using data on demand and supply for forest resources and the backlog created by lack of regular replanting of clear-felled plantations, the Kenya Forestry Master Plan (1994) predicted serious shortages in forest products by the year 2020. The master plan also provided guidelines to increasing forest cover among which were promoting tree planting on farms to reduce pressure on both natural and planted forests. Tree planting on farms, or agroforestry as it is commonly referred to, has mainly focused on fast growing species such as *Grevillea robusta* and *Eucalyptus* spp. that give products within a short time. Though faced with many challenges such as small land sizes, diseases and pests, agroforestry practices have become popular because they provide varied products on a continuous basis.

1.2 An overview of agroforestry

Agroforestry is a system of land use that combines planting of trees or shrubs with other farm practices such as agricultural production and livestock. Agroforestry systems are common throughout the tropics, subtropics (Huxley, 1996) and are also becoming popular in the temperate areas (Gordon and Newman, 1997). Three types of agroforestry are recognized: silvopastoral which combines trees, pasture or forage and livestock; agrisilviculture which combines crops and trees or shrubs; agrosilvopastoral which combines crops, trees, pastures and livestock (Nair, 1985).

Agroforestry systems are viewed as viable land-use systems that diversify farm production by providing various ecological and economic benefits to alleviate poverty. Shibu (2009) re-evaluated information on agroforestry systems and found that the major ecosystem services and environmental benefits fell into four broad categories: carbon sequestration, soil enrichment, biodiversity conservation and improved air and water quality. At the household and community levels, these systems are designed to meet subsistence needs such as provision of timber, fuel-wood, poles, shade, shelter, food, fodder, fruits and mulch. Other services include soil fertility improvement by nitrogen-fixing trees, aesthetic values and environmental amelioration (Tengnäs, 1994; Huxley, 1996).

The most common agroforestry practices are: 1) alley cropping or hedgerow intercropping which involves planting crops in between rows of trees or alleys, 2) windbreaks or shelter belts where trees are planted in multiple rows along an edge of an agricultural field to reduce wind speeds, 3) riparian buffer strips and terraces for soil erosion and water quality control and 4) forest farming or farm forestry which involves growing high-value multipurpose trees together with crops and or livestock (Nair, 1985; Huxley, 1996).

1.3 Agroforestry in relation to pests and diseases

Agroforestry systems have been viewed as having the potential to alter environments both physically and biologically which may favor or discourage some pests or diseases. However, it is known that a new tree species introduced in a new environment, such as agroforestry practices, may become attacked by new pathogens already existing in the area or attacked by pathogens brought in together with the tree species. There is also the risk that native species could be attacked by a new pathogen brought in by the non-native species (Boyce, 1961). These relationships become even more

complicated because agricultural crops are grown near trees in agroforestry systems.

The arrangement and management of trees in relation to crops within an agroforestry technology influences micro-climatic factors and thus may modify the environment and pest and disease incidence (Huxley and Greenland, 1989). The structural and temporal variations in agroforestry systems may act in different ways either to reduce or enhance disease incidence. For example Schroth *et al.,* (1995) found that shading of groundnuts by trees increased incidences of rust (*Puccinia arachidis* Speg.) and the late leaf spot fungus (*Phaeoisariopsis personata* (Berk. & M.A. Curtis) Arx). Shading also increases humidity by prolonging the period of leaf wetness thus facilitating the infection process (Waller, 1972). Stressful conditions that may be created in agroforestry systems such as competition for water, light or nutrients may increase the plants' susceptibility to infection by latent pathogens (Slippers and Wingfield, 2007).

In most cases, the heterogeneity brought by the biodiversity in agroforestry has been suggested to reduce outbreaks of diseases and pests in different ways for example by providing protection against some host specific pests. Scheffer and Siva, (1990) reported that mixed planting of the iroko tree, *Chlorophora exelsa* Welw., and the umbrella tree, *Maesopsis eminii* Engl. reduced the infestation of *C. exelsa* by the gall bug *Phytolyma lata* in eastern Usambaras in Tanzania. However, it is also argued that these systems may provide pests or diseases with alternative hosts and hence increase diseases or pest problems. *Sesbania sesban* (L.) Merr. is known to increase the incidence of root knot nematodes *Meloidogyne* spp. in maize farms (Ong and Rao, 2001). Other studies have also shown that heterogeneity does not necessarily produce stable ecosystems (Mchowa and Ngugi, 1994; Rao *et al.*, 2000; Schroth *et al.,* 2000).

Plurivorous pathogens pose serious risks in agroforestry. An example is *Phytophthora cinnamomi* Rands, which has proved to be devastating in many ecosystems (Shearer *et al.*, 2004). Also the canker-forming and dieback pathogen, *Botryosphaeria australis* Slippers, Crous & M.J. Wingf., sp. nov., was isolated from unrelated *Acacia* spp. Miller in Australia and showed plurivorous behavior on three related *Eucalyptus* species within the same area. The same pathogen was also proved to be associated with *Eucalyptus gomphocephala*, *E. marginata*, *E. globulus*, *Banksia grandis*, *Agonis flexuosa*, *Acacia rostellifera*, *Acacia cochlearis*, *Santulum acuminatum*, *Allocasuarina fraseriana*, *Callitris preissii*, *Olea* spp. *Acacia* sp., and *Vitis vinifera* in Western Australia (Taylor *et al.,* 2005; Burgess *et al.*, 2006b). Although there is considerable evidence about the role of agroforestry systems in relation to pest

populations, there is little information regarding the role of diseases in agroforestry systems (Schroth *et al.,* 2000).

1.4 *Grevillea robusta* and agroforestry in Kenya

Grevillea robusta Cunn ex R. Br., the silky-oak, locally known as *mukima* is a native of Eastern Australia belonging to the family Proteaceae. In its native range, the species has a limited distribution in the warm temperate eastern Australia (26°-30°S) extending 100–160 km inland from the coast where the annual rainfall range from 720-1710 mm (Harwood, 1988). The species was introduced into Kenya as a shade and windbreak tree for tea and coffee plantations more than 100 years ago (Harwood, 1989) and was primarily cultivated in the tea and coffee growing zones. According to Booth and Jovanovic (2002) these zones are characterized by conditions regarded as optimal for the growth of the species.

G. robusta is popular among farmers due to its fast growth, ability to tolerate heavy pollarding and pruning of branches and because it mixes well with other crops (Muchiri *et al.*, 2002). Furthermore, the species has a proteoid root system (cluster of roots that develop in soils deficient of phosphorus) and hence is believed to compete less for minerals with crops making it ideal for planting on small farms sizes (Akycampong *et al.*, 1999). The species provides important goods and services including construction material, fuel wood, shade, fodder, soil erosion control and soil fertility improvement (FAO, 2001). It has been very well adopted and forms a near monoculture in central Kenya highlands, particularly in Kirinyaga district where it was reported to be grown on about 96% of the farms (Tyndall, 1996). Currently it is a major timber species in small-scale farms where it significantly contributes to household income (Holding *et al.*, 2006).

