Chapter 1

The biology of fungal endophytes, with specific reference to the Botryosphaeriaceae on *Eucalyptus* trees

1.0. Introduction

Fungal endophytes form an integral part of microbial communities that are commonly associated with plants. They have many different ecological roles that include mutualism, commensalism and parasitism (Carroll 1988; Arnold 2007; Saikkonen 2007; Sieber 2007). They also occur on a variety of hosts that include trees, shrubs, grasses, mosses, ferns and lichens (Stone *et al*. 2000; Zhang *et al*. 2006). Although there is abundant evidence for the positive effects that endophytes confer on some hosts such as grasses, there is little information on the ecological role that endophytes play on other hosts such as trees.

Most work on fungal endophytes of plants has been done on grasses, most notably tall fescue grasses and with a focus on either *Acremonium* or *Epichloë* species. These fungi are important for host survival and abundant evidence supports this view (Siegel *et al*. 1987, 1993; Clay 1988, 1990; Funk *et al*. 1993; Saikkonen *et al*. 1998). However, it has been proposed that this association depends on environmental conditions and nutritional availability (Müller and Krauss 2005). If nutritional resources are limited, endophyte-host association may change from mutualism to commensalism and even to antagonism or parasitism. For example, improved performance was observed on endophyte-infected *Festuca pratensis* as compared to uninfected plants on highly fertilized and watered soil. On the contrary, under low nutrient and water conditions, few tillers and lower root and total biomass were observed in *F. pratensis* infected with endophytes. In this case, an endophyte shifted from being a mutualist to an antagonist under limited nutritional resources (Ahlholm *et al*. 2002*b*). Under conditions of stress, the endophytes are hypothesized to act as commensalists or antagonists (Stanosz *et al*. 2001; Desprez-Loustau *et al*. 2006; Slippers and Wingfield 2007).

The host genotype plays an important role in the endophyte-host association. An endophyte might thus act as a mutualist, antagonist or commensalist based on the host genotype. For example, Redman *et al*. (2001) demonstrated that well-known plant pathogens in the genus *Colletotrichum*, i.e. *C. magna*, *C. coccodes*, *C. orbiculare*, *C. musae, C. lindemuthianum, C. gaminicola, C. glioesporiodes* and *C. acutatum*, were observed in a non-pathogenic/mutualistic lifestyle in tomato and pepper cultivars, which were known not to be susceptible to these pathogens.

The existence of endophytes in trees was first reported in *Picea canadiensis* in the Pacific North West (Lewis 1924). Since that time, there have been many reports focusing on fungal endophytes on trees (e.g. Petrini 1986; Rodrigues 1994; Azevedo *et al*. 2000; Frohlich *et al*. 2000; Stone *et al*. 2000; Arnold *et al*. 2003). A predominant view is that these fungi are highly diverse in most hosts. For example, Gamboa and Bayman (2001) found 38 morphospecies of endophytes from leaf segments of two populations of the tree species *Guarea guodonia*. These included *Phomopsis*, *Colletotrichum*, *Xylaria* and *Rhizoctonia* like fungi. These fungi were found in 95 % of leaf segments examined. More recently nine fungal genera were identified in both young and mature leaves of teak (*Tectona grandis* L.) and the rain tree (*Samanea saman* Merr.) in the study of foliar endophytes on these trees (Chareprasert *et al*. 2006). A diverse assemblage of endophytes has also been reported from *Lucuala* sp. (palm) in Brunei Darassalam and from *L. ramsayi* palm in Australia (Frohlich *et al*. 2000). Furthermore, 418 endophyte morphospecies were obtained from 83 leaves segments of two tree species *Heisteria concinna* (Olacaceae) and *Ouratea lucens* (Onchnaceae) (Arnold *et al*. 2000). Unfortunately only a few of these studies have considered the infection biology and transmission of these endophytes in trees (Deckert and Peterson 2000; Arnold and Herre 2003; Kaneko and Kaneko 2004). This will be essential if we are to better understand the role of these fungi and how they are spread in the environment.

Members of the Botryosphaeriaceae are well-known endophytes in a variety of tree hosts. Some of the better studied hosts include species in the families Myrtaceae (*Eucalyptus* spp.), Proteaceae (*Protea* spp.), Leguminoceae (*Acacia* spp.) and Pinaceae (*Pinus* spp.) (Fisher *et al*. 1993; Smith *et al*. 1996*a*; Stanosz *et al*. 1997; Burgess *et al*. 2001; Denman *et al*. 2003; Mohali *et al*. 2006). The Botryosphaeriaceae do not usually cause disease symptoms on hosts with which they are associated, unless the hosts are predisposed through environmental stress, in which case they act as latent pathogens (Schoeneweiss 1981; Swart and Wingfield 1991; Blodgett and Stanosz 1997; Desprez-Loustau *et al*. 2006).

