CHAPTER 1

DIPLODIA PINEA SENSU LATO AS PART OF THE BOTRYOSPHAERIACEAE AND ASSOCIATED MYCOVIRUSES

1. INTRODUCTION

Most species belonging to the Botryosphaeriaceae are well-known pathogens causing disease symptoms such as die-back and cankers on numerous woody and non-woody hosts (Eldridge 1961; Buchanan 1967; Punithalingam & Waterston 1970). Some of these well-recognized species are *Diplodia pinea* (Desm.) Kickx. (=*Sphaeropsis sapinea* (Fr.) Dyko & Sutton), the conifer pathogen (Swart *et al*. 1985), *Botryosphaeria dothidea* (Moug. Fr.) Ces. & De Not. and *N. eucalyptorum* Crous, H. Smith & M.J. Wingf., pathogens of *Eucalyptus* L'Hér. (Smith *et al*. 1994, 2001) and *D. seriata* De Not. (="*Botryosphaeria*" *obtusa*) and *D. mutila* (Fr.) Mont., fruit tree pathogens (Phillips *et al*. 2007; Slippers *et al*. 2007).

Diplodia pinea is an asexual fungus but is clearly recognized as a species of the Botryosphaeriaceae. Together with other species of *Diplodia* and *Lasiodiplodia*, it forms one of the ten lineages of the Botryosphaeriaceae as described by Crous *et al*. 2006. The taxonomy of the fungus has been complex and confused mainly due to the lack of a clear distinction between *Diplodia* and *Sphaeropsis*. The description of four distinct forms of *D. pinea* (A, B, C and I morphotypes) has furthermore, confused the taxonomic status of this fungus (Wang *et al*. 1985; Palmer *et al*. 1987; Hausner *et al*. 1999; De Wet *et al*. 2000). For the purpose of this chapter, the term *D. pinea sensu lato* has been applied to refer to the A, B and C morphotypes of this fungus. Members of the *D. pinea sensu lato* complex are able to infect various *Pinus* spp. as well as, species of *Larix*, *Cedrus*, *Picea* and *Pseudotsuga* belonging to different sub-families of the Pinaceae (Stanosz *et al*. 1999; Zhou & Stanosz 2001). The different morphotypes of *D. pinea* have different host ranges, but they can overlap. The host association patterns of *D. pinea sensu lato* and other species of the Botryosphaeriaceae might reveal some interesting observations with regards to the driving forces of evolution in this group.

Diplodia pinea can persist in a latent form in healthy pine tissue (Smith *et al*. 1996; Stanosz *et al*. 1997; Flowers *et al*. 2001, 2003) but in association with unfavorable environmental conditions or harsh physical factors, it gives rise to many different disease symptoms (Laughton 1937; Buchanan 1967; Swart *et al*.1987a; Stanosz *et al*. 2001). The most common of these symptoms are die-back, whorl cankers, crown wilt, a root disease and blue stain of timber or logs (Laughton 1937; Da Costa 1955; Gilmour 1964; Punithalingham & Waterston 1970; Wingfield & Knox-Davies 1980a; Chou 1984; Swart & Wingfield 1991b). The morphotypes of *D. pinea sensu lato* differ in their virulence. Isolates of the A morphotype are more virulent than those of the B morphotype (Palmer *et al*. 1987) while the C morphotype is considered to be the most virulent (De Wet *et al*. 2002).

The genetic structure of *D. pinea* populations plays an essential role when considering optimal management and quarantine strategies for the fungus. As an asexual fungus, populations are expected to be almost clonal due to a lack of recombination. The fungus is, however, seed-borne (Rees & Webber 1988; Fraedrich & Miller 1995; Vujanovic *et al*. 2000) and multiple introductions of seed from different sources can result in a complex genetic structure (Burgess *et al*. 2001a). The different morphotypes of *D. pinea* also have different population structures (Burgess *et al*. 2004a, 2004b). Populations of the A morphotype have limited gene and genotypic diversities (Burgess *et al*. 2004a), while those of the B morphotype have high allelic diversity with considerable genetic distance between populations (Burgess *et al*. 2004b).

Current management strategies for *Diplodia*-induced disease symptoms are based on planting of resistant host species and the implementation of optimal management strategies and silvicultural practises (Lückhoff 1964; Swart *et al*. 1985). Chemical control is only viable in nurseries and against blue stain. Therefore, great economic losses due to *D. pinea* infections are still incurred

especially in plantations of non-native pine species in the southern hemisphere (Laughton 1937; Lückhoff 1964; Zwolinski *et al*. 1990a, 1990b). Integration of biological control strategies with conventional management strategies, are emerging as a potentially feasible management of fungal pathogens such as *D. pinea*.

Biological control using dsRNA-mediated hypovirulence has been implemented against the chestnut blight pathogen, *Cryphonectria parasitica* (Murrill) M. E. Barr. (Anagnostakis 1988). Hypovirulence refers to a condition where cytoplasmic determinants such as dsRNA elements that occur naturally in fungi attenuate virulence (Anagnostakis 1988; McCabe & Van Alfen 2002; Nuss 2005). Most dsRNA elements in fungi are, however, latent but their biology and simple genomic structure make them ideal candidates for genetic manipulation.

In the *D. pinea sensu lato* species complex, several dsRNA elements have been isolated (Wu *et al*. 1989; Preisig *et al*. 1998; Steenkamp *et al*. 1998; De Wet *et al*. 2001; Adams *et al*. 2002). Of these two have been characterized and are known as *Sphaeropsis sapinea* RNA virus 1 and 2 (Preisig *et al*. 1998). The characterization of a third dsRNA element mainly associated with the B morphotype of the fungus is addressed as part of this dissertation. Although none of the dsRNA elements in *D. pinea* have been shown to confer hypovirulence (Wu *et al*. 1989; Preisig *et al*. 1998; Steenkamp *et al*. 1998; De Wet *et al*. 2001; Adams *et al*. 2002), the distribution patterns and interaction between multiple infections needs to be assessed and their role ascertained.

This literature review and the chapters of the dissertation that follow it, deal mainly with two issues pertaining to *D. pinea sensu lato*. One relates to the appropriate identification of *D. pinea* morphotypes and other *Diplodia*-like isolates encountered during this study and their phylogenetic relationship with other genera of the Botryosphaeriaceae are considered. The

second subject concerns the identification and characterization of a novel dsRNA element and the distribution of these dsRNA elements in members of the *D. pinea sensu lato* species complex.

2. TAXONOMY OF THE *DIPLODIA PINEA SENSU LATO* SPECIES COMPLEX

2.1 Taxonomic history

In 1842, a pathogen was isolated from *P. sylvestris* L. trees in France and described as *Sphaeria pinea* Desm. by Desmazières (Waterman 1943). In 1867, the fungus was transferred to *Diplodia* by Kickx as *Diplodia pinea* (Desm.) Kickx (Waterman 1943). Some years later, Petrak & Sydow (1927) proposed the name *Macrophoma pinea* (Desm.) Petrak & Syd., as an earlier epithet of *S. pinea*. Petrak (1961), however, found *M. pinea* to be a later homonym of *M. pinea* Pass. (=*Dothiorella pinea* (Pass.) and renamed the fungus *M. sapinea* (Fr.) Petrak. In the CMI descriptions of pathogenic fungi and bacteria, Punithalingam & Waterston (1970) presented *D. pinea* as a synonym of *S. pinea*, *M. pinea* and *M. sapinea*.