1.5 Disease status of *Grevillea robusta*

For a long time the species was regarded as having "no disease or pest of economic importance worldwide" (FAO, 2001). In Kenya, however, canker and dieback symptoms were first reported on *G. robusta* in 1960 (Smith, 1960) and later in the 1980s (Milimo, 1988). No isolations were performed to isolate the pathogens and the species continued to be regarded as disease free. Initially, the use of the species was restricted to the humid and semi-humid areas primarily where the tea and coffee plantations grow (Harwood, 1989). Its desirable characteristics as an inter crop with other agricultural crops has led to the species spreading to semi arid areas (Muthuri *et al.*,

2005) that are clearly not suitable for the species according to the requirements set by Booth and Jovanovic, (2002). There have been numerous reports of canker and dieback symptoms in these areas prompting initiation of disease-monitoring surveys in the semi-arid areas by the Kenya Forestry Research Institute (KEFRI).

Preliminary reports from the monitoring surveys of on-farm experiments for the period 2001 - 2003 showed that the incidence of canker and dieback symptoms in *G. robusta* increased from 17% to 65% between 2001 and 2003 in Kitui district (Njuguna, 2003). Mortality of the trees also increased from 2% to 18% within the same period. Infected trees showed poor growth and were characterized by cracks on stems or branches leading to rupturing of the bark followed by resin exudation. As cankers increased in size and numbers, girdling of young stems, branches and twigs led to dieback of shoots, branches and death of trees. Similar symptoms were reported on this species in Uganda where *Neofusicoccum parvum* (Pennycook & Samuels) Crous, Slippers & Phillips [*Botryosphaeria parva* Pennycook & Samuels] were isolated and also shown to be pathogenic on *G. robusta* seedlings (Toljander *et al.*, 2007).

Reports from other regions on pathogenic fungi infecting *G. robusta* include dieback caused by *Botryosphaeria dothidea* (Moug.) Ces. & De Not. in Guatemala (Schieber and Zentmeyer, 1978), and dieback caused by *Botryosphaeria quercuum* (Schwein.) Sacc., *Diplodia* sp. Fr. and *Botryosphaeria* sp. on *Grevillea* spp. Florida, USA (Marincowitz *et al.*, 2008). Other disease reports for *G. robusta* include leaf spots caused by *Amphichaeta grevilleae* Loos and dieback caused by *Corticium salmonicolor* Berk. & Broome in India (Nayar, 1987), as well as *Phyllosticta telopeae* H.Y. Yip and *Cercostigmina protearum* var. hakeae U. Braun & Crous in Australia (Crous *et al.*, 2000). Within East Africa, *N. parvum, L. theobromae* and a *Botryosphaeria* sp. were recently reported on *G. robusta* in Uganda (Toljander *et al.*, 2007). At the time of this study, there were no records of serious disease on *G. robusta* in its native range of south eastern Australia (Ian Smith, Angus Carnegie and Geoff Pegg personal communications). To date, no systematic study had been carried out to isolate the causes or to determine the magnitude of the canker and dieback disease on *G. robusta* in Kenya. This study therefore highlights the distribution, magnitude and causes of the canker and dieback disease in *G. robusta*-based agroforestry systems in Kenya.

2 Aims of the project

The main aim was to evaluate the health status of *Grevillea robusta* in Kenya and to isolate and describe the pathogens associated with the species. The specific objectives of the study were to:

(1) Assess the distribution and magnitude of the canker and dieback disease on *G. robusta* on small-scale farms in five agro-ecological zones and also the role of biotic and environmental factors influencing disease incidence and severity.

(2) Determine the diversity of fungi associated with healthy and diseased *G. robusta* trees and also determine their distribution in the agro-ecological zones since little information was available on fungi associated with the species in Kenya.

(3) Characterize the main canker pathogens, Botryosphaeriaceae species associated with *G. robusta*.

(4) Fulfill Koch's postulates by testing the ability of the potential canker-forming fungi to cause canker symptoms in healthy seedlings of *G. robusta* in cool and hot conditions.

(5) To evaluate if the pathogens found on *G. robusta* could cause disease on other commonly associated tree species, *Senna siamea*, *Azadirachta indica* and *Melia volkensii* in the semi-arid areas.

3 Materials and Methods

3.1 Study area and agro-ecological zone (AEZ) characteristics (Paper I-III)

The study was conducted in 2005 along an ecological gradient covering five AEZs spread across three districts. The zones stretched from the humid slopes around Mount Kenya in Kirinyaga district (longitude 37° 10' and 37° 30' East and latitude 0° 10' and 0° 50', South) and the semi-humid zones in Maragua district (longitude 36° 42' and 37° 15' East and latitude 0° 46' and 1° 0' South) to the semi arid areas in Kitui district (37° 5' and 39° 10' East and latitude 1° 5' and 3° 5' South). The altitudes of the farms sampled varied from 2040 to 680 meters above sea level (a.s.l.), (Figure 1). Similar to other areas in Kenya, the study areas experience a bimodal type of rainfall with two rainfall periods; the long rains (around 700 mm) occur from March to May and the short rains (around 376 mm) occur from September to November (Jaetzold and Schmidt, 1983) and, in both periods, rainfall fluctuates greatly (Biamah *et al.*, 2005). The characteristics of the five AEZs (Table 1) from which ninety-five farms were randomly selected for the study are described in Njuguna *et al.* (2011).

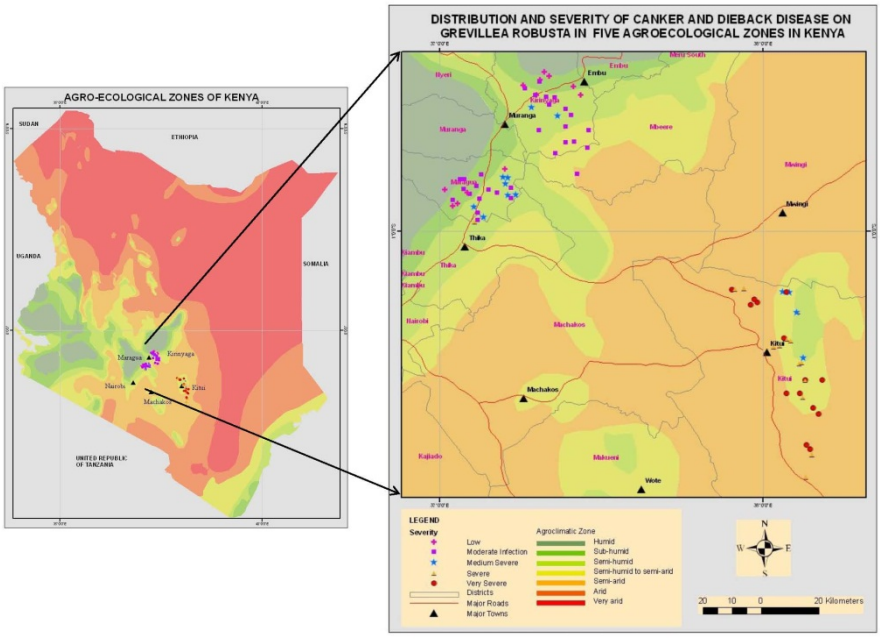

Figure 1: Distribution of the farms in the study area. The farms are indicated by different colors and shapes indicating average disease severity in each farm. Map adapted from Maundu andTengnäs (2005).