Some species of Botryosphaeriaceae are known to be serious latent pathogens on a wide range of agricultural crops of economic importance including fruit trees such as apples, peaches, pears, avocado, mango, macadamia and pome fruits (Hartill 1991; Johnson *et al*. 1991, 1992; Biggs 1995; Ogata *et al*. 2000; Hartill and Everett 2002; Slippers *et al*. 2005;

Slippers *et al*. 2007). To give just two examples, there is a high frequency of *B. dothidea* as a latent pathogen on fruit clusters of Pistachio, as well as some latent infection of leaves (Ahimera *et al*. 2003). *Botryosphaeria dothidea* is also known to quiescently infect mangoes causing stem end rot post-harvest disease (Johnson *et al*. 1992). The species, however, also occurs on numerous other fruits and forest trees (Slippers and Wingfield 2007). Even though some species of the Botryosphaeriaceae are known as latent pathogens on many different hosts, the ecological role of most species as endophytes, especially in native ecosystems, is far from fully understood.

Given the ecological and economic importance of endophytes of trees, a clearer understanding of their lifestyle is needed. This review, therefore, aims to examine the different ecological roles of endophytes and thus to provide a basis for their further study. A specific focus is on the Botryosphaeriaceae as a key endophyte group in tree hosts.

2.0. Defining fungal endophytes

The term endophyte is loosely used in the literature to refer to fungi that occur inside plant tissue. The association is sometimes mutualistic where both partners benefit (Clay 1991) or commensalistic where one partner benefits and the other remains unaffected (Seiber 2007). Some endophytes may spend part or all their life cycle in the host without causing disease (Carroll 1988; Clay 1988, 1991; Petrini 1996). Others, typically referred to as latent pathogens, may exist as endophytes for part of their life cycle, but they can cause disease symptoms under certain conditions (Sinclair and Certauskas 1996). Disease symptoms often occur after the onset of environmental stress such as drought, hail damage, frost, hot or cold winds (Schoeneweiss 1981; Pusey 1989; Old *et al*. 1990). The term endophyte thus often refers to fungi with vastly different ecological roles (see Fig. 1).

Hawksworth *et al*. (2001) noted that "no agreement exists as to whether the term endophyte should be restricted only to completely asymptomatic and / or mutualistic fungi within aerial plant parts or to fungi that grow endophytically regardless of their symptoms or disease effects". Some authors have argued that endophytes are often closely related or have evolved from plant pathogenic fungi, and that makes their differences more difficult to interpret (Carroll 1988). The life cycle of endophytes, which at some point becomes parasitic, also makes the distinction between the terms mutualism and parasitism difficult as

it relates to them (Carroll 1988). On the other hand, some case studies on specific hosts have shown that there is no close relatedness between their endophytes and known pathogens with a latent phase. For example, endophytes of *Pinus monticola* that were compared to parasites based on sequence homology, were found not to be closely related (Ganley *et al*. 2004).

Rodriguez and Redman (1997) propose that there is an evolutionary change between parasites, mutualists, and / or saprophytes (commensalists). This evolutionary change is multi-directional, i.e. the fungi can evolve from mutualists to saprophytes and to pathogens or from pathogens to mutualists and to saprophytes. Schulz and Boyle (2005) refer to this evolution as a "developmental and evolutionary continuum". A mutualism-parasitism continuum for example exists in the symbiosis between *Epichloë* sp. and grasses, where the fungus possess both the mutualistic and parasitic nature in its life cycle (Müller and Krauss 2005; Schardl *et al*. 2004). The assumption is that the change from mutualism to parasitism might be due to some balanced antagonism or equilibrium in endophyte-host interaction. If the balance is disturbed, disease development occurs (Kogel *et al*. 2006).

3.0. Endophytes of grasses
3.1. Fungal endophytes as mutualists on grasses

Much research has been undertaken to understand the interaction of endophytes and grasses. Among the best studied of the fungal endophytes are those residing in the tribe *Balansieae*, which are Ascomycetes fungi belonging to the family Clavicipitaceae (Clay 1988, 1990; Bacon 1995). These include *Epichloë* and *Neotyphodium* species, previously known as *Acremonium*. These endophytes have been reported to persist asymptomatically in grasses with some mutualistic effect. Dingle and Mcgee (2003) also observed the reduction of leaf rust disease in wheat infected with *Chaetomium* and *Phoma* species as fungal endophytes. Due to the abundant indications of the positive effect that these endophytes confer on their hosts, effort has been made to also understand the mutualistic role they play in other grasses, specifically wild grasses or grasses in natural stands (Saikkonen *et al*. 2000; Brem and Leuchtmann 2002).