Diplodia pinea was transferred to *Sphaeropsis* Sacc. as *S. sapinea* (Sutton 1980) and the relevance of *Macrophoma* for the fungus best known as *D. pinea* was reconsidered. This change in name was supported by the percurrent proliferation of the conidiogenous cells of the fungus that were considered to be more typical of *Sphaeropsis* than of *Diplodia* (Sutton 1980). Phillips (2002), however, showed that percurrent proliferation is also common in the conidiogenous cells of *Diplodia* spp. Sutton (1980) also used septation to separate species of *Sphaeropsis* and *Diplodia*. He considered conidia of *Sphaeropsis* as aseptate with a faint septum developing prior to germination, while his interpretation was that conidia of *Diplodia* become euseptate as they mature. The distinction between *Sphaeropsis* and *Diplodia* was thus, never clearly defined resulting in considerable controversy with regards to the taxonomy of this fungus.

Sphaeropsis and *Diplodia* Fr., as well as, *Fusicoccum* Corda and *Lasiodiplodia* Ellis & Everh. were all considered as anamorph genera of the Botryosphaeriaceae based on morphology. Combining, morphology with DNA sequence data, later resulted in only two anamorph genera being recognized namely *Diplodia* and *Fusicoccum* (Jacobs & Rehner 1998; Denman *et al*. 2000, Zhou & Stanosz 2001). Species with dark, wide conidia were thus shown to reside in the *Diplodia*-group and those with light coloured, narrow conidia in the *Fusicoccum*-group. These two groups were also referred to as section *Brunnea* and section *Hyala* (Zhou & Stanosz 2001). A teleomorph state has never been observed for *S. sapinea* but it has been shown using ribosomal DNA (rDNA) and protein-coding gene sequence data that this fungus consistently groups with *Diplodia*-anamorphs of Botryosphaeriaceae (Jacobs & Rehner 1998; Denman *et al*. 2000; Zhou & Stanosz 2001; Zhou *et al*. 2001). The recommendation was, therefore, made to revert to the name *D. pinea* (Denman *et al*. 2000; Chapter 2 of this thesis).

The two anamorph system proposed for anamorphs of Botryosphaeriaceae (Jacobs & Rehner 1998; Denman *et al*. 2000; Zhou & Stanosz 2001; Zhou *et al*. 2001) was simplistic and clearly not a representation of the natural classification of this group. A recent phylogenetic study has changed the taxonomy of the Botryosphaeriaceae markedly (Crous *et al*. 2006). Ten lineages have been recognized (Fig. 1) namely *Diplodia*/*Lasiodiplodia*/*Tiarosporella* (no designated teleomorph), *Botryosphaeria* (*Fusicoccum* anamorphs), *Macrophomina* (teleomorph unknown), *Neoscytalidium* (teleomorph unknown), *Dothidotthia* (*Dothiorella* anamorphs), *Neofusicoccum* (*Botryosphaeria*-like teleomorphs, *Dichomera*-like synanamorphs), *Pseudofusicoccum* (teleomorph unknown), "*Botryosphaeria*" *quercuum* (*Diplodia*-like anamorph), *Saccharata* (*Fusicoccum*-like teleomorph, *Diplodia*- and *Fusicoccum*-like synanamorphs) and *Guignardia* (*Phyllosticta* anamorphs). In the above mentioned study, the *Diplodia*/*Lasiodiplodia* clade was

unresolved and accommodates all *Diplodia*-like isolates with dark, >10 µm broad, thick-walled conidia. The *Dothiorella* clade also include species with *Diplodia*-like conidia but these conidia are dark and single-septate early in development unlike those of *Diplodia* and *Lasiodiplodia* turning dark and multi-septated over time. The *Botryosphaeria* clade accommodates species like *B. dothidea* that has *Botryosphaeria* teleomorphs and *Fusicoccum*-like anamorphs with light, <10 µm broad and thin-walled conidia and no synanamorphs. The *Neofusicoccum* clade also accommodates species with *Botryosphaeria*-like teleomorphs but has *Fusicoccum*-like anamorphs and *Dichomera*-like synanamorphs. The *Pseudofusicoccum* clade accommodates the *Fusicoccum*-like anamorphs with unusually large conidiomata, conidia that are thick-walled and surrounded by a mucous sheath. The other clades all have their unique features that easily distinguish them from the rest (Crous *et al*. 2006). The Botryosphaeriaceae was consequently re-classified under a new order the Botryosphaeriales (Schoch *et al*. 2006), better suited for this group than the Dothideales (Miller 1928; VonArx & Müller 1975). The *D. pinea sensu lato* species complex is well placed in this group.

2.2 Taxonomy of *Diplodia pinea* and its morphotypes

Diplodia pinea is an asexual fungus belonging to the Coelomycetes. Asexual spores or conidia are produced in pycnidia (Haddow & Newman 1942; Waterman 1943). Conidia are oblong to clavate with blunt basal ends, rounded apices and develop monoblastically via percurrent proliferation of the conidiogenous cells (Sutton 1980). A range of conidial sizes, 30-45 x 10-16 µm, have been reported for *D. pinea* (Punithalingam & Waterston 1970; Sutton 1980). The huge range of conidial sizes is apparently influenced by the age of the conidia and the existence of different morphotypes for *D. pinea*. Juvenile conidia are hyaline, thick-walled and non-septate, while mature conidia are dark brown with up to three septa. Spermatia and spermatiophores

have been reported in the fungus (Wingfield & Knox-Davies 1980b). They are hyaline, independent of age, and smaller than conidia. It could be an indication of *D. pinea* having a reduced or lost sexual state.

Four morphotypes have been described for *D. pinea*. These have been defined based on morphological characteristics, particularly of the conidia and cultural characteristics. These four morphotypes have been referred to as the A, B, C and I morphotypes (Wang *et al*. 1985; Palmer *et al*. 1987; Hausner *et al*. 1999; De Wet *et al*. 2000, 2002). Isolates of the A morphotype are characterized by fluffy, aerial mycelium, conidia (34 µm x 16 µm) longer than those of the B morphotype but shorter than those of the C morphotype, smooth conidial walls and usually no or only one septum (Wang *et al*. 1985, 1986; Palmer *et al*. 1987). Isolates of the B morphotype are characterized by appressed mycelium, growing close to the surface or in the agar, conidia (32 µm x 15 µm) shorter than those of the other morphotypes, pitted conidial walls and up to three septa (Wang *et al*. 1985, 1986; Palmer *et al*. 1987). Isolates of the C morphotype are closely related to those of the A morphotype also having fluffy, aerial mycelium, smooth conidal walls, none or only one septum but with conidia (37 µm x 15 µm) longer than both those of the A and B morphotypes (De Wet *et al*. 2000, 2002). Isolates of the I morphotype were described having characteristics of both the A and B morphotypes with neither fluffy nor appressed mycelium and smooth or pitted conidial walls (Hausner *et al*. 1999).