Table 1: Characteristics of the agro-ecological zones included in the study

Agro-ecological zone (AEZ)*)					
	Humid (AEZ1)	Sub-humid (AEZ2)	Semi-humid (AEZ3)	Semi-humid/semi-arid (AEZ4)	Semi-arid (AEZ5)
Altitude m above sea level (a.s.l)	>2000	1500-2000	1000-1500	700-1000	400-700
Annual rainfall (mm) bimodal	1200-1400	1100-1200	900-1100	500-900	<500
Mean annual temp OC	13-15	5-18	18-20	21-24	23-24
Mean min temp OC	Seasonal frost	9	12.5	14	16
Mean max temp OC	18	22	25	32	34
Soils; fertility	Moderate to high	Moderate to high	Moderate	Moderate to variable to low	Low to very low
Soil type	Humic andosols, partly lithic	Ando-humic nitisols, with humic andosols	Mixtures of nitisols-cambisol-acrisols	Mostly regosols with lithosols, humic cambisols and luvisols	Mostly lithosols/regosols/ xerosols-ferasols
Production potential	High	High–moderate	Moderate	Low-marginal	Marginal

*Agro-ecological zone characteristics were adapted from Jaetzold & Schmidt (1983).

3.2 Disease incidence and severity assessment (Paper I)

Disease incidence was assessed in the farms based on the occurrence of three main disease symptom types: (1) dieback of shoots, branches and cankers, (2) stem (trunk) cankers with severe resin flow and (3) diseased leaves (leaf spots and blights). Disease incidence was estimated as the total number of trees with canker and dieback disease symptoms expressed as the percentage of the total number of trees counted per farm.

Disease severity was recorded as the percentage of the tree showing canker and dieback disease symptoms, rated on a 1–6 severity scale as follow: (1) healthy trees, no canker or dieback symptoms (2) no canker or resin flow, 1–5% of the tree showing dieback of shoots (low infection), (3) 1–5 cankers, up to 25% of the tree crown showing dieback, low levels of resin flow, (4) 5–25 cankers, up to 50% of the tree showing cankers and dieback of bigger branches and moderate levels of resin flow, (5) 15–30 cankers, up to 65% of the tree showing shoot dieback and severe resin flow, (6) >30 cankers and over 65% of the tree showing very severe shoot dieback, very severe resin flow or dying (modified from Sharma and Sankaran, 1988). The severity of each tree was based on an average of three observations made by three people standing about 10 meters from the tree, observing the tree from three different directions using binoculars. Disease index per farm was calculated as described by Sharma and Sankaran (1988) and expressed as a percentage (PSI):

$$PSI = \frac{(1xa) + (2xb) + (3xc) + (4xd) + (5xe) + (6xf)}{N \text{ (Total number of trees assessed per farm)}} \times 100$$

Where: 1, 2, 3, 4, 5 and 6 are severity categories and a, b, c, d, e and f are the number of trees examined in each severity category.

Tree mortality was expressed as a percentage of the number of dead trees out of the total trees per farm. Disease index and mortality were also calculated for each type of tree planting or niches and the ages of the trees assessed. The farm altitude and length of drought period in each zone were assessed and analyzed as described in **Paper I**.

3.3 Isolation and identification of fungal flora associated with *Grevillea robusta* (Paper II and III)

Samples were collected from leaves, branches and stems from ten diseased trees and two healthy ones from each of the 95 farms selected. In total, 1,140 trees, (950 diseased, 190 healthy) were sampled. Three samples were collected from the prevailing disease symptom from each tree and also from healthy trees from which twelve pieces were cut for isolation of fungi (Table 2). The samples were placed in paper bags, labeled and stored in a cool box with ice and transported to the laboratory for the isolation of fungi within 24 hours. After each sampling, the cut section of the tree was sprayed with a fungicide to avoid re-infection by other pathogens. The sampling tools (an axe or a machete) were sterilized after each sampling by cleaning with 70% ethanol.

Isolations were made from the disease leading edge of cankers on branches, stems and dieback symptoms on shoots and branches and also from inner bark and woody tissues of healthy trees following standard laboratory procedures on isolation of fungi from plants (Agrios, 2005). The pieces were surface sterilized by immersing them in 70% ethanol for 1 minute, and then immersed in 33% hydrogen peroxide for one minute, after which they rinsed three times in sterile distilled water, blotted dry with sterile filter papers and aseptically plated on 9-cm Petri plates containing 2% malt extract agar (MEA) (Sigma-Aldrich Chemie, the Netherlands) amended with streptomycin sulfate (100 mg/l) (Merck, Germany) to inhibit bacterial growth. The plates were incubated at 25°C under alternating light and dark cycles of 12 hours and were monitored every day for fungal growth for the first 15 days.

Each emerging fungal colony was marked, assigned with a unique number and isolated onto fresh culture media. The number of times each colony was encountered per sample was counted. Fungal isolates were then grouped based on colony color and mycelial texture using a stereozoom microscope (NIKON SMZ-2B, Japan). The isolates were purified by isolating single spores as described by Slippers *et al.* (2004a). Spores were mounted on microscope slides in 85% lactic acid and examined microscopically to further group and identify the fungi using established protocols (Booth, 1971; Ellis, 1971, 1976; Barnett and Hunter, 1972; Nelson *et al.*, 1984; Alexopoulos *et al.*, 1996; Jacobs and Rehner, 1998; Leslie and Summerell, 2006; Phillips *et al.*, 2002, 2008; Slippers *et al.*, 2004; Pavlic *et al.*, 2007; Alves *et al.*, 2008). Representative cultures from single spore isolates of each morphological group were selected for further studies.

A set of four replicates were grown on malt yeast extract agar (MYA) (2% malt extract, 0.2% yeast and 2% agar, Sigma-Aldrich Chemie, the Netherlands) and stored for further use, another set was grown on half strength potato dextrose agar (Sigma-Aldrich) and another set on 2% liquid media for DNA extraction.

The Botryosphaeriaceae species, being the most frequent were distinguished by their grayish-black pigmentation in cultures and were grouped based on conidial morphology under the light microscope. Growth rates of the Botryosphaeriaceae were studied using three isolates from each group grown on 2% MEA under continuous darkness at 10°, 15°, 20°, 25°, 30° or 35°C as described in Paper III. Growth measurements were taken every 24 hours. Colony color change was examined every 24 hours and colors were determined using the color charts of Rayner (1970). The experiment was monitored over 15 days and was repeated once.

Table 2: Number of samples examined from four disease symptom types and number of fungal colonies isolated from each disease symptom type

Disease symptom type	Number of samples collected	Number of fungal colonies realized
Diseased leaves (spots and blights)	270	1,137
Dieback of shoots, branches with cankers	1,020	5,071
Stem (trunk) cankers with severe resin flow	1,551	7,951
Healthy		
Leaves	120	129
Shoots /Branches	244	347
Stems	215	236
Total	3,420	14,871

3.4 DNA amplification, purification, sequencing and sequence analysis (**Paper II and III**)

Single spore cultures from selected isolates were grown on 2% liquid MEA for four days. Genomic DNA was extracted using Cetyltrimethyl ammonium bromide (CTAB) (3%) – chloroform method as described by Gardes and Bruns (1993) with modifications according to Ihrmark *et al.* (2002). A fragment of the internal transcribed spacer (ITS) rDNA was amplified using the fungal-specific primers, ITS1F (5'-CTTGGTCATTTAGAGGAAGTAA-3') (Gardes and Bruns, 1993) and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (White *et al.*, 1990). Part of the translation elongation factor 1-alpha (Tef 1-α) gene was also amplified with the primers EF1-728F (5' CATCGAGAAGTTCGAGAAGG 3') and EF1-986R (5' TACTTGAAGGAACCCTTACC 3') (Carbone *et al.* 1999) to confirm the identity of key fungal groups. The amplified PCR products were purified either with AMPURE PCR Purification kit (Beckman Coulter, USA) or 6% Sephadex G-50 columns with 50–150-mm bead size (Sigma–Aldrich) following the manufacturer's instructions.