Mutualism is attributed to the ability of fungal endophytes to produce one or more compounds or secondary metabolites that enhance the host's vigour, photosynthetic rate and increases the host's resistance against abiotic and biotic stress. Insect herbivory, pathogens

and drought are some of the factors that are most likely to cause stress and these are affected by endophytes (Siegel *et al*. 1987; Clay 1988, 1990, 1993; Funk *et al*. 1993; Saikkonen *et al*. 1998). For example, *Lolium multiflorum* produced significantly more vegetative tillers and allocated more biomass to roots and seed when infected with *Neotyphodium* spp. as compared to uninfected individuals (Vila-Aiub *et al*. 2005). Larvae of the fall armyworm (*Spodoptera frugiperda*) showed low pupal mass, as well as low rate of survival and mass gain, when fed on grass infected with endophytes (Clay 1988). Likewise, Tunali and Marshall (1995) demonstrated that culture filtrates of some *Acremonium* spp. and *Neotyphodium* spp. caused abnormal elongation of the hypha, lysis of the conidia and abnormal germ tubes of some pathogens. In turn, the fungi acquire nutrition, long lasting protection and dissemination via seed or vegetative material from the host (Latch 1993; Siegel 1993). Such mutualistic roles of endophytic fungi imply that they can be used as biological control organisms against pests and pathogens.

3.2. Fungal endophytes parasitic on grasses

Grass endophytes can be parasitic, where pathogens have a latent phase in their life cycle such as the smut fungi (Petrini 1986; Latch 1998). One example showing endophyte-parasitism is that of *Epichloë* endophytes, where the sexual state is able to sterilize hosts such as *Brachypodium sylvaticum* by preventing flowering and seed formation (Siegel 1993; Meijer 2001). Faeth and Sullivan (2003) also demonstrated that a *Neotyphodium starrii* endophyte on *Arizona fescue* plants reduced host growth and reproduction, and fewer germinated seeds were also observed. Fungal pathogens with a latent phase normally referred to as endophytes have been reported on a number of grass hosts including rice (*Oryza sativa*) and maize (*Zea mays*) and their negative effects have been well documented (Fisher and Petrini 1992; Pinto *et al*. 2000). These parasitic fungi fall into class three (3) based on a fungal lifestyles classification and ecosystem dynamics defined by Rodriguez and Redman (1997). This classification states that "fungi that are quickly 'walled off' or inhibited from colonization by plant defence responses or metabolic inhibitors, remain quiescent until the host becomes senescent".

Environmental factors such as nutritional deficiencies and other potential pathogens that bring about stress on host plants play an important role in either sustaining an

endophyte-host association as mutualistic or turning it into a parasitic relationship. If the host is stressed or where there are insufficient nutrients available for growth, the mutualistic association often becomes parasitic (Müller and Krauss 2005). Plant defence response or metabolic inhibitors with minimal effect prevent these fungi from causing disease symptoms and the endophytes can consequently live in a latent form within a plant host until it dies. Therefore, the early assumptions that endophytes do not cause disease have definitively been shown to be false and the fungi are clearly able to adopt a parasitic lifestyle under suitable conditions (Stanosz *et al*. 2001; Faeth 2002; Faeth and Fagan 2002).

3.3. Infection and transmission of grass endophytes

Grass endophytes reproduce both sexually and asexually in their life cycle (Schardl *et al*. 2004). For example, *Epichloë sylvatica* that infects *Brachypodium sylvaticum* was reported to reproduce both sexually and asexually (Meijer 2001). In such cases, *Epichloë* spp. (sexual form) has an antagonistic effect because flowering and seed formation is suppressed. This is due to the formation of the stroma followed by the fruiting structure around the developing inflorescence. *Neotyphodium* spp. (asexual form) has a mutualistic effect and the fungi grow vegetatively into the seed from one plant to the other.

Grass endophytes are commonly vertically transmitted and the hyphae grow internally and intercellularly in the host tissue (Wilson 1993; Saikkonen *et al*. 2004). The infection is thus systemic throughout the whole plant. Saikkonen *et al*. (2004) proposed that the morphological arrangement of grasses allows easy systemic growth of the fungi throughout the plant tissue. Vertical transmission to seed progeny in most cases promotes single species dominance where a single fungal genotype is transmitted from one generation to the next.

Studies demonstrating vertical transmission of endophytes have mostly been done on cultivated or controlled tall fescue and perennial grasses (Clay 1998; Saikkonen *et al*. 2004). However, recent work on wild grasses has also revealed the possibility of horizontal transmission. For example, no evidence of vertical infection was observed when perennial ryegrass plants were infected with *Epichloë typhina*. All seeds were found not to be infected with this endophyte (Chung and Schardl 1997). Therefore, a possibility of horizontal

transmission cannot be excluded especially when dealing with endophytes on natural grasses.

4.0. Endophytes of trees

4.1. Fungal endophytes as mutualists on trees

Considerably less is known regarding fungal endophytes of trees than those of grass endophytes with regard to their protective mutualistic role (Carroll 1995). Recently, evidence suggesting potentially mutualistic effects in tree-fungal interactions has been reported (Arnold *et al*. 2003). The presence of endophytes (common genera *Colletotrichum*, *Xylaria* and *Fusarium*) on *Theobroma cacao* L. seedlings reduced the incidence of leaf necrosis and mortality when challenged with pathogenic *Phytophthora* species. Furthermore, Rubini *et al*. (2005) demonstrated the antagonistic effect of *Gliocladium catenulatum* against *Crinipellis perniciosa*, the cause of Witches' Broom Disease of *T. cacao* under greenhouse conditions. The presence of endophytic fungi on *T. cacao* was also shown to increase the host defence system against *Phytophthora palmivora* (Herre *et al*. 2007). Clearly not much research has been conducted on the protection to trees conferred by endophytes. Future research should also focus on other mutualistic roles that an endophyte might play, other than protection against pathogenic fungi.