Various techniques have been used to authenticate the existence of the morphotypes of *D. pinea*. Isozyme profiles were initially used to distinguish between the A and B morphotypes of the fungus (Palmer *et al*. 1987; Swart *et al*. 1991). Random amplified polymorphic markers (RAPDs) were later applied to this question and resulted in the distinction of the A, B and C morphotypes (Smith & Stanosz 1995; Stanosz *et al*. 1996; De Wet *et al*. 2000). Restriction

fragment length polymorphisms (RFLPs) resulted in distinction of the A, B and I morphotypes (Hausner *et al*. 1999). More recently, short sequence repeats (SSRs) have made it possible to distinguish among the A, B and C morphotypes of *D. pinea* and have shown that isolates of the I morphotype represent the anamorph of "*Botryosphaeria*" *obtusa* (d.i. *D. seriata*) (Burgess *et al*. 2001b). The above mentioned techniques could distinguish between the morphotypes of *D. pinea* only after pure cultures were obtained.

Overcoming the constraints of first having to produce pure *Diplodia* cultures, followed by DNA extractions and DNA sequence comparisons to determine the identity of the *Diplodia* morphotype or closely related species have resulted in the development of various rapid identification assays. Fingerprinting profiles generated through MSP-PCR (microsatellite-primed polymerase chain reaction) and rep-PCR (repetitive-sequence-based polymerase chain reaction) are able to distinguish between the morphotypes of *Diplodia*, as well as, 25 other species belonging to the Botryosphaeriaceae (Alves *et al*. 2007). Some other techniques for the quick differentiation of morphologically similar species of the Botryosphaeriaceae include ISSR (inter simple or short sequence repeats fingerprinting (Zhou *et al*. 2001) and ARDRA (amplified ribosomal DNA restriction analysis) (Alves *et al*. 2005).

As part of this dissertation, isolates representing the B morphotype of *D. pinea* were shown to represent a distinct taxon, which has been provided the name *Diplodia scrobiculata* J. de Wet, Slippers & M.J. Wingf. This review precedes the scientific study and thus refers to the fungus as the B morphotype of the *D. pinea sensu lato* species complex.

3. PATHOGEN BIOLOGY

3.1 Distribution and host range

Diplodia pinea sensu lato has a worldwide distribution and a host range that includes various *Pinus* spp. and some other conifers (Fisher 1912; Waterman 1943; Eldridge 1961; Buchanan 1967; Punithalingam & Waterston 1970; Gibson 1979; Swart *et al*. 1985; Stanosz *et al*. 1996, 1999). Isolates of the different morphotypes of the fungus differ in their host specificity and distribution (Wang *et al*. 1985; Smith & Stanosz 1995). The A morphotype of *D. pinea* is the most common form, occurring in all conifer-growing regions of the world and on a wide range of *Pinus* and other conifer species (Stanosz *et al*. 1999). Until recently, isolates of the B morphotype were thought to be restricted to *P. banksiana* Lamb. and *P. resinosa* Sol. ex Aiton in the north central United States (Palmer *et al*. 1987), but have since been reported from other hosts and from Europe (Smith & Stanosz 1995; Stanosz *et al*. 1999; Zhou & Stanosz 2001). The C morphotype of *D. pinea*, has been reported only from Indonesia on *P. patula* Schiede ex Schlthdl. & Cham. (De Wet *et al*. 2000). This type appears not to have moved out of South East Asia and extensive sampling worldwide make it reasonably certain to say it does not occur in other pine-growing countries.

3.2 Disease symptoms

Diplodia pinea is an opportunistic pathogen and the most common disease symptoms associated with it are die-back and shoot blight (Fisher 1912; Nicholls 1977; Peterson 1977), whorl cankers and crown wilt (Gilmour 1964; Marks & Minko 1969; Nicholls 1977; Chou 1984, 1987; Swart *et al*. 1985; Palmer 1991), collar rot (Punithalingham & Waterston 1970; Swart *et al*. 1985), a root disease (Wingfield & Knox-Davies 1980a) and blue stain (Laughton 1937; Da Costa 1955; Eldridge 1961). Disease symptoms normally appear four to ten days after initiation of infection

providing that conditions are favorable for germination of conidia and proliferation of the fungus (Brookhouser & Peterson 1971; Peterson 1977; Chou 1984; Swart *et al*. 1987a, 1987b).

3.2.1 Die-back and shoot blight

Die-back and shoot blight due to *D. pinea* infection result in the loss of normal growth of terminal shoots. The terminal shoots become brittle, stunted and resin is exuded (Fisher 1912; Eldridge 1961; Nicholls 1977; Peterson 1977). The needles turn brown and are shed. Internally, the woody tissue discolors, pycnidia are formed and the tissue becomes necrotic (Swart *et al*. 1985).

3.2.2 Whorl cankers and crown wilt

Diplodia pinea-induced cankers reduce the normal growth of trees (Nicholls 1977). Cankers are elongated, depressed areas at the whorl of the tree as a result of the cambium and cortical tissue being infected by the fungus (Gilmour 1964; Marks & Minko 1969; Swart *et al*. 1985). In severe cases, cankers can girdle the trees, causing mortality or crown wilt where only a portion of the crown is killed (Chou 1984, 1987).

3.2.3 Collar rot and a root disease

Diplodia pinea causes collar rot in pine seedlings (Waterman 1943; Punithalingam & Waterston 1970). The root collar area discolors followed by foliage chlorosis and resin exudation (Swart *et al*. 1985). Wingfield & Knox-Davies (1980a) also reported a root disease on *P. elliottii* Engelm. and *P. taeda* L. as a result of *D. pinea* infection. The lateral roots of infected trees have dark blue, resinous lesions on the radial parts that can extend to a height of two meters up the tree trunks.

3.2.4 Blue stain

Blue stain normally refers to the discoloration of the sapwood due to dark pigmented sap-staining fungi growing in the ray parenchyma. It is a cosmetic, non-degrading defect that results only in the devaluation of timber. Blue stain of pine logs and timber is not only due to *D. pinea* but rather a combination of fungi that can also include *Lasiodiplodia theobromae* (Pat.) Griffon & Maubl., *D. mutila* as well as, several *Ophiostoma* and *Ceratocystis* species (Laughton 1937; Da Costa 1955; Eldridge 1961; Butcher 1968; Swart & Wingfield 1991b; Seifert 1993; Mohali & Encinas 2001). Sometimes the undesirable staining of the sapwood is not only due to the mycelium of these blue stain fungi but the result of host cells reacting to metabolites produced by the fungi as was reported by Butcher (1968) for *Ceratocystis piceae* (Munch) Bakshi. and *Phialophora fastigiata* (Lagerb. and Melin) in red beech, or the production of pigments. Saprophytic *D. pinea* and *L. theobromae* infections can occur through bark abrasions caused during felling, bark butts after pruning or exposed ends of cut logs (Laughton 1937; Eldridge 1961; Gilmour 1964; Lückhoff 1964; Marks & Minko 1969; Peterson 1977; Swart *et al*. 1985; Swart & Wingfield 1991b; Zwolinski *et al*. 1995). *Ophiostoma* and *Ceratocystis* species on the other hand, are insect-vectored, mainly by bark beetles (Harrington 1993; Siefert 1993; Paine *et al*. 1997). Blue stain-associated fungi utilize only the extractives of the tree as carbon source while lignified walls and structural carbohydrates remain intact leaving the strength of the wood unaffected (Da Costa 1955; Eldridge 1961; Schirp *et al*. 2003).