The samples were sequenced in both directions at least twice using the PCR Big Dye® Terminator v.1 Cycle sequencing kit. The PCR reaction mix was prepared according to the manufacturer's instructions. The sequences were analyzed with an ABI 310 genetic analyzer (Applied Biosystems, USA). SeqMan II™ 5.05 (DNASTAR Inc, USA) was used to edit the nucleotide sequences. Edited sequences were submitted to the National Center for Biotechnology Information (NCBI) GenBank sequence database and identified by comparing them with relevant sequences of the ITS region (ITSI-5.8S-ITS2) rDNA and (Tef 1- α) using BLASTN algorithm (www.ncbi.nlm.nih.gov/BLAST/blast.cgi) (Altschul *et al.,* 1997). Sequences that showed 98–100% similarity were considered to be the closest matches to the sequences submitted (**Table 2 in Paper II**) and were assigned the names with highest species identity from GenBank. The sequences were then downloaded from the database for further analysis. The nucleotide sequences obtained from this study were deposited in GenBank and their accession numbers are shown in **Table 2 in Paper II**. In paper III, more ITS sequences of the Botryosphaeriaceae were downloaded from GenBank and used to compare Botryosphaeriaceae isolates from this study with those from other studies (**Table 1 in Paper III**). Selected cultures of the identified fungal species were deposited at the Forestry and Agriculture Biotechnology Institute (FABI), University of Pretoria and also at the Dept of Forest Mycology and Pathology (SLU).

3.5 Phylogenetic analyses (Paper II and III)

Edited sequences of both ITS and Tef *1-α* obtained from this study and those retrieved from GenBank (http://www.ncbi.nlm.nih.gov/genbank/) were aligned using CLUSTALW of MEGA4 (Tamura *et al.*, 2007). Phylogenetic analyses were also performed using MEGA4). The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). In both phylogenies, branches corresponding to partitions reproduced in less than 50% of bootstrap replicates were collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (2000 replicates) is shown above the branches (Felsenstein, 1985).

The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al.*, 2004) and are in the units of the number of base substitutions per site. All positions containing alignment gaps and missing data were eliminated from the dataset (Complete deletion option).

There were 161 positions in the final data set comprising of 103 taxa (**Paper II**) and the resulting neighbor joining tree is shown in **Figure 3 in Paper II**. There were 397 positions in the final dataset comprising of 44 taxa and the tree is shown in **Figure 3 in Paper III.** The trees were drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. Both phylogenies were performed on ITS data only.

3.6 Pathogenicity of five canker forming fungi on four tree species (Paper IV)

This experiment was performed using one isolate of the four of the most abundant canker-forming fungi on *G. robusta* under cool and hot conditions. Under hot conditions, three other tree species were included because they were commonly planted with *G. robusta* in the semi-arid areas and they showed similar symptoms.

3.6.1 Experimental sites

The tree seedlings were raised following KEFRI's tree nursery procedures under glasshouse conditions at KEFRI headquarters, Muguga (Site 1, cool conditions), altitude 2099 m, latitude 36° 38′ 60 E and longitude 1° 10′ 60 S and at the Kitui Regional Research Center (Site 2, hot conditions), altitude 1151 m, latitude 1° 22′ 0 S and longitude 38° 1′ 0 E. Site 1 received a mean annual rainfall of >1,200 mm, experienced temperatures that varied from a mean minimum of 13°C to a mean maximum of 23°C. The cool climatic conditions at Site 1, together with fertile soils favored the growth of *G. robusta*. Site 2 is hot and represented the semi-arid zones characterized by low and erratic rainfall, usually less than 500 mm per year and high temperatures with a mean maximum of >31°C and a mean minimum of 17°C and low fertility soils (Jaetzold and Schmidt, 1983). Site 2 was chosen because *G. robusta* was recently introduced and disease incidence around this site was severe on all farms (Njuguna *et al.*, 2011).

3.7 Fungi used in the pathogenicity tests

The fastest growing isolate from four of the most abundant Botryosphaeriaceae fungi was chosen for inoculation on seedlings of the four tree species: *Neofusicoccum parvum* (FJ904892), *Lasiodiplodia theobromae* (FJ904889), *Botryosphaeria* sp. (FJ904864) and *Diplodia seriata* (FJ904868). *Phomopsis* sp. (FJ904852) was added to the test. *Phomopsis* sp. has been proved to be a dieback pathogen on *A. indica*, a common agroforestry tree species in the semi-arid areas of Kenya. Isolates were deposited at FABI and KEFRI.

3.7.1 Tree species used in the pathogenicity

Four tree species: *G. robusta, S. siamea, A. indica and M. volkensii* were selected. The latter three are commonly inter-planted with *G. robusta* in agroforestry systems in the semi-arid areas and showed similar disease symptoms. Seedlings at both sites were raised and maintained in glasshouses for 8 months before inoculation. The temperatures in the glasshouses at both sites were not regulated. Daily temperatures were recorded every day between 12 and 1 pm for the whole period. During the experimental period the temperatures varied between 19 ± 5°C at Site 1 and between 31 ± 5°C at Site 2. During the growing and experimental periods, seedlings were watered to field capacity as needed at both sites.

3.7.2 Inoculation of seedlings

Healthy seedlings with an approximate root collar diameter of 0.8–1.0 cm and approximately 30–35 cm in height were chosen for the pathogenicity tests and inoculated by stem wounding as described in **Paper IV**. At Site 1 (cool conditions), the total number of *G. robusta* seedlings inoculated was 180 (30 seedlings × 5 fungal species + 1 control of 30 wounded but un-inoculated seedlings). The inoculated seedlings and one set of un-inoculated controls were arranged in three replicates (blocks) each replicate comprised ten seedlings of each fungal species. The fungal isolates were randomized within each replicate, making it a completely randomized block design and maintained as described above in a cool glasshouse where temperatures varied between 19 ± 5°C.

At Site 2 (hot conditions), four tree species were used and the total number of seedlings inoculated was 720 (4 tree species × 6 treatments × 30 seedlings). The inoculated seedlings and one set of un-inoculated but wounded control seedlings for each isolate were arranged in three replicates (blocks) using a split plot design in which the tree species formed the main plot and the fungal species formed the split plot with each replicate comprising ten seedlings of each fungal species. The seedlings were assessed regularly for 10 months at both sites. The time taken by each fungal species to show early canker symptoms was recorded and after ten months, the number of seedlings showing various diseases symptom types and the number of dead seedlings were counted in each treatment. Fifteen seedlings were randomly selected from each treatment, slit longitudinally and the length of the internal lesion was measured upwards and downwards from the point of inoculation and recorded.