4.2. Fungal endophytes parasitic on trees

Fungal pathogens with a latent phase are common factors that affect tree health. For example the pathogenic fungus, *Guignardia citricarpa* was recovered as an endophyte from leaves of *Citrus limon* in Argentina (Durán *et al*. 2005). This fungus has, however, been shown to be a pathogen where environmental conditions are unfavourable to the tree. Likewise, *Cytospora chrysosperma*, a well-known pathogen of poplar (Chapela 1989), was frequently isolated from both healthy and dying tissues of *Populus tremula* from northern Spain (Santamaria and Diez 2005). In another similar study, *Colletotrichum gloeosporioides*, which is pathogenic on orchids in the northeast Argentina (Cabrera *et al*. 2003), was isolated as endophyte from *Taxus mairei* in Taiwan (Wang *et al*. 2008). Some fungi can thus exist as endophytes on one host and potential mutualists on another (Espinosa-Garcia and Langenheim 1990). A significant issue here is that such studies are plagued by taxonomic

issues. For example, what is called *C. gloeosporioides* refers to a complex of species which can be confusing and other examples are treated later. More knowledge is clearly needed on the role of factors such as host genotype, environmental stress and evolutionary changes in order to thoroughly understand the ecological relevance of an endophyte in endophyte-tree associations.

4.3. Infection and transmission of tree endophytes

Fungal endophytes of woody plants are typically non-systemic infections as compared to the systemic infections of grasses. Factors such as tree size, the complex morphology of the tree and time taken for trees to reach maturity probably all limit the potential for systemic growth of endophytes (Saikkonen *et al*. 2004). Tree endophytes are mainly transmitted horizontally (Arnold *et al*. 2003) and tend to show high diversity as compared to single species dominance of endophytes in grasses (Carroll 1991; Petrini 1991). This is attributed to multiple infections caused by different fungal endophytes on a particular part of a host plant (McCutcheon *et al*. 1993). Fungal endophytes on trees also tend to show high levels of specificity to the host genotype they infect or to a particular host tissue of plant part in which they occur (Carroll 1988; Saikkonen 2007). Thus, Ahlmolm *et al*. (2002*a*) proposed that successful infections of host-genotypes by certain endophyte-genotypes rely on co-evolution that started from the time the hosts' fitness was affected by the endophyte. It is clear that more studies on the infection and transmission of these fungal endophytes are needed before we can fully understand the relationship between trees and their endophytes.

5.0. Botryosphaeriaceae as endophytes of trees

Many species belonging to the Botryosphaeriaceae have been reported as endophytes on various tree hosts (Slippers and Wingfield 2007). However, not much is known regarding their ecological role on these tree hosts. Their existence as latent pathogens has been documented worldwide (although mostly in plantation or fruit trees), with no information on the role they might play as mutualists. There is especially little information about their role in natural environments.

5.1. Botryosphaeriaceae as latent pathogens on *Eucalyptus*

Eucalyptus spp. are amongst the most important commercially planted tree crops in many countries around the world. Various fungal pathogens are known to cause diseases on this tree. Among them are species of Botryosphaeriaceae. Species of Botryosphaeriaceae have also been reported as latent pathogens on *Eucalyptus* worldwide (Smith *et al*. 1996*a*; Slippers *et al*. 2004*c*, Mohali *et al*. 2006).

In the native environment of these trees, species of Botryosphaeriaceae (then reported as *B. dothidea*) were isolated as endophytes from leaves, xylem and bark of *E. nitens* in Australia (Fisher *et al*. 1993). Furthermore, *N. eucalypticola* and *N. eucalyptorum* were isolated from native *Eucalyptus* trees in eastern Australia (Slippers *et al*. 2004*c*). More recently, *N. australe* was found to be the most common endophyte species in the leaves of *E. globulus* in plantations in Western Australia and from native *Eucalyptus* spp. in Western Australia (Burgess *et al*. 2005). In addition, *N. parvum*, *D. eucalypti* and *N. macroclavatum* were also isolated as endophytes in the latter study. More recently *Lasiodiplodia rubropurpurea* was isolated from cankers of *Eucalyptus grandis* in Queensland (Burgess *et al*. 2006*a*).