3.3 Spore development, dispersal and infection

Pycnidia containing *D. pinea* conidia are formed on dead needles, bark, scales of two-year-old pine cones and the forest litter (Laughton 1937; Waterman 1943; Peterson 1981). Mature conidia are dispersed through water (Eldridge 1961; Brookhouser & Peterson 1971) or wind if

long distance dispersal is necessary (Swart *et al*. 1985). Conidia germinate and enter the host through wounds or stomata (Brookhouser & Peterson 1971), or through direct penetration of the epidermis of non-lignified shoots (Chou 1978). Hyphal aggregates in crevices at the needles bases and on the surfaces of the needles and bud scales, furthermore suggest that infection can potentially originate there (Waterman 1943; Rees & Webber 1988; Flowers *et al*. 2006). Mycelium grows into the mesophyll from which it colonizes and spreads to the phloem and cortical tissue of the host (Laughton 1937). Latent *D. pinea* infections are localized in the outer stem cortex, while pathogenic infections occur throughout the shoot stem tissue (Flowers *et al*. 2006). The normal cambial function of the host is disrupted leading to bark and cambial necrosis, discoloration of the needles, girdling of the shoots and shedding of the needles (Laughton 1937; Marks & Minko 1969; Brookhouser & Peterson 1971; Brown *et al*. 1981). Infection levels are high during the active elongation phase of the shoots and when environmental conditions are favorable for spore germination and penetration. This is especially true when the host is more susceptible either genetically or as a result of stress conditions (Millikan & Anderson 1957; Brookhouser & Peterson 1971; Minko & Marks 1973; Chou 1978, 1982). Optimal environmental conditions for infection include temperatures between 24 ° and 30 ºC and a relative humidity higher than 90 % (Brookhouser & Peterson 1971; Chou 1982). Infection is typically more severe in actively growing trees and it decreases with tree age (Laughton 1937; Marks & Minko 1969; Chou 1977, 1982). This is probably due to the change in the microenvironment and nutrition of the tree as it increases in size and complexity, making it less suitable for infection by *D. pinea* (Chou 1977). In older hail-damaged trees, infection was however, more severe than in younger trees (Smith *et al*. 2002a). A possible explanation for this is the presence more seed cones on older trees already containing latent *D. pinea*, which is able

to initiate a pathogenic infection with the onset of stress through hail (Smith *et al*. 2002a). Altitude also plays a role in infection. *Diplodia pinea* infections are less in higher altitudes as the microenvironment necessary for infection is less suitable than in lower altitudes (Chou 1977; Zwolinski *et al*. 1990b).

3.4 Wounding, stress, virulence and host susceptibility

For many years, it was assumed that *D. pinea*, especially the B morphotype, required wounds for infection (Wang *et al*. 1985; Palmer *et al*. 1987). These wounds would typically originate during pruning, hail damage or through insect feeding (Laughton 1937; Gilmour 1964; Lückhoff 1964; Marks & Minko 1969; Wright & Marks 1970; Peterson 1977; Swart *et al*. 1985; Zwolinski *et al*. 1995). This view has changed with various reports of both morphotypes of the fungus being able to infect unwounded stems and leaves through direct penetration of the stomatal pits (Waterman 1943; Brookhouser & Peterson 1971; Palmer 1991; Blodgett & Stanosz 1997a). *Diplodia pinea* has also been shown to persist in healthy, asymptomatic host tissue and mature, unopened seed cones in a latent state (Smith *et al*. 1996; Stanosz *et al*. 1997; Flowers *et al*. 2001, 2003). Stanosz *et al*. 1997 demonstrated that both the A and B morphotypes of *D. pinea* isolated from asymptomatic shoots of *P. resinosa* and *P. banksiana* were able to develop characteristic Diplodia die-back symptoms when artificially inoculated.

Diplodia pinea sensu lato, like most species of the Botryosphaeriaceae, occur as latent infections in healthy tissue (Slippers & Wingfield 2007). One of the major obstacles in dealing with these latent infections is the lack of ability to detect them easily and to distinguish them from other epiphytic infections. Various quick assays have therefore, been developed to detect latent infections directly from asymptomatic host tissue. Flowers *et al*. (2003) developed a nested PCR using nuclear rDNA ITS primers to detect the presence of latent *D. pinea* and *B. obtusa*

infections in pine tissue but without being able to differentiate between these closely related species and the morphotypes of *D. pinea*. This was followed by a Real-time quantitative PCR assay based on the small ribosomal subunit able to rapidly detect and quantify *D. pinea* infections in inoculated *P. nigra* Arnold shoots (Luchi *et al*. 2005), as well as asymptomatic *P. nigra* shoots (Maresi *et al*. 2007). These assays were also unable to distinguish between the morphotypes of the fungus. A species-specific PCR assay, based on polymorphisms in the mitochondrial small subunit ribosome gene (mtSSU rDNA), has since been developed that is able to differentiate between the A and B morphotypes of *D. pinea* and *D. seriata* directly from dead red and jack pine tissue (Smith & Stanosz 2006).

Latent *D. pinea sensu lato* infections are hypothesized to be a survival mechanism of the fungus awaiting opportunity to overcome host defense responses and cause visible disease symptoms (Stanosz *et al*. 1997; Flowers *et al*. 2006). The onset of host stress as a result of adverse environmental or physical factors initiates pathogenic *D. pinea* infections (Laughton 1937; Waterman 1943; Buchanan 1967; Minko & Marks 1973; Brown *et al*. 1981; Swart *et al*. 1987a; Stanosz *et al*. 2001). Hail, drought, overstocking, poor site conditions and nutrient deficiencies are general predisposing factors (Laughton 1937; Lückhoff 1964; Wright & Marks 1970; Minko & Marks 1973; Chou 1977, 1982; Bega *et al*. 1978; Brown *et al*. 1981; Bachi & Peterson 1985; Stanosz *et al*. 2001). Maresi *et al*. (2007) demonstrated how water stress can potentially be a trigger that enables the fungus to switch from a latent phase to that of a more active pathogenic phase when they found a positive correlation between the presence of *D. pinea* and the normalized insolation index. The normalized insolation index is a measure of the amount of heat at a point that ultimately is an indication of water stress at a specific site. These predisposing factors decrease the rate of the host defense responses and consequently the growth of the

pathogen increases as a result of a larger carbohydrate pool available to it (Schoeneweiss 1981; Bachi & Peterson 1985).

Hail damage followed by *D. pinea*-induced die-back is a serious problem in South Africa resulting in huge economic losses (Zwolinski *et al*. 1990a, 1990b). The highest degree of mortality occurs four months after a hailstorm and can last for up to a year, after which regeneration of foliage normally occurs (Zwolinski *et al*. 1990b). Smith *et al*. (2002a) mapped the colonization of *D. pinea* in hail-damaged *P. patula* trees from the cone pith, through the stipe (connection between the cone and the branch), the branch and finally into the branch pith. In undamaged *P. patula* trees no discoloration due to *D. pinea* was found in the branch pith but *D. pinea* was present latently in the cone pith and in the stem.