3.7.3 Estimation of pathogenicity of the five fungal species

Ranking of pathogenicity was estimated from the results obtained from three variables: (1) average number of days to show early canker symptoms, (2) mean size of internal lesions and (3) percentage mortality. Occurrences under each variable were categorized on a 1–6 scale from the highest to the lowest for each fungal species on the four tree species at both sites. This was done as follows: (1) time to show symptoms from fastest (rank 6) to slowest (rank 1); (2) internal lesion size >10 cm (rank 6) to healthy plants or lesion size <0.5 cm (rank 1); (3) Percentage mortality from highest (rank 6) to lowest (rank 1).

3.7.4 Re-isolations

At the end of the experiment, four plants from each treatment and four plants from each control were randomly selected for re-isolation of the inoculated pathogens at both sites. A 1 cm^2 piece was cut off from the discolored regions between the infected and healthy tissues (disease leading edge) of each sample, sterilized and plated on 2% malt extract agar. The plates were incubated at 25°C for 14 days after which fungal identity was confirmed by its colony and conidia morphology.

3.8 Data analyses

Minitab Version 15 (Minitab Inc. 2009) and GenStat Version 12 were used to analyze the data as needed for each experiment (**Papers I–IV**). Summary statistics were used to calculate means and standard errors of different variables. In the field survey, data on disease incidence, severity and tree mortality were $\log_{10}(x + 1)$ transformed before analysis. Analysis of variance (ANOVA) was used to test the significance of AEZs, type of tree planting and farm altitude with disease index and mortality of *G. robusta* trees. Pearson's correlation was used to show the relationships between disease index and tree mortality. The relationships between disease index and mortality with farm altitude and number of continuously dry months (drought) were determined using simple linear regression. One-way ANOVA using Minitab was used to test the differences between occurrences of the Botryosphaeriaceae fungi on farms, trees and disease types on *G. robusta* (**Paper II**) and data were pooled for all farms in each AEZ.

In the pathogenicity experiments, data on internal lesion sizes were square root transformed before analysis. Generalized linear model (GLM) using GenStat was used to test interactions between fungal species and tree species and sites for the variables assessed. Least significant differences between treatment means were calculated using Fisher's least significant difference method. One-way ANOVA was also used to test differences in internal lesion lengths and also to test differences between percentage mortality caused by the five fungal species at the two sites. The number of seedlings showing symptoms of wound healing, cankers and dieback and also dead were counted and expressed as a percentage of total number of seedlings inoculated per tree species per site.

Pathogenicity of each fungal species was estimated using a ranking approach by averaging all the ranks for the two variables assessed for each fungal species. The ranks obtained were analyzed using a Kruskal Wallis test [non-parametric one-way ANOVA where total N = 180 (6 treatments × 30

seedlings) at Site 1 and N = 720 (6 treatments × 4 species × 30) at Site 2] (Theodorsson-Norheim 1986). The isolate showing the highest rank from all variables was suggested to be the most pathogenic.

4 Results and discussion

4.1 Distribution of the disease in the five agro-ecological zones (Paper I)

There was no doubt that the disease was widespread in whole area of investigation. The canker and dieback disease affected about 36% of the 17,994 trees assessed with the most severe attacks being in the drier AEZs. The average size of the 95 farms investigated was 2.5 ha and based on the average age of the trees found in each zone we concluded that planting of *Grevillea robusta* was a relatively new introduction in the semi-arid areas. Majority of the trees in these zones were on average 5–7 years old and a few were less than 3 years or over 15 years old at the time of the study. *G. robusta* was probably taken to Kitui district during the by the Kenya/Japan Social Forestry Training Project in the late 1980s (Yamashita *et al.* 1990). Tree mortality increased from 0.3% in the humid zones to 20.1% in the semi-arid zones, and trees <5 years accounted for 34.7%. The disease index in the semi-arid zones was 67% whereas in the humid zones it was 18%. However, dieback symptoms can easily be mistaken for drought problems and in this way a disease could be present for a long time without a correct diagnosis.

The first sign of the disease was dieback: death of infected tissues resulted in girdling of shoots and branches causing dieback. Internally the fungal infection followed and destroyed growth rings (**Fig.1 in Paper I**), which is also characteristic of stem cankers caused by Botryosphaeriaceae species on *Eucalytpus* spp., (Roux *et al.*, 2001; Gezahgne *et al.*, 2004; Slippers and Wingfield, 2007). The infection in a growth ring might have originated from an infection that started when the growth ring emerged, and this could explain a connection between shoot dieback and inner canker formation in

stems. Although the inner infection followed the growth ring it was also obvious that the infected growth ring could collapse and that the infection could spread to the next growth rings, thereby producing the resin pockets mentioned by (Roux *et al.*, 2001).

Increased tree planting density influenced disease severity. Disease index was high in the woodlots (50%) compared with other types of planting, and was lowest on trees planted along farm boundaries (27%). The close spacing of trees in woodlots (<2 m) may have facilitated the spread of the disease between trees. Under such conditions, stress from competition for water, light and nutrients might have further predisposed the trees to infection and disease development. Pruning of trees, which was observed at many farms, may have aided disease spread within farms through contaminated pruning tools. Fresh cuts or open wounds provide points of direct entry for many fungi into the host system (Rumbos, 1997; Slippers and Wingfield, 2007; Davison and Tay, 2008).

Disease index and tree mortality showed strong negative correlations with farm altitude (**Figures 2A and B in Paper I**). There was a clear increase in disease index and tree mortality below 1,200 m a.s.l. Available rainfall data showed that the number of continuously dry months (<10 mm rainfall) increased between 1990–2005 (Table 3). In addition, the length of the drought period in the zones positively correlated with the disease index (p <0.001, Pearson corr. = 0.89). These results showed that the prevailing biophysical characteristics correlated with the magnitude of the canker and dieback disease in the AEZs. In the lowland semi-arid areas, there were prolonged periods of drought and high temperatures coupled with low fertility soils (Table 1). These factors which clearly deviated from the optimal growth conditions for *G. robusta* (Booth and Jovanovic, 2002) may have predisposed *G. robusta* trees to infection by the canker-forming and dieback pathogens. In addition, the high negative correlations between altitude, disease index and tree mortality showed that the hot and dry lowlands were not suitable for growing *G. robusta* trees, contradicting the suggestions made by Raju (1992) that planting *G. robusta* should be encouraged in the arid and semi-arid areas (ASALs). Furthermore, given that the disease occurred in all AEZs and was severe where prolonged drought occurred, planting *G. robusta* in these areas should be avoided.