The presence of species of Botryosphaeriaceae as endophytes and pathogens has also been reported from countries where eucalypts are not native. In Venezuela, *Neofusicoccum andinum*, *Pseudofusicoccum stromaticum* and *B. mamane* were isolated from asymptomatic branches and stems of a *Eucalyptus urophylla* x *Eucalyptus grandis* hybrid, as well as from *Acacia* spp. (Mohali *et al*. 2006). In Ethiopia, Congo and Chile, *N. parvum* was found to be the major cause of *Botryosphaeria* stem canker in *Eucalyptus* plantations (Roux *et al*. 2000; Ahumada 2003; Gezahgne *et al*. 2004). In South Africa, members of the genus *Botryosphaeria* were observed on *Eucalyptus* foliage in the 1980's (Crous *et al*. 1989). *Botryosphaeria dothidea*, which was later identified as either *N. parvum* or *N. eucalyptorum*, was regarded to be amongst the most important canker and dieback pathogens of this tree species in the country (Smith *et al*. 1996*a*; Slippers *et al*. 2004*c*; Crous *et al*. 2006).

Eucalyptus spp. are affected by some species of the Botryosphaeriaceae that are also endophytes and cause diseases on various other hosts. Thus, *Lasiodiplodia theobromae* (= *B. rhodina*), a well known latent pathogen, has been reported from more than 500 hosts (Punithalingam 1976). For example, *L. theobromae* was found to be the cause of stem end

rot on mango, and to cause wood discoloration on *Pinus*, *Acacia* and *Eucalyptus* spp. in Venezuela (Johnson *et al*. 1992; Jacobs 2002; Mohali 2005; Burgess *et al*. 2006*a*). Diseases such as reduced germination of seeds, stem canker and dieback in various African countries have been reported to be associated with this fungus on *Pinus* and *Eucalyptus* spp. (Cilliers *et al*. 1993; Roux *et al*. 2001). *Neofusicoccum parvum* (= *B. parva*) is another common fungus known to cause diseases on variety of hosts (Slippers *et al*. 2004*c*; Crous *et al*. 2006). It is known to cause canker and dieback and stem end rots on hosts such as *Eucalyptus*, Avocado (*Persea americana*) and Mango (*Mangifera indica*) in countries like South Africa, Australia, Chile and New Zealand (Jacobs 2002; Hartill and Everett 2002; Ahumada 2003; Slippers *et al*. 2004*c*; Burgess *et al*. 2005). Given the wide host range of some Botryosphaeriaceae, movement of such fungi with *Eucalyptus* germplasm or seeds to new areas could be detrimental to the native plant communities in those areas.

5.2. Taxonomic history of Botryosphaeriaceae endophytes on trees

The Botryosphaeriaceae have been plagued by considerable taxonomic confusion in the past. This confusion has had a significant negative effect on studies of the endophytic nature of the members of Botryosphaeriaceae and thus deserves discussion here. The taxonomic confusion regarding these fungi arose from the fact that their taxonomy initially relied on teleomorph morphology and the host association. This approach proved to be unreliable because the species have overlapping morphological characteristics especially in the case of their teleomorph structures. Furthermore, many species also colonise more than one host and naming new species based on the hosts on which they occur was particularly misleading. In more recent studies, identification of species of the Botryosphaeriaceae has been based predominantly on anamorph morphology which is more frequently observed in nature. Studies combining both DNA sequence data and morphological characteristics has, however, made the greatest contribution to reliable species identification (Jacobs and Rehner 1998; Denman *et al*. 2000; Slippers *et al*. 2004*a*; Crous *et al*. 2006).

The most recent and extensive attempt to resolve the taxonomy of the Botryosphaeriaceae based on DNA sequence data and morphology was by Crous *et al*. (2006), which separated multiple lineages within the Botryosphaeriaceae into distinct genera. These genera include *Diplodia* / *Lasiodiplodia*, *Botryosphaeria*, *Macrophomina*,

Neoscytalidium, *Dothidotthia*, *Botryosphaeria*, *Neofusicoccum*, *Pseudofusicoccum*, "*Botryosphaeria*" *quercuum*, *Saccharata*, *Guignardia*, *Camarosporium* and *Stenocarpella*. *Botryosphaeria dothidea* provides an excellent example of how modern tools have affected studies of Botryosphaeriaceae as endophytes. This fungus was previously thought to be common as an endophyte infecting *Eucalyptus* in South Africa (Smith *et al*. 1996*b*). It was later shown that this identification rather represents *Neofusicoccum parvum*, *N. eucalyptorum* and *N. eucalypticola* (Slippers *et al*. 2004*c*). These species all occur as endophytes and are also associated with canker and dieback of *Eucalyptus* in South Africa (Smith *et al*. 2001; Slippers *et al*. 2004*c*). A number of previously unknown species were also discovered in other countries by combining morphological and molecular data. In Venezuela, for example *N. andinum* and *Pseudofusicoccum stromaticum* were discovered and described from asymptomatic branches and stems of *Eucalyptus* and *Acacia* spp. (Mohali *et al*. 2006). Likewise, in Western Australia, *N. macroclavatum* was described from *Eucalyptus globulus* as an endophyte (Burgess *et al*. 2005).