Insects are commonly associated with *D. pinea* infection. They play a role in facilitating the colonization of healthy cambial tissue and thus, enhance the severity and impact of the infection rather than playing a role in the dissemination of the fungus (Haddow & Newman 1942; Wingfield & Palmer 1983; Zwolinski *et al*. 1995). Examples of insects that have been associated with *D. pinea* are the pine spittle bug (*Aphrophora parallela* Say) (Haddow & Newman 1942; Waterman 1943), the pitch nodule moth (*Petrova albicapitana* Busk) (Hunt 1969), the deodar weevil (*Pissodes nemorensis* Germar), the bark beetle (*Orthomicus erosus* Woll.) (Wingfield & Palmer 1983; Zwolinski *et al*., 1995), the cone bug (*Gastrodes grossipes* De Geer) (Feci *et al*. 2002) and the pine shoot moth (*Dioryctria* sp.) (Feci *et al*. 2003). Zwolinski *et al*. (1995) made interesting observations regarding the association of *P. nemorensis* and *O. erosus* with post-hail associated *Diplodia* infections in South Africa. *Orthomicus erosus* is found only on post-hail *Diplodia*-infected trees while *P. nemorensis* can occur on healthy trees but exacerbates the spread of the fungus in post-hail *Diplodia*-infected trees.

Wounding and adverse environmental conditions, together with differences in host susceptibility and virulence of the pathogen play a role in *D. pinea* infections (Burdon *et al*. 1980; Palmer *et al*. 1987; Zwolinski *et al*. 1990b). Generally, *Pinus* species of the subgenus *Haploxylon* (white or soft pines, lacebark and foxtail pines) are less susceptible than those of the subgenus *Diploxylon* (yellow or hard pines) (Vujanovic *et al*. 2000). In the north hemisphere, *P. nigra* and *P. mugo* Turra seed cones were found to be more susceptible to *D. pinea* infection than *P. sylvestris*, and *P. resinosa* was the most tolerant species (Vujanovic *et al*. 2000). In South Africa, *P. radiata* D. Don was found to be the most susceptible species followed by *P. pinaster* Aiton (Swart *et al*. 1985). More resistant species are *P. taeda*, *P. elliottii* and *P. patula*, with the latter being more susceptible than the former two species (Swart *et al*. 1985). The northern *P. greggii* Engelm. ex Parl. provenance (*P. greggii* var. *greggii*) was also reported to be significantly more resistant to *D. pinea* than the southern provenance (*P. greggii* var. *australis*), even after hail damage (Smith *et al*. 2002b). These differences observed in host susceptibility are hypothesized to be the result of secondary metabolites like monoterpenes and phenolic compounds that have a fungistatic effect on *D. pinea* (Chou & Zabkiewicz 1976; Brown *et al*. 1981; Chou 1981; Blodgett & Stanosz 1997b).

The morphotypes of *D. pinea* differ in virulence. Isolates of the A morphotype are more virulent as those of the B. morphotype (Palmer *et al*. 1987; Palmer 1991; Blodgett & Stanosz 1997a). Interestingly, the C morphotype, which is known only from Northern Sumatra, has been shown to be the most virulent form of the fungus (De Wet *et al*. 2002). The differences in virulence observed for the A and B morphotypes was linked to the defense chemistry of the host (Blodgett & Stanosz 1997b). The phenolic extracts of *P. resinosa* reduced mycelial growth of the B

morphotype, resulting in a weak, localized infection, while the growth of the A morphotype was unaffected by these phenolics resulting in more aggressive infection that spread quickly.

4. POPULATION GENETICS

The genetic structure of *D. pinea sensu lato* populations is relevant to the management and quarantine of Diplodia die-back and other *Diplodia*-associated diseases. A pathogen population with a highly diverse genetic structure can more easily adapt and overcome resistance. Because *D. pinea* is an endophyte (Smith *et al*. 1996; Stanosz *et al*. 1997; Burgess *et al*. 2001a; Flowers *et al*. 2001) and found on pine seed collected from cones in seed orchards (Peterson 1977; Fraedrich & Miller 1995; Vujanovic *et al*. 2000), it is fair to assume that wherever pine seed or seedlings have been introduced the fungus is likely to have been introduced with it.

In a study conducted by Smith *et al*. (2000), genotypic diversity of two *D. pinea* populations was assessed using vegetative compatibility groups (VCGs). They found the genotypic diversity of an introduced South African population to be unexpectedly higher than that of a native Indonesian population (Smith *et al*. 2000). In a subsequent study, simple sequence repeats (SSRs), were used to determine the genotypic diversity of four *Diplodia* populations from native and introduced *P. radiata* (Burgess *et al*. 2001a). The same trend was observed with higher genotypic diversities for the introduced South African, New Zealand and Australian populations, with those of South Africa being the highest, followed by New Zealand and Australia compared to a native Californian population (Burgess *et al*. 2001a).

With *D. pinea* being an asexually reproducing fungus, populations would be expected to be almost clonal with very low genotypic diversities. In the absence of sexual recombination and selective pressure, the assumption was made that the observed genotypic diversity reflects the number of introductions of the fungus into a region (Burgess *et al*. 2001a; Burgess & Wingfield

2002). Therefore, the high genotypic diversity observed for the introduced *D. pinea* populations is accounted for by multiple introductions of the fungus together with pine seed into the southern hemisphere (Smith *et al*. 2000; Burgess *et al*. 2001a; Burgess & Wingfield 2002). The much higher genotypic diversity calculated for the South African population was linked to the fact that afforestation in South African started about 100 years before Australia and New Zealand and that there has been little control on the importation of seed into the country (Burgess *et al*. 2001a; Burgess & Wingfield 2002). In contrast, Australia and New Zealand have strict quarantine regulations that significantly restrict the introduction of pine seed into those countries (Burgess *et al*. 2001a; Burgess & Wingfield 2002).

In the previous two studies, the genetic diversity of *D. pinea* populations were determined but the existence of *D. pinea sensu lato* as representing two different morphotypes and potential cryptic speciation were not considered. As previously discussed, the morphotypes of *D. pinea* differ with regards to their taxonomy, biology and virulence. Populations of the two morphotypes also have different genetic structures. The A morphotype or *D. pinea sensu stricto* is the main species associated with most *Pinus* spp. outside their native range (Burgess *et al*. 2004a). While the B morphotype is almost exclusively associated with *P. radiata* in its native range (Burgess *et al*. 2004b). The *D. pinea sensu stricto* populations have very low gene diversities, little population differentiation and share multilocus genotypes between populations on different continents (Burgess *et al*. 2004a). This suggests a long asexual history and constant selection pressure as selection is linked to the success of the endophyte. *D. pinea sensu stricto* populations are thus highly unlikely to overcome host resistance and breeding for resistance in the host will be a durable option. In contrast, populations of the B morphotype have high allelic diversity and no multilocus genotypes are shared between populations. The huge genetic

distance between populations with limited gene flow suggests a recent history of recombination and/or mutation as well as the presence of a cryptic sexual stage (Burgess *et al*. 2004b).