Table 3: Number of dry months in each agro-ecological zone between 1990 and 2005; Data missing for 1998 from the Meteorological department

	Number of dry months in each year				
Year	AEZ1	AEZ2	AEZ3	AEZ4	AEZ5
1990	0	0	2	3	3
1991	0	1	2	4	4
1992	0	1	2	7	7
1993	2	0	4	5	6
1994	0	1	1	6	6
1995	0	2	0	5	7
1996	1	2	3	8	5
1997	2	3	4	7	7
1999	1	1	2	7	8
2000	0	2	3	8	8
2001	1	2	3	6	7
2002	1	1	1	5	5
2003	1	1	4	8	9
2004	0	2	4	8	9
2005	1	3	3	8	8
Average	0.7	1.5	2.5	6.3	6.6

Source: Kenya Meteorological Department; Kenya Forestry Research Institute, Kitui Research center and Tiva

4.2 Fungi associated with *G. robusta* in Kenya (Paper II)

This was the first extensive study of the fungal flora of *Grevillea robusta* trees growing outside of its natural range in areas with optimal and non-optimal growth conditions. The aim of this study was to identify potential canker pathogens and other pathogens that may have implications on other crops. In all, 14,871 isolates were obtained from 13,680 isolation attempts. Molecular analysis of the ITS sequence data identified 38 species (**Table 3 in Paper II**). Thirty two species were identified as new occurrences on *G. robusta* in Kenya. The species richness in the AEZs was high, ranging from 2.9 to 3.3 according to the Shannon-Weaver indices. The species accumulation curve showed that most of the fungal species associated with *G. robusta* were isolated in each AEZ (**Fig. 2 in Paper II**) and varied between 31 and 38 species in the AEZs.

Most of the fungi occurred on diseased stems (49.4 %), followed by shoots and branches (33.5 %), leaves (9.2 %) and healthy parts (7.9%). As the same species occurred in dieback symptoms as in cankers, a connection between these two symptoms seemed probable. *G. robusta* played host to a wide range of fungal species, some of which are known to cause disease in other hosts that are of economic importance to agriculture (Agrios, 2005). The occurrence of 24 of the species was infrequent and very low (<1%); these species were not investigated further because they were probably not involved in the disease under investigation. Seventeen fungal species showed an occurrence of <1% and therefore were suggested as potentially important on *G. robusta* (**Table 5 in Paper II**).

Members of the Botryosphaeriaceae occurred in both symptomatic and asymptomatic tissues and comprised 42% of the total isolations. *Neofusicoccum parvum* comprised 17.2% of the isolations and *Lasiodiplodia theobromae* 15.2%. The occurrence of these two fungi was significantly higher in the semi-arid zones compared with the humid zones and positively correlated with the higher disease index in the semi-arid areas (**Table 5 in Paper II**). *Diplodia seriata* De Not., which comprised 6.9% of isolations, was more prevalent in the humid zones where *N. parvum* seemed to be less abundant. Our observations were consistent with those of previous studies showing that *D. seriata* grew best under cool humid conditions (Swart and Botes, 1995; Úrbez-Torres *et al.,* 2006) similar to those found in the humid to semi-humid zones.

Among the Nectriaceae, *Fusarium solani* was the most frequently isolated species, followed by *Gibberella zeae*, *Nectria haematococca* and *Fusarium*

oxysporum, all showed a slightly greater abundance in diseased tissues compared with healthy tissues. Apart from *F. oxysporum*, which occurred in AEZ4 and AEZ5 only, the occurrence of the other Nectriaceae was not significantly different among the zones. The occurrence of the Amphisphaeriaceae species *Pestalotiopsis* sp. was also not significantly different among the zones but was still more common on diseased than healthy tissues.

The occurrence of the Diaporthaceae species *Phomopsis* sp., a dieback and canker pathogen on some woody tree species (Janse van Rensburg *et al.*, 2006; Prasad *et al.*, 2009), increased significantly from the humid to the semi-arid areas and occurred in higher frequencies in diseased tissues than in healthy tissues. *Phomopsis* sp. was regarded as a potential pathogen of the dieback and canker disease. A few species with an occurrence of 2%, *Cladosporium* sp. and *Glomerella cingulata* also occurred in both healthy and diseased tissues with *Cladosporium* sp. showing greater abundance in diseased tissues. *Penicillium citroenigrum, Alternaria* sp., *Paecilomyces sinensis* and *Xylaria* sp. did not show differences in occurrence between healthy and diseased tissues. *Trichoderma viride* occurred only in diseased tissues and decreased in the semi-arid areas and was higher in diseased leaves. Given that its low occurrence in the semi-arid zones was correlated with a high disease index it could not be of importance in the disease. The four Basidiomycetes *Coriolopsis cinerea, Coriolopsis polyzona* and *Armillaria* sp. and *Wrightoporia tropicalis* were not associated with healthy plant parts, indicating pathogenic or opportunistic growth, although the incidences of their occurrence were too low to be analyzed. High abundances particularly of generalist pathogens are reported to increase the pathogens' inter-specific competitive advantage by increasing their chances for substrate colonization and dispersal by producing large amounts of inoculums into the atmosphere (Gilbert, 2002).

Phylogenetic analysis of the fungi isolated and their closest matches in GenBank showed that the 38 of the fungal species identified grouped into 17 clades, corresponding to 17 fungal families all with good bootstrap supports of >90% (**Fig. 3 in Paper II**). The analysis revealed that *G. robusta* has the potential to share different types of pathogens with many other plant species, some of which are of economic importance (Holliday, 1995; Agrios, 2005; Sinclair and Lyon, 2005; Marasas *et al.,* 2006). The presence of these pathogens on *G. robusta* on small-scale farms may pose a threat to the production potential of agricultural crops.

In this study, we hypothesized that fungal species that were associated with canker and dieback symptoms and whose abundances positively correlated with high disease index in the AEZs had a bearing on the disease under investigation. Given that some of these fungi, *Fusarium solani,*

Gibberella zeae, Nectria haematococca, Alternaria spp., *Cladosporium* sp., and *Glomerella cingulata*, are pathogens of agricultural importance, further investigation is required to determine if they play a role in the canker and dieback disease on *G. robusta*. The species regarded as potential pathogens and chosen for pathogenicity tests were *N. parvum*, *L. theobromae*, *D. seriata*, *Botryosphaeria* sp. and *Phomopsis* sp.

4.3 Characterization of Botryosphaeriaceae species associated with canker and dieback disease on *G. robusta* (Paper III)

Based on culture and conidia morphology, seven fungal morphological groups belonging to the Botryosphaeriaceae were identified. Further categorization grouped these fungi into the three genera *Neofusicoccum, Lasiodiplodia* and *Diplodia*. These Botryosphaeriaceae fungi were isolated from the majority of diseased trees (94%) and from 74% of healthy trees. About 63% of the 950 diseased trees sampled yielded one Botryosphaeriaceae species, 27% yielded two species, 4% yielded three or four species and the remaining 16% did not yield any Botryosphaeriaceae species. Co-occurrence of the Botryosphaeriaceae species was also observed by Brown and Britton (1986). The tendency of two or more species to occur together increased from the humid to the semi-arid areas.

