Characterization of latent Botryosphaeriaceae on diverse *Eucalyptus* species

by

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MAGISTER SCIENTIAE

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Declaration

I, the undersigned, hereby declare that the thesis submitted herewith for the degree *Magister Scientiae* to the University of Pretoria contains my own independent work. This work has hitherto not been submitted for any degree at any University.

Happy Mamodise Maleme

December 2008

I dedicate this thesis to my late father Matthews, my mother Florence and my sisters Modiehi, Dimakatso and Nthabeleng

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SUMMARY

The Koala bears housed and maintained by the Pretoria Zoo are relying on 20 different *Eucalyptus* species in the *Eucalyptus* arboretum in Pretoria for food. Species of the Botryosphaeriaceae are well known endophytes and pathogens of *Eucalyptus* in South Africa and elsewhere. This study was aimed at characterizing the Botryosphaeriaceae infecting these trees planted for the Koala bears. The experimental set-up also provided the opportunity to broaden our knowledge regarding the *Eucalyptus* species preferences of the Botryosphaeriaceae, as well as to reveal their potential movement to and from surrounding trees.

In Chapter 1, the literature is reviewed regarding the biology of fungal endophytes, with a specific focus on Botryosphaeriaceae on *Eucalyptus*. Such a review clearly shows that, despite much previous work, we still only have a limited understanding of the biology and ecology of most fungal endophytes. This is particularly true for tree endophytes. Botryosphaeriaceae are clearly important and widespread canker and dieback pathogens of *Eucalyptus*. The taxonomic confusion that plagued the group, however, hindered a clear understanding of their diversity and biology. The use of molecular tools together with morphological characteristics has improved the ability to separate these fungi at species level. These tools are important for future work to better understand the true diversity. This would be a prerequisite if we are to gain a better understanding of the biology of these fungi and design with management strategies to control them.

In Chapter 2, five species of Botryosphaeriaceae were identified from *Eucalyptus* species in the Pretoria arboretum, South Africa. Two species were described here as new species of Botryosphaeriaceae, within *Neofusicoccum*, namely *N. ursorum* prov. nom. and *N. crypto-australe* prov. nom. This is the first report of the latter species on *Eucalyptus* in South Africa. The other species included *N. parvum*, *N. eucalypti* comb. nov. (previously known as *Dichomera eucalypti*) and *B. dothidea*. *Neofusicoccum parvum* was the most common species isolated, followed by *N. ursorum* and *N. eucalypti*. The identifications were all based on the morphological characteristics, including cultural and conidial morphology, and DNA sequence data of the internal transcribed spacer region (ITS 1 and 4), and the translation elongation factor 1-α. The closely related species *N. parvum* and *N. ribis* were distinguished using a previously designed PCR-RFLP technique. As for the distinction between the cryptic species *N. crypto-australe* and *N.*

australe, it was necessary to analyze a third gene region (β- tubulin) to confirm their separation using the phylogenetic species concept.

All isolates obtained from this study produced lesions on stems of *Eucalyptus camaldulensis* (clone ZG-14) in the pathogenicity trial conducted under green house conditions. Of all species, *N. eucalypti* and *N. crypto-australe* were found to be the most pathogenic and *B. dothidea* the least pathogenic. There was, however, also significant variation in virulence between isolates of the same species. The results clearly show the potential threat of species of Botryosphaeriaceae to *Eucalyptus*. Field trials should be conducted in future studies to validate the findings in the greenhouse trials.

Isolates representing different species identified in Chapter 2, were used for designing molecular tools for the *in vitro* and *in vivo* identification of Botryosphaeriaceae (Chapter 3). Sequences of the translation elongation factor 1-α were compared, and unique polymorphisms identified. Species specific primers were designed around these polymorphisms. All the primers designed were proven to be specific enough to distinguish the five different species from each other. The sensitivity of all primers were shown to detect fungal DNA concentration between 50 ng/µL and 0.01ng/µL. Preliminary tests of these primers on *Eucalyptus* leaves were done, and latent infections of *N. parvum* (the most common species) could be identified. More samples are likely to reveal the latent infections of other species using these tools. Future studies could now use these tools for the rapid identification of the fungi on *Eucalyptus*. It could be expanded to other hosts and more species of the Botryosphaeriaceae as well.