5. DISEASE MANAGEMENT

5.1 Conventional disease management

Management of *Diplodia*-associated diseases has most commonly been based on planting resistant pine species in combination with effective silvicultural practices (Lückhoff 1964; Wright & Marks 1970; Brookhouser & Peterson 1971; Peterson 1977; Gibson 1979; Swart *et al*. 1985; Swart & Wingfield 1991b). Although, these management strategies are implemented vigorously, substantial losses due to *D. pinea* infections are still experienced, especially in plantations of non-native *Pinus* spp. (Laughton 1937; Lückhoff 1964; Chou 1976; Zwolinski *et al*. 1990a). In South Africa, hybridization of *P. patula*, the most widely planted species, with *P. greggii* var. *greggii* for its drought tolerance, altitude adaption and *D. pinea* resistance even after hail damage, has been proposed as a robust solution to post-hail associated Diplodia die-back (Smith *et al*. 2002b). Breeding for *Diplodia* resistance in *Pinus* spp. under variable environmental conditions is, however a slow process and it is far from implementation in commercial forestry plantations.

In the meantime, management of *Diplodia*-induced diseases is being achieved by replacing susceptible *Pinus* spp. with more resistant species, especially in areas likely to be favorable for the initiation and spread of infection by this fungus (Lückhoff 1964; Wright & Marks 1970; Gibson 1979; Burdon *et al*. 1980; Palmer & Nicholls 1983). In South Africa, the very susceptible *P. radiata* is restricted to the winter rainfall areas where hailstorms are rare (Swart *et al*. 1987a, 1987b, 1988). The highly susceptible *P. patula* has been replaced by *P. elliottii* in hail sensitive areas of the summer rainfall region (Swart *et al*. 1987a, 1987b, 1988). There have

however, been reports of the tolerant *P. elliottii* experiencing post-hail associated Diplodia die-back and its replacement with *P. greggii* var. *greggii* might prove more resistance under those particular stress conditions (Smith *et al*. 2002b).

Appropriate silvicultural practices and sanitation are essential in managing *D. pinea*-associated diseases. Pruning must be done carefully to prevent wounds and should be scheduled for times when the density of fungal inoculum is low and environmental conditions are unfavorable for the dispersal and germination of the conidia (Gilmour 1964; Palmer & Nicholls 1983; Swart *et al*. 1985; Swart & Wingfield 1991a). The inoculum source can be reduced by removing slash after thinning and pruning ((Nicholls 1977; Bega *et al*. 1978; Gibson 1979). Premature thinning also reduces *D. pinea* infection as it lowers the atmospheric humidity and competition for water and nutrients is less (Bega *et al*. 1978; Gibson 1979). In nurseries, good sanitation practices are essential in reducing *D. pinea* infections (Nicholls 1977).

Fertilization has a profound influence on the incidence and severity of diseases. Generally, the application of fertilizers is believed to alleviate physiological stress on the host, ensuring overall well-being and vigorous growth. In the case of *D. pinea*, fertilization does not always lower the impact of the pathogen (Blodgett *et al*. 2005). The incidence of *D. pinea* was found to increase in areas with high rates of atmospheric ammonium deposition or when treated with fertilizers (De Kam *et al*. 1991; Blodgett *et al*. 2005). Blodgett *et al*. (2005) found that chemicals such as lignin and soluble phenolics, previously reported to play a role in host defense (Chou & Zabkiewicz 1976; Brown *et al*. 1981; Chou 1981; Blodgett & Stanosz 1997b), decrease when fertilizers were applied. The increased susceptibility of the host to *D. pinea* infections was thus hypothesized to be the result of a more suitable growth environment for the fungus or a tradeoff between growth and defense in the host.

Chemical control of *D. pinea* in commercial plantation forestry is not practical but has been useful in nurseries and in the case of small ornamentals like Christmas trees (Palmer & Nicholls 1983). Fungicides shown to be efficient in controlling *D. pinea* outbreaks usually have Benomyl (=Benlate) or thiophanate-methyl as active ingredient (Palmer & Nicholls 1983; Palmer *et al*. 1986; Stanosz & Smith 1996). These fungicides belong to the chemical family benzimidazoles. Control of blue stain is difficult as a combination of fungi is responsible for the unwanted discoloration of the sapwood and it is aggravated by storage conditions conducive to fungal growth. In the past, logs were forced-air dried to lower the moisture content necessary for fungal growth, followed by chemical dips like sodium azide and sodium petachlorophenate (Butcher 1968). These chemical dips are enzyme inhibitors that inhibit fungal metabolism. More recently, antisapstain chemicals that have been applied to exposed surfaces of felled logs through spraying or dipping are copper-8-quinolate and didecyldimethyl ammonium chloride (DDAC) (Thwaites *et al*. 2004). These chemicals form a protective layer on the exposed surfaces preventing fungal spores from germination and penetration for up to 10 weeks (Thwaites *et al*. 2004). No protection is however, provided against fungi that have already penetrated the wood or against chemical-tolerant sapstain fungi. A more successful antisapstain chemical that enables protecting against a broad spectrum of blue stain fungi for longer periods is a combination of two fungicides, registered under the name Sentry® (Wakeling *et al*. 2000). This is a solubilised concentration of methylenebisthiocyanate (MBT) and 2-*n*-octyl-4-isothiolin-3-one (OIA) formulated to form a micro-emulsion. An integrated approach, combining chemical control with biological control agents, has however been proposed as the best method for combating the undesirable effect of blue stain fungi (Behrendt *et al*. 1995b).

5.2 Biological control

Biological control is based on the ability of naturally occurring microorganisms that inhibit the growth or metabolic activity of pathogenic microorganisms (Cook 1993; Duffy *et al*. 2003; Howell 2003). Mechanisms of biocontrol can include antibiosis, competition, mycoparasitism, induction of defense responses in plants or hypovirulence (Day *et al*. 1977; Cook 1993; Duffy *et al*. 2003; Howell 2003). Biological control has environmental advantages over chemical control as it is safe and there is a reduced likelihood of the pathogen overcoming the control due to resistance (Duffy *et al*. 2003).

Biological control of blue stain fungi, mainly *D. pinea* and *Ophiostoma* spp., has been extensively studied and in combination with antisapstain fungicides proved to be highly effective (Behrendt *et al*. 1995b). Two methods of biocontrol can be employed. The first is based on inhibiting the growth of blue stain fungi by another fungus e.g. *Trichoderma* spp. or *Trichothecium roseum* (Vanneste *et al*. 2002). The second method is based on inhibition of blue stain fungi by secondary metabolites produced by plants or microorganisms. Oxygenated monoterpenes such as oxygenated alcohol or phenolic monoterpenes are secondary metabolites produced by the tree that are able to inhibit blue stain fungi preventing the unwanted discoloration of sapwood for up to nine months (Vanneste *et al*. 2002). Pine oil derivates containing oxygenated monoterpenes is being developed for commercial use for the treatment of wood and wood products against blue stain fungi.

Several biocontrol agents specifically against blue stain-associated *Ophiostoma* species have been reported (Behrendt *et al*. 1995a) and two US patents have been registered (Patent no. 5096824; 5518921). These are based on Cartapip-97®, a commercially available formulation of a non-pigmented strain of *O. piliferum* (Behrendt *et al*. 1995a). The non-pigmented *O. piliferum*

strain competes with the blue stain-associated *Ophiostoma* spp. reducing the impact of the blue stain. Another biocontrol agent is *Phlebiopsis gigantea*, a white rot fungus that is able to parasitize on blue stain-associated *Ophiostoma* spp. reducing the undesirable blue stain (Behrendt & Blanchette 1997).