Sequence and phylogenetic analyses of the ITS rDNA data revealed that the *Neofusicoccum* group was composed of two species, *Lasiodiplodia* comprised three species and *Diplodia* comprised two species, giving a total of seven species. Blast results of the ITS data set showed that isolates from the two *Neofusicoccum* groups were related to *N. parvum* and *Botryosphaeria* sp. The three morphological groups of *Lasiodiplodia* were related to *L. theobromae, L. pseudotheobromae* and *L. parva* and the two morphological groups of *Diplodia* were related to *D. seriata* and *Diplodia* sp. Exploratory phylogenetic analyses showed that isolates belonging to the same species grouped together in the same clades irrespective of the zone of origin, and also with others from different hosts and regions. *Botryosphaeria* sp. and *Diplodia* sp. were not identified down to the species level. The Tef1-α sequence data analysis was useful in identifying a species previously identified as *Lasiodiplodia* sp. to the species level as *L. parva*. In the phylogenetic analyses, *Botryosphaeria* sp. grouped closely with *N. parvum* and *Diplodia* sp. grouped closely with *D. seriata*.

Neofusicoccum parvum, *L. theobromae* and *D. seriata* were widely distributed in the five AEZs and their significance increased on canker and dieback symptoms in all zones. Their relative incidences increased significantly from the humid to the semi-arid areas **(Table 4 in Paper III**). Isolates of *N.*

parvum and *L. theobromae* were dominant and formed more than 70% of the total isolations among the Botryosphaeriaceae. These two species also seem to be widely distributed within eastern Africa on native and non-native species (Roux *et al*., 2001; Gazahgne *et al*., 2004; Gure *et al*., 2005; Tojlander *et al*., 2007). Three species, *Botryosphaeria* sp., *L. pseudotheobromae* and *L. parva* only occurred in the semi-arid areas and their roles in the humid zones were not clear. Four out of the seven Botryosphaeriaceae species identified occurred on all plant parts, including healthy parts.

From this and other studies, it seemed that *N. parvum* is increasing in importance on woody species in the African region, being associated with stem cankers and dieback symptoms on Myrtaceous, Proteaceous hosts including *G. robusta* and other hosts in South Africa, Uganda and Ethiopia and other tropical countries (Roux *et al* 2001; Gezagne *et al.,* 2004; Gure *et al.,* 2005; Pavlic *et al*., 2007; Mohali *et al*., 2009, Heath *et al*., 2011). *Lasiodiplodia theobromae,* the second most abundant species has a wide host range throughout the tropics and the temperate regions, occurring on over 500 host species (Punithaligham, 1980). It is well known as a latent pathogen on many host species and is known to cause serious disease symptoms when the hosts are stressed (Shoeneweiss, 1981; Slippers and Wingfield, 2007). Under laboratory conditions, these two species also tolerated high temperatures ranging from 25°C to 35°C correlating closely with their isolation abundances which increased from the humid to the semi-arid areas (Njuguna *et al*., 2011).

Other studies have reported that *Lasiodiplodia* species are prevalent within the African continent (Pavlic *et al*., 2004; Damm *et al*., 2007; Begoude *et al*., 2010a). In this study, *L. pseudotheobromae* and *L. parva* were not detected in healthy tissues, occurred only in the semi-arid areas and were least abundant. However, they have been reported to be widely distributed as endophytes and also pathogens of other woody species in other regions (Burgess *et al*., 2006a; Alves *et al*., 2008; Begoude *et al*., 2010a, 2010b). The roles of these fungi on *G. robusta* need to be investigated further.

The *Diplodia* group, like the *Neofusicoccum* group seemed to be less diverse. Only two species of each group identified; *Diplodia seriata* and *Diplodia* sp. and were detected in healthy tissues and diseased tissues in almost equal proportions (**Table 1 in Paper III**). Both species achieved optimal growth rates at 25°C, although the growth rate of *Diplodia* sp. was much lower than that of *D. seriata* (**Table 3 in Paper III**). They did not grow well above 25°C. This group of pathogens, which also has a wide host range, seemed to prefer the cool conditions in the humid regions. *Diplodia seriata* is a serious pathogen of grapes (Úrbez *et al*., 2008).

This study indicated an endophytic relationship between the four major Botryosphaeriaceae fungi and *G. robusta* given that the disease incidence was low in the humid areas compared with the semi-arid areas (Njuguna *et al.*, 2011, **Paper I**). This endophytism may partly explain why the disease has gone undetected for a long time in the humid areas. We also noted that tree planting was a relatively new practice in the semi-arid areas and *G. robusta* is primarily propagated through seeds and seedlings. Given that the Botryosphaeriaceae are indicated to be seed-borne (Gure *et al.*, 2005), it is possible that these pathogens may have been transferred to the semi-arid areas in this way (Cilliers *et al.* 1995) where the species seemed to grow well initially but the canker and dieback disease intensified in the 1990s (Njuguna, 2003). Many reports indicate that endophytic Botryosphaeriaceae almost always attack their hosts when grown under stress conditions, and the onset of the disease was linked to stressful environments that reduce the growth vigor of the host plants (Desprez-Loustau *et al.*, 2006; Slippers and Wingfield, 2007).

Phylogenetic analyses showed that the Botryosphaeriaceae isolates identified in this study had a wide host range (**Fig. 3 in Paper III**) as also observed in other studies (Britton and Hendrix, 1986; Pavlic *et al.*, 2007, 2009; Taylor *et al.*, 2009). The benefits of co-occurrence among the pathogens were not clear although Slippers and Wingfield (2007) proposed that co-occurrence may increase their ability to overcome the host's resistance, particularly under stressful conditions. In this study, the hot conditions in the semi-arid areas seemed to favor the occurrence of many pathogens on the same host plant. However, co-occurrence was also likely to obscure their isolation and identification, resulting in a disease complex that required further studies.

4.4 Pathogenicity of five potential canker pathogens on four tree species (Paper IV)

The ability of four Botryosphaeriaceae fungi, *N. parvum, L. theobromae, D. seriata* and *Botryosphaeria* sp, and the Diaporthaceae fungus *Phomopsis* sp. to cause canker and dieback disease was tested on four tree species: *G. robusta, S. siamea*, *A. indica* and *M. volkensii*. The latter three are commonly inter-planted with *G. robusta* in agroforestry systems in the semi-arid areas and showed similar disease symptoms on the farms, necessitating testing of the fungi under hot conditions. *G. robusta* was tested under both cool and hot glasshouse conditions to ascertain the roles of different temperatures on the ability of the fungi to cause disease.

The pathogenicity of the fungi was evaluated using three factors: first signs of canker development, final internal lesion length and seedling mortality after 10 months. Seedlings inoculated with *N. parvum* were the first to show early canker development, closely followed by seedlings inoculated with *L. theobromae*. Canker development was slower in seedlings inoculated with *Botryosphaeria* sp. and *D. seriata*. *G. robusta*, which is most susceptible to the disease, was the first species to show signs of infection, closely followed by *S. siamea* and *A. indica*. *M. volkensii*, which was least susceptible to infection, showed a late response to infection. Similar relationships between the Botryosphaeriaceae pathogens and the trees were observed for internal lesion length and seedling mortality. These factors were compiled in a ranking analysis, which showed that *N. parvum* seemed to be the most virulent on all tree species, supporting earlier findings in Uganda (Toljander *et al.*, 2007). *Lasiodiplodia theobromae* and *Botryosphaeria* sp. were moderately virulent whereas *D. seriata* showed slightly lower virulence.