Many species of the Botryosphaeriaceae known as endophytes of trees other than *Eucalyptus* have also undergone taxonomic changes in recent years. The fungus previously treated as *Diplodia pinea* (= *Sphaeropsis sapinea*) on pine, has been shown to represent a species complex including two forms of *D. pinea* and *D. scrobiculata* (de Wet *et al*. 2003). Furthermore, the Botryosphaeriaceae that had previously been identified as *Dothiorella* or *Natrassia* from mango (Johnson *et al*. 1992) were re-examined and found to represent *N. parvum*, *N. mangiferum*, *F. aesculi* (teleomorph *B. dothidea*) and an undescribed *Neofusicoccum* sp. (Slippers *et al*. 2005). Clearly, wide variety of hosts are affected by members of the Botryosphaeriaceae and a thorough taxonomic resolution of these species will serve as a basis for understanding their biology as endophytes.

Studies based on ITS sequence data, together with morphological characteristics, paved the way towards more consistent identification and more reliable interpretation of the biology of the Botryosphaeriaceae as endophytes (Jacobs and Rehner 1998; Smith *et al*. 2001; Pavlic *et al*. 2004). It is important, however, to note that in the case of closely related species, a single gene region might not be sufficiently variable to distinguish these cryptic species. For this reason, multiple gene sequence data are needed to clearly distinguish between these cryptic species (de Wet *et al*. 2003; Slippers *et al*. 2004*a, b, c*; Burgess *et al*.

2005; Phillips *et al*. 2005, Alves *et al*. 2008). Future studies will need to focus on the correct identification of unrecognized species and cryptic species using tools of appropriate resolution.

Earlier work on the Botryosphaeriaceae as endophytes is confusing as the species names used do not reflect the current nomenclature of these fungi. For example, it is almost impossible to use reports that were made prior to the application of DNA sequence data for the taxonomy of the Botryosphaeriaceae, to understand the specificity, infection and distribution of these fungi as endophytes. The early literature should, therefore, be interpreted with caution or at least with an understanding that the names are probably not accurate.

5.3. Mode of infection, transmission and dispersal

Botryosphaeriaceae infect their hosts through both natural openings and wounds, although wounds were previously thought to be the primary mode of entry into the host for many of these fungi (von Arx and Müller 1954; Schreiber 1964; Punithalingam and Holliday 1973; McGlohon 1982; Michailides 1991; Smith 1995, 2001). Smith (1995) showed *E. grandis* leaf infection by *Neofusicoccum* spp. (identified as *B. dothidea*), where germ tubes from conidia entered through the stomatal openings. The same situation was also observed on leaves, rachises and shoots of pistachio (Michailides 1991). This enables a fungus to be established inside a leaf tissue with no apparent disease symptoms, therefore causing a latent infection.

Species of Botryosphaeriaceae reproduce sexually and asexually in their life cycle through the production of ascospores and conidia, respectively (Sutton 1981). Conidia are more frequently encountered in nature than ascospores (Michailides 1991; personal observations). The conidia are thus expected to be the primary source of inoculum and are transmitted horizontally from one plant to another (Smith 2001). These conidia are mostly wind dispersed or are dispersed through rain splash (Weaver 1974; Michailides and Owaga 1986; Cresswell and Milholland 1988; Pusey 1989). This is consistent with the findings of Stanosz *et al*. (2005) who reported 88 % higher frequency detection of *D. pinea* in seedlings that were near a source of *D. pinea* conidia than those that were distant from it in nurseries.

Germination and infection is strongly influenced by environmental conditions (Swart *et al*. 1987; Ntahimpera *et al*. 2002; Ahimera *et al* 2003). For example, Michailides and Owaga (1986) reported that infections on pistachio caused by *B. dothidea* developed rapidly in the late spring and summer when maximum temperature ranged from 21–40 ºC. Everett and Pak (2002) on the other hand reported that in latent pathogens such as *N. parvum*, which causes fruit rot on avocado, spore germination could occur at low temperatures. Low temperatures were shown to influence rapid proliferation of '*Botryosphaeria' ribis* that invades stems of *Melaleuca quinquenervia* (Rayachhetry *et al*. 1996). There are other factors that also contribute to ascospore and conidial germination of the Botryosphaeriaceae. Relative humidity has thus been shown to influence ascospore and conidia germination of *Diplodia seriata* (= *Botryosphaeria obtusa*) and most spores achieve maximum germination at 95 % RH or higher. If free water and relative humidity decrease, germination is also decreased (Arauz and Sutton 1989). Closer attention needs to be paid to the role of temperature and moisture in influencing the mode of infection, transmission and dispersal of these fungi.

Physical response to Botryosphaeriaceae infection has been documented on hosts such as *Melaleuca quinquenervia* (Rayachhetry *et al*. 1996). Callus cells are formed around the invaded tissue and these limit pathogen invasion. However, there are mechanisms that exist by which the pathogens can overcome host resistance. In the case of *D. pinea*, this is aided by enzymatic activity that degrades the cell walls in the initial phase of pathogenicity (Chou 1978) and this work was done before the fungus was known to exist as an endophyte. However, this topic has been poorly studied and more intensive investigations on the plants' response to Botryosphaeriaceae infections is needed if we are to better understand their mode of infection and how the plants' resistance is overcome by these fungi.