Biological control against *Diplodia*-induced disease symptoms other than blue stain is a relatively unexplored area. In this review, the focus is therefore, on biological control using fungal viruses as they have been studied in *D. pinea* in the past and they form a part of the research that makes up this dissertation.

5.2.1 Virus-like particles in fungi

Virus-like particles (VLPs) associated with fungi were first isolated in 1950 from commercially produced mushrooms (*Agaricus bisporus*) associated with La France disease (Hollings 1962). These VLPs are normally associated with the cytoplasm of their hosts, have dsRNA genomes and are commonly known as mycoviruses (Buck 1986). VLPs associated with the mitochondria are referred to as mitoviruses (Polashock & Hillman 1994; Hong *et al*. 1999). The genetic composition of these viruses is very basic and therefore, cellular factors of the hosts sometimes play an important role in their transcription and replication (Lai 1998). Multiple infections with different viruses are common in all major classes of fungi (Hollings & Stone 1971; Barton & Hollings 1979; Buck 1986).

Mycoviruses are classified into virus families based on their nucleic acid composition. Those with dsRNA genomes, constituting the majority of mycoviruses thus far discovered, belong to the *Hypoviridae* (Nuss *et al*. 2005), *Totiviridae* (Wickner *et al*. 2005), *Partitiviridae* (Ghabrial *et al*. 2005), *Chrysoviridae* (Ghabrial & Castón 2005) and the genus *Mycoreovirus* (Mertens *et al*. 2005). Mycoviruses with ssRNA genomes belong to the *Barnaviridae* or *Narnaviridae* (Buck *et*

al. 2005). Mitoviruses that are associated with the mitochondria are classified in the genus *Mitovirus* belonging to the *Narnaviridae*. Mycoviruses with reverse transcribed RNA genomes belong to the *Metaviridae* (Boeke *et al*. 2005b) and *Pseudoviridae* (Boeke *et al*. 2005a) and those with dsDNA genomes to the genus *Rhizidiovirus* (Ghabrial & Buck 2000) (Fig. 2).

The origin of mycoviruses, especially the more common dsRNA mycoviruses, is believed to be polyphyletic (Koonin *et al*. 1989; Ghabrial 1998). This assumes there are multiple origins at different times, presumably from other cellular organisms (Hollings 1982; Koonin *et al*. 1989). Mycoviruses and mitoviruses co-evolved with their fungal hosts and co-adapted over time (Lemke 1976; Hollings 1982; Koonin *et al*. 1989; Ghabrial 1998). The closest relative of fungal viruses is believed to be (+) ssRNA plant viruses of supergroups I and II (Koonin *et al*. 1989; Ghabrial 1998) (Fig. 3).

Most mycovirus infections are latent, having no affect on the phenotype or pathogenicity of the fungus they infect (Lemke & Nash 1974; Ghabrial 1980). Some VLPs do however, exhibit a range of phenomena in their hosts, such as killer traits in *S. cerevisiae* (Bevan *et al*. 1973) and *U. maydis* (Koltin & Kandel 1978), hypovirulence in *C. parasitica* (Day *et al*. 1977), *O. novo-ulmi* (Brasier 1983) and *H. victoriae* (Ghabrial 1986), modulation of virulence in *R. solani* (Tavantzis 1988) and gene silencing in a variety of hosts.

Multiple infections with different cytoplasmic and/or mitochondrial dsRNA elements are common in fungi (Hollings 1962; Barton & Hollings 1979; Buck 1986). These multiple virus infections can be from different or the same virus families. A few examples are, two viruses found in a single *Helminthosporium victoriae* isolate i.e. a totivirus (Huang & Ghabrial 1996) and a chrysovirus (Ghabrial *et al*. 2002). In *Gremmeniella abietina* var. *abietina* type A, three viruses were found i.e. a totivirus (*G. abietina* RNA virus L2 or GaRV-L2), a partitivirus (*G.*

abietina RNA virus MS2 or GaRV-MS2) and a mitovirus (*G. abietina* mitochondrial RNA virus S2 or GaMRV-S2) (Tuomivirta & Hantula 2005). Two viruses were found in a single *Rhizoctonia solani* isolate i.e. an unclassified virus related to plant bromoviruses (Jian *et al*. 1998) and a mitovirus (Lakshman *et al*. 1998). Four mitoviruses were found in a single *Ophiostoma novo-ulmi* isolate (Hong *et al*. 1998, 1999) and two partitiviruses in a single *Helicobasidium mompa* isolate (Osaki *et al*. 2004).

The interaction of multiple virus infections and their combined effects are less well studied. In *C. parasitica*, Sun *et al*. (2006) demonstrated the synergistic effect of dual infections with a hypovirus (CHV1-EP713) and a mycoreovirus (MyRV1-Cp9B21). More severe reductions in growth rate and sporulation were observed, relative to single infections with either virus (Sun *et al*. 2006). The dual infection however, only enhanced the replication and transmission of the mycoreovirus, while that of the hypovirus was unaffected.

DsRNA elements are ideal for genetic manipulation to potentially mediate biological control due to their small, elementary genomes and basic composition (Buck 1986; Nuss & Koltin 1990; Ghabrial 1994; Nuss *et al*. 2002). The only obstacle is the construction of transformation or transfection systems with which dsRNA-free isolates of the plant pathogenic fungus can be infected with the manipulated dsRNA elements. Transfection systems have however, been successfully developed for the *C. parasitica* hypovirus (CHV-1) (Dawe & Nuss 2001) and the *Diaporthe* RNA virus (DaRV) (Moleleki *et al*. 2003). These infectious cDNA-based reverse genetic systems enable detailed studies of virus-host interactions, fungal pathogenesis mechanisms, fungal signaling pathways, evolution of RNA silencing and engineering of mycoviruses for enhanced biocontrol properties (Nuss 2005).

5.2.2 Hypovirulence-mediated dsRNA elements as biocontrol agents

DsRNA elements that confer hypovirulence are increasingly being considered as biocontrol agents for plant pathogenic fungi. Hypovirulence refers to the spontaneous reduction in virulence of the pathogen and is linked to the presence of dsRNA elements (Anagnostakis 1988; McCabe & Van Alfen 2002). In order for this type of biocontrol to be effective, an understanding of the interaction among the tree, fungus and virus is essential. All factors reducing the rate of the disease epidemic and those enhancing the establishment of the hypovirus need to be considered (Heiniger & Rigling 1994; Milgroom & Cortesi 2004).

Effective transmission of the dsRNA elements is essential to allow natural spread of the virus through a population (Heiniger & Rigling 1994; Milgroom & Cortesi 2004). Transmission of mycoviruses occurs either horizontally (hyphal anastomosis) or vertically (cell division and spore production) and is controlled through vegetative incompatibility (*vic*) genes (Buck 1986; Liu & Milgroom 1996; Milgroom & Brasier 1997; Cortesi & Milgroom 1998; Milgroom 1999). If all the *vic*-genes of two fungal isolates are identical, they can anastomose, transmit dsRNA elements and produce heterokaryons (Buck 1986; Liu & Milgroom 1996). If all their *vic*-genes are different, they are incompatible, no heterokaryons are produced and programmed cell death occurs. Partial transmission of dsRNA elements has been reported where a few *vic*-genes are different (Liu & Milgroom 1996). *Vic* loci from several fungi have been characterized e.g. in *C. parasitica* in Europe, six unlinked *vic* loci with 2 alleles were described, which can result in 64 genotypes (Cortesi & Milgroom 1998).