The three factors used in the ranking analyses seemed useful in determining pathogenicity. Early canker development gave a quick response, which became clearer as the other two factors were assessed at the end of the experiment. Seedling mortality as a single factor gave a clear differentiation between the five fungal species and also between the four tree species. *Phomopsis* sp. did not seem to be a pathogen of either *G. robusta* or *M. volkensii*. However, given that a few seedlings of *S. siamea* and *A. indica* were killed by *Phomopsis* sp., this fungus could be a weak pathogen on these two species.

An unexpected result recorded at the end of the experiment was wound healing at the inoculation point. Out of the four tree species compared under hot conditions, *Melia volkensii* seedlings showed the highest level of wound healing in response to inoculation by each of the five pathogens, particularly in response to *Phomopsis* sp. infection. The four tree species responded in the same way to infection by each of the Botryosphaeriaceae species. No wound healing occurred in *G. robusta* and *S. siamea*, and moderate wound healing occurred in *A. indica*. Wound healing was an active defense mechanism by the plants to resist disease and was taken as an indication of the relative susceptibility of the tree species to the fungi or the virulence of the pathogen. In regard to wound healing, *M. volkensii* could be considered as the least susceptible to infection by the five fungal species given that the highest incidences of wound healing and lowest mortality was recorded on *M. volkensii* seedlings.

Grevillea robusta was more sensitive to infection by the four Botryosphaeriaceae fungi under hot conditions than under cool conditions, a

clear indication that *G. robusta* was better suited to the cool climates. Furthermore, Britton and Hendrix (1986), and Swart and Botes (1995) showed that the incidences of Botryosphaeriaceae depended on the season but that all were active during warm conditions. Hot conditions increased the mortality of inoculated *G. robusta* seedlings, supporting our field observations of high tree mortality in the semi-arid areas (Njuguna *et al.*, 2011). The highest level of seedling mortality was recorded for seedlings inoculated with *Neofusicoccum parvum* and grown under hot conditions.

It is possible that hot conditions increased the susceptibility of *G. robusta* to infection by the Botryosphaeriaceae fungi, which would support previous observations by Shoeneweiss (1981), Pusey 1989 and Desprez-Loustou *et al.* (2006) that water stress predisposes plants to infection and increases the aggressiveness of canker pathogens. In this respect, we found that *N. parvum* was the most virulent pathogen and *M. volkensii* was the least susceptible host. *Melia volkensii*, the only native species, was therefore better adapted to the semi-arid conditions, which lead to less strain. The non-native *G. robusta* was able to resist infection better in a cool climate than under hot conditions where 93% of the seedlings were killed by *N. parvum*. In all treatments, 90–100% of the inoculated fungi were re-isolated from the inoculated plants and we were thus able to fulfill Koch's postulates.

Since the four Botryosphaeriaceae caused similar symptoms on the four tree species, it was not possible to isolate a primary cause of the disease, supporting previous observations by other authors on the Botryosphaeriaceae (Denman *et al.*, 2003; Mohali *et al.*, 2006, 2009; Pavlic *et al.*, 2007; Alves *et al.*, 2008; Slippers *et al.*, 2009). We therefore concluded that the canker and dieback disease is associated with the Botryosphaeriaceae species complex and that *N. parvum* was the most important pathogen on *G. robusta* in Kenya. It was also clear that the pathogens isolated from *G. robusta* are also strongly virulent on *S. siamea* and *A. indica*.

5 General conclusions

i. Agroforestry using *G. robusta* faces challenges because the species, which forms a near monoculture on small-scale farms in Kenya, is under threat from a widespread canker and dieback disease. The disease is milder in the humid areas where the conditions favor the growth of the species but is severe in the semi-arid areas.

ii. A connection between symptoms on the trees and occurrence of Botryosphaeriaceae pathogens was established. The four Botryosphaeriaceae species: *N. parvum, L. theobromae, Botryosphaeria* sp. and *D. seriata* were individually capable of causing canker, dieback and mortality on the four tree species. However the symptoms were indistinguishable and led to the conclusion that they formed a "Botryosphaeria disease complex".

iii. In the pathogenicity tests, together with *G. robusta, Senna siamea* and *Azadirachta indica* were susceptible to the disease but *Melia volkensii* was least susceptible. *M. volkensii* the only native species was a more suitable species for agroforestry systems in the semi-arid areas. *G. robusta* should be restricted to the cool climates, thus emphasizing that proper species site matching as an important disease management strategy to reduce losses by disease agents.

iv. *G. robusta* was host to over forty fungal species, some of which cause serious diseases of other woody species as well as agricultural species. These results over turn the long held misconception that *G. robusta* was "pests and disease free". The species could be regarded as a potential risk to other plant species in agroforestry systems because it could act as a source or reservoir of fungal inoculums.

v. The endophytic nature of the pathogens identified in this study enable them to be transferred easily via seeds or seedlings to new areas without being noticed. Their plurivorous characteristics allow them to easily

expand their host range, particularly under conditions that are stressful to the host species.

vi. This study concluded that the emergence of unspecific plurivorous pathogens coupled with stressful environmental factors for the host can mean that a tree mix as in agroforestry practices can be as vulnerable to infection as in monocultures because new environments may add to stress factors and therefore, increase their susceptibility to opportunistic pathogens (Mitchell, 1989; Wingfield *et al.*, 2001).

vii. From this and other studies, it is clear that environmental stress factors, such as high temperatures and drought periods, as envisaged in global warming, could play a role in increasing the virulence and expansion of the Botryosphaeriaceae pathogens to other hosts.

6 Future perspectives

i. The results form a baseline on which other studies could be performed. The rush to intensify reforestation in the semi-arid areas and the lack of a clear understanding of the biology of introduced tree species in new environments has led to trees being planted in the wrong places, which reduces their chances of survival as has been proved in this study. To enhance the sustainability of agroforestry systems, it is important to carry out thorough eco-physiological studies before introducing tree species in new environments.
ii. The population dynamics of the major pathogens identified studied on different provenances of *G. robusta* would further develop the conditions needed for *G. robusta* in different agroforestry systems.
iii. This study did not establish whether the disease was native or introduced. Therefore further mycological studies of the native vegetation surrounding small-scale farms are needed to ascertain whether the pathogens are native or introduced. As native species have been shown to be more resistant to infection, we emphasize that agroforestry should focus on using more native species.
iv. Where is the disease spreading from? It is possible that some fungal pathogens could have spread from other woody species to *G. robusta* or from agricultural crops on the farms. Studying such relationships would shed light on the host–pathogen dynamics for successful disease management programs.
v. *G. robusta* leaves are commonly used as mulch on annual agricultural crops. The use of leaves is likely to increase the inoculum level of the pathogens thereby making it difficult for susceptible crops to grow. Unconfirmed reports and field observations have shown that disease

control on small-scale farms has proved difficult, requiring the use of many and strong fungicides with time. The study proposes urgent investigation of this aspect.

vi. Is the disease seed-borne? This is an important aspect to study because *G. robusta* is primarily propagated through seeds. Our visits to some tree nurseries showed that most seedlings were generally weak, and isolations also revealed the presence of *Neofusicoccum* and *Lasiodiplodia* species.

vii. Finally, many studies have linked Botryosphaeriaceae diseases to stressful environmental factors, such as drought and extremes of temperature, but few if any has attempted to link these diseases with soil nutritional status. Studies of this aspect would give a holistic view of the influencing role of the environment on these pathogens.

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