The results presented in this study provided detailed information on species of the Botryosphaeriaceae from the 20 different species of *Eucalyptus* in a Pretoria arboretum. It yielded unique species, as well as well known pathogens of this host. All species isolated in this study were found to be pathogenic on *Eucalyptus*. The knowledge foundation, data and tools provided by this study can now be applied to characterize the infection biology, fine scale distribution and population diversity of these fungi. It is likely to bring new insights into the ecology of these organisms, their potential origin and movement between hosts. The presence of these fungi in other parts of South Africa should also be considered.

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PREFACE

Since the description of *Botryosphaeria dothidea* by Cesati and De Notaris in 1863, Botryosphaeriaceous fungi have become well known for some serious diseases of agricultural crops, fruit trees, and trees in plantation forests. More recently, this group of fungi have also become known as endophytes causing latent infections on their hosts. These latent infections normally change to active disease when conditions turn unfavourable for the host, causing stress on the plant. Amongst the woody hosts affected by these fungi are members belonging to the Myrtaceae Family, including *Eucalyptus*. *Eucalyptus* diseases caused by Botryosphaeriaceae have been documented in various parts of the world, with canker and dieback being the most commonly reported symptoms.

Eucalyptus have been established as exotics in plantation forests in South Africa for more than a century. Recently, a variety of *Eucalyptus* species were established for feeding Koala bears housed by the Pretoria Zoo. For this project an *Eucalyptus* arboretum was established, including 20 different species. A preliminary investigation indicated that species of Botryosphaeriaceae were present and causing diseases in the arboretum. This provided an opportunity to study the Botryosphaeriaceae diversity in this defined location, investigate potential host preference of different species of the Botryosphaeriaceae and their ability to cause latent infection, and lastly to compare their relation to Botryosphaeriaceae infecting naturally regenerated *Eucalyptus* spp. surrounding the arboretum. Such information would provide a foundation for dealing with these diseases in future.

As background to the thesis, the literature was reviewed pertaining to endophytic fungi (Chapter 1). The focus was on both grasses and trees, with a particularly focus on Botryosphaeriaceae infecting *Eucalyptus* spp. It also highlights the poor definition of the term ‘endophyte’ in the literature regarding the ecological role of these fungi. Endophytes are able to adopt different ecological roles depending on a number of factors. The literature on Botryosphaeriaceae as endophytes and/ or latent pathogens on *Eucalyptus* is assessed in the light of recent taxonomic changes, their mode of infection, transmission and dispersal, stress as an inducer for disease expression, the symptoms they cause, as well as the need for prevention of their spread.

The first and main aim of the project was to identify species of the Botryosphaeriaceae from 20 different *Eucalyptus* species from the *Eucalyptus* arboretum

in Pretoria (Chapter 2). This was achieved by sampling branches and leaves from trees from each row representing the different species. Both asymptomatic and diseased material were sampled. Corresponding samples were also randomly taken from naturally regenerated, wild *Eucalyptus* surrounding the arboretum. All isolates obtained that were typical of the Botryosphaeriaceae in culture morphology were identified to the species level. DNA based tools, together with morphology, were used to characterize different species of Botryosphaeriaceae. Apart from known *Neofusicoccum* and *Botryosphaeria* species, two undescribed *Neofusicoccum* spp. were identified and are described here for the first time. A taxonomic revision is also made for a *Neofusicoccum* sp. which was previously described under *Dichomera*. The pathogenicity of all the species to eucalypts was tested. Two-year old trees of *Eucalyptus camaldulensis* (clone ZG-14) were used for this purpose in a trial conducted under green house conditions.

Most Botryosphaeriaceae were obtained from asymptomatic leaves. There is a need for tools to quickly and accurately identify these latent infections *in vivo*. A second aim of the thesis was therefore to develop molecular tools for the *in vivo* identification of the five different species of Botryosphaeriaceae identified in Chapter 2. These markers were tested on DNA extracted from both purified cultures and *Eucalyptus* leaves. The development and testing of these markers are presented and discussed in Chapter 3.

The thesis is finalised by a summary discussion of the data obtained and conclusions made in this study. Insights from this work that could guide future studies to better understand the Botryosphaeriaceous fungi, their mode of transmission, ecological role and prevention of spread is also included in this section.