Some species of Botryosphaeriaceae are known to be seed-borne. For example, *D. rosulata* has been isolated from healthy seeds of *Podocarpus falcatus* and *Prunus africana*, while *L. theobromae* and *D. pinea* has been isolated from coniferous seeds (Cilliers *et al*. 1993, 1995; Smith *et al*. 1996*a*; Gure *et al*. 2005). It has been suggested that the seed-borne nature of these fungi has led to the global movement of the Botryosphaeriaceae around the world (Smith *et al*. 2000; Burgess *et al*. 2001, 2004). There is no conclusive evidence that endophytic infections result in systemic infection of the entire plant, but most evidence

points to the plant being continuously re-infected with these endophytes from the environment (Slippers and Wingfield 2007). There is clearly a need for additional studies to address these questions and thus to better understand the infection biology and distribution of species of Botryosphaeriaceae as endophytes.

5.4. Stress as an inducer of disease expression

Environmental conditions are important for disease expression of Botryosphaeriaceae living as latent pathogens. Visual disease symptoms are typically observed as soon as conditions become unfavorable for the host. This probably results in a lowering of the hosts natural defenses, which in turn results in their becoming susceptible to fungal colonization and disease development (Schoeneweiss 1981; Pusey 1989; Old *et al.* 1990; Smith *et al.* 1994; Brown-Rytlewski and McManus 2000). It has also been shown that a decrease in water potential increases colonization of plants by Botryosphaeriaceae (Schoeneweiss 1981). In addition, it has been shown that freezing and defoliation stress are important factors that favour disease development (Schoeneweiss 1981). Death of *E. radiata* trees in species selection trials has been attributed to increased susceptibility resulting from drought or low rainfall (Shearer *et al.* 1987). Likewise, 92 % of dead *Pinus* seedlings with collar rot symptoms, which develop due to water stress, were infected with *D. pinea* (Stanosz *et al.* 2001). The seedlings displayed symptoms of collar rot, which normally develops due to internal water deficit. Similarly ring necrosis that develops under dry conditions has been shown to promote susceptibility to species of Botryosphaeriaceae causing post harvest disease of avocado fruits in New Zealand (Hartill 1991). The damage that these fungi can cause to their hosts under stressful conditions can be quite severe, and is expected to increase with increasing stress on trees due to climate change (Desprez-Loustau *et al.* 2006).

5.5. Disease symptoms

Species of Botryosphaeriaceae have been associated with diseases of many woody plants and economically important agricultural crops (Sutton 1980; Ciesla *et al.* 1996; Old 2000; Old and Davidson 2000). Disease symptoms are often associated with environmental stress resulting from abiotic and biotic factors (Schoeneweiss 1981; Swart and Wingfield 1991; Smith *et al.* 1994; Paoletti *et. al.* 2001). Symptoms associated with the

Botryosphaeriaceae infections are also very variable. They can for example include twig and branch dieback of walnut trees induced by *N. ribis* (Rumbos 1987), root diseases of *Pinus taeda* and *P. eliottii* caused by *B. dothidea* in Hawaii (Hodges 1983), gummosis of peach trees caused by three species of Botryosphaeriaceae (*B. dothidea*, *D. seriata* and *L. theobromae*) (Britton and Hendrix 1989), and bleeding necrosis of sweet gum caused by *N. ribis* in Illinois and India (Neely 1968).

On *Eucalyptus,* disease symptoms associated with the Botryosphaeriaceae have been reported in many countries of the world. Members of this family of fungi are known to cause diseases such as seed capsule abortion and twig dieback of *E. camaldulensis* in South Florida, USA (Webb 1983), twig, branch and upper trunk cankers of *E. marginata* in Western Australia (Davison and Tay 1983), death of *E. radiata* in species selection trials in Australia (Shear *et al*. 1987), basal cankers and coppice failure of *E. grandis* in Florida (Barnard *et al*. 1987). In South Africa, *B. dothidea* (later shown to represent *N. parvum* and *N. eucalyptorum*) has been shown to cause cankers and dieback on this host throughout South Africa (Smith *et al*. 1996*a*; Smith *et al*. 2001; Slippers *et al*. 2004*c*).

6.0. Endophytes and quarantine

Many endophytic fungi are seed-borne or can be present on asymptomatic host material. Their movement around the world is of great concern and they must be considered when developing quarantine strategies (Wingfield *et al*. 2001; Burgess and Wingfield 2002*a*, 2002*b*). As latent pathogens occur in the absence of symptoms for part of their life cycle, they can easily be overlooked. This must clearly facilitate their introduction into new areas. For example, Slippers *et al*. (2004*c*) found *N. eucalyptorum* and *N. eucalypticola* to be the most common species on Eucalypts in eastern Australia. They are probably native to that region. Their occurrence on *Eucalyptus* spp. in South Africa and Chile was possibly due to the introduction of these exotic hosts into these two countries (Smith *et al*. 2001; Ahumada 2003).

Species of Botryosphaeriaceae have the ability to jump hosts and to co-infect native as well as closely related introduced tree species. For example, *N. parvum* has been reported to occur on *Eucalyptus* spp. in many countries around the world, but it is also common on native trees in South Africa (Ahumada 2003; Nakabonge 2002; Gezahgne *et al*. 2004;

Slippers 2003, 2004*c*). Pavlic *et al*. (2007) identified eight species of Botryosphaeriaceae on the native species *Syzygium cordatum* in South Africa and some of these fungi have also been reported to occur on *Eucalyptus* (Slippers *et al*. 2004*a*, *b*).

Neofusicoccum australe was found to commonly occur on different hosts native to Australia, including *Eucalyptus*, but has been isolated from native *Acacia* spp., fruit trees (apple, pear, plum and almond), *Syzygium cordatum* and grapevine in South Africa (Slippers *et al*. 2004*b*; van Niekerk *et al*. 2004; Pavlic *et al*. 2007; Slippers *et al*. 2007). No barriers were detected in the movement of *N. australe* between commercial *E. globulus* plantations and native forests in Western Australia (Burgess *et al*. 2006*b*). Since it is known that introduction of exotics can introduce pathogens into new areas, stringent quarantine measures are needed to control detrimental effect that these pathogens could have on tree hosts, both native and introduced.

7.0. Conclusions

Fungal endophytes can assume many different ecological roles. They can be beneficial (mutualism), exist without being harmful (commensalism) or can be harmful to the hosts at some point in their lifecycle (parasitism). Much of the work done on grasses focused on endophytes as mutualists. More research is clearly is needed to better understand other ecological roles that these fungi might have. Endophyte infected grasses in the natural, undisturbed stands have also been less well studied, and such studies could substantially expand the understanding of the biology of endophytes.

Many reports show that endophytes of trees can become parasitic. These species include members of the family Botryosphaeriaceae that are well known latent pathogens of many woody hosts. However, most studies focused on planted and managed trees or tree crops and there is little information available on the ecological role these fungi play as endophytes in natural ecosystems. The possibility of these species existing as mutualists needs to be further investigated, more so now that evidence of other mutualistic endophytes on woody hosts is slowly emerging.

Factors such as environmental stress clearly contribute to the change from an endophytic lifestyle to a parasitic one. Many agricultural crops and hosts of commercial importance are planted on marginal sites, and increasingly so due to limited space and

climate change. These plants will be under constant attack from latent opportunistic pathogens such as the Botryosphaeriaceae. Future studies should focus on understanding the interaction of these pathogens with their hosts under varying environmental conditions. The mode of reproduction, infection and distribution of these fungi under such conditions can contribute substantially in assessing risk and developing control measures. Resistant cultivars could also be selected based on a better understanding of the tree-fungal-environment interaction.

The existence of fungi such as the Botryosphaeriaceae as latent pathogens, pose a threat to indigenous flora, plantation forestry and agricultural crops, especially because these fungal pathogens often go undetected by quarantine procedures. Rapid identification tools should thus be designed for early detection of these fungal pathogens in order to help address this problem. This process should be based on a fuller understanding of the true diversity of species belonging to the Botryosphaeriaceae. Databases on fungal pathogens should also be frequently up-dated, to keep track of newly emerging latent pathogens with the aim of trying to minimize their spread.

8.0. References

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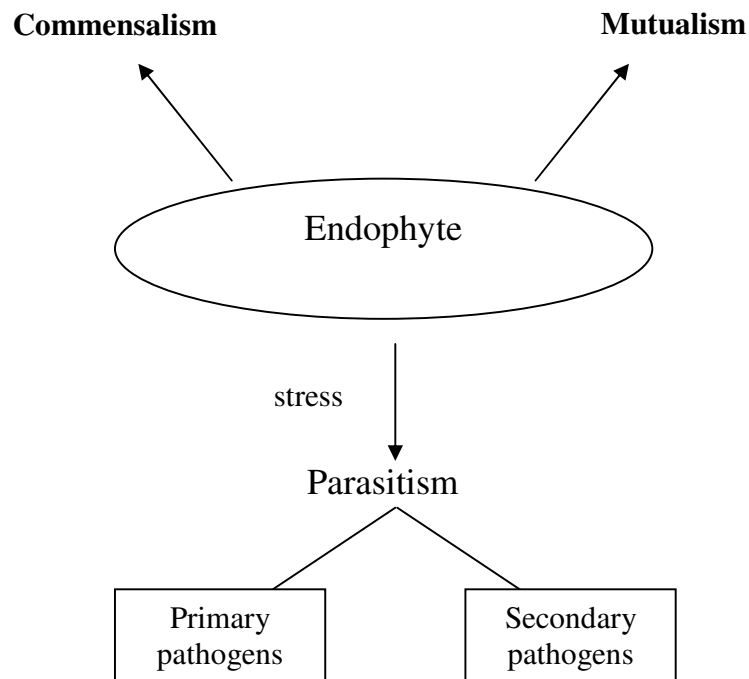**Commensalism**   **Mutualism**

Fig. 1. Different ecological roles that an endophyte can adopt.