The first successful implementation of dsRNA elements as hypovirulence-mediated biocontrol agents was achieved in the chestnut blight pathogen, *C. parasitica* (Anagnostakis 1982; Van Alfen 1982; Fulbright *et al.* 1983; Griffin 1986; MacDonald & Fulbright 1991; Nuss 1992;

Heiniger & Rigling 1994; Dawe & Nuss 2001; Milgroom & Cortesi 2004). Hypovirulence-inferring viruses of *C. parasitica* mainly belong to the family *Hypoviridae* (Shapira *et al*. 1991a; Hillman *et al*. 1994; Smart *et al*. 1999) and four species have been described namely *Cryphonectria parasitica hypovirus* (CHV-I, CHV-2; CHV-3; CHV-4) (Hillman & Suzuki 2004). However, only CHV-1, CHV-2 and CHV-3 confer hypovirulence (Milgroom & Cortesi 2004). Four other viruses with apparently no phenotypic effects on *C. parasitica* have also been found, of which one is a mitochondrion-associated dsRNA element (Peever *et al*. 1997). All the dsRNA elements associated with *C. parasitica*, as well as the presence of defective and satellite dsRNAs contribute to the complexity of the hypovirulence-associated phenotype (Shapira *et al*. 1991b).

Common phenotypic changes observed in CHV-infected *C. parasitica* isolates are a reduction in growth, reduction or absence in sexual or asexual reproduction, changes in pigment production and changes in virulence (Nuss & Koltin 1990; Nuss 1992; McCabe & Van Alfen 2002; Nuss *et al*. 2002). These phenotypic changes are the result of dsRNA elements disrupting the normal developmental processes of the fungus by producing secondary metabolites like antibiotics and toxins (Nuss 1996; McCabe & Van Alfen 2002). These foreign viral metabolites interact with the fungal G proteins (GTP-binding proteins) and consequently disrupt normal signal transduction pathways (Nuss 1996; McCabe & Van Alfen 2002).

Recently, the first evidence of RNA silencing as an antiviral defense mechanism was demonstrated in *C. parasitica* (Segers *et al*. 2007). RNA silencing refers to the RNA-mediated sequence-specific suppression of gene expression. In the study conducted by Segers *et al*. (2007), the effects of dicer gene disruptions upon mycovirus infections were examined. The dicer genes code for endonucleases that process structured or dsRNA into small interfering RNA

(siRNAs) of 21-24 nt. These siRNAs are incorporated into the RNA-induced silencing complex reversing the effect hypovirulence-associated dsRNA elements normally have on this fungus. Hypovirulence-mediated biocontrol using CHV-1 in controlling chestnut blight was only successful in Europe and in a few isolated cases in North America. This is primarily due to the larger number of vegetative incompatibility (*vc*) groups in the North American *C. parasitica* population compared to that of the European population (Anagnostakis *et al*. 1986; Heiniger & Rigling 1994; Nuss *et al*. 2002; Milgroom & Cortesi 2004). The diversity of the host thus gave an early indication of the success of the hypovirulence-associated dsRNA viruses as they are dependent on the host for migration (Peever *et al*. 1997). Any factors that further enhance the establishment of the hypovirus, like environmental conditions and host species play a role in ensuring successful biocontrol (Milgroom & Cortesi 2004).

5.2.3 DsRNA elements in *Diplodia pinea*

Several dsRNA elements ranging from 400 bp – 9 kb in size have been reported from *D. pinea sensu lato* (Wu *et al*. 1989; Preisig *et al*. 1998; Steenkamp *et al*. 1998; De Wet *et al*. 2001; Adams *et al*. 2002). Two of these elements, isolated from a single, South African *D. pinea* isolate, have been characterized and are known as *Sphaeropsis sapinea RNA* virus 1 and 2 (SsRV1 and SsRV2) (Preisig *et al*. 1998). These two viruses belong to the family *Totiviridae* and the genus *Totivirus* (Fig.1). They are characterized by monopartite dsRNA genomes in the 5 kb size range with two open reading frames (ORFs), one coding for a capsid polypeptide and the other for a RNA-dependant RNA polymerase (RdRp).

Members of the *Totiviridae* are hypothesized to have the most ancient origin, most probably a non-infectious, single cell, virus progenitor that predates the differentiation of protozoans and fungi (Koonin *et al*. 1989; Bruenn 1993; Ghabrial 1998) (Fig. 3). The ability of this family of

viruses to infect a wide host range including yeasts, fungi and protozoa is furthermore, an indication of its ancient origin. Mycoviruses in the *Totiviridae* (SsRV1, SsRV2 and Hv190SV) have a higher degree of sequence homology to one another than to members of the same family infecting protozoa (*Giardiaviruses* and *Leishmaniaviruses*) or yeasts (*S. cerevisiae* L-A virus) (Preisig *et al*. 1998). These mycoviruses are also hypothesized to be the ancestors of those belonging to the *Partitiviridae* (Oh & Hillman 1995; Ghabrial 1998) (Fig. 3).

DsRNA elements in *D. pinea sensu lato* are transmitted via hyphal anastomosis. Transmission can also occur via conidia and Adams *et al*. (2002) reported a 70 - 100 % transmission rate. As mentioned previously, the genetic diversity of the host population is a restrictive factor in the transmission of dsRNA elements. Therefore, the implementation of dsRNA elements as biocontrol agents would be limited to populations of *D. pinea* with low genetic diversities.

DsRNA elements associated with *D. pinea sensu lato* have been reported to have no effect on the virulence of their hosts or result in any phenotypic changes (Wu *et al*. 1989; Preisig *et al*. 1998; Steenkamp *et al*. 1998; De Wet *et al*. 2001). Adams *et al*. (2002) did, however find one A morphotype isolate that was significantly more virulent when cured of its dsRNA, thus showing potential of having dsRNA-mediated hypovirulence. They used the AMMI (additive main effects and multiplicative interaction) model to obtain a more accurate estimation of relative virulence by partitioning the effects of genotype and environmental factors on the virulence. This model quantifies the sensitivity of the response of an isolate or tree species to the year, in the host-pathogen-year interaction. Interestingly, they found that dsRNA-containing parent strains could either be more or less virulent than dsRNA-free subcultures depending on year (environmental factors) and specific pine species. DsRNA-free subcultures also tended to be

more virulent in one year and less in the following year. DsRNA infections thus tend to moderate interactivity in *D. pinea* isolates of both the A and B morphotypes (Adams *et al*. 2002). Co-infections with both SsRV1 and SsRV2 are known to occur in *D. pinea* (Preisig *et al*. 1998) but the interaction between multiple infections and their cumulative effect has not been studied. Therefore, the unknown dsRNA elements associated with *D. pinea sensu lato* need to be characterized and their distribution in the different morphotypes of the fungus accessed, before they can be considered for exploitation as potential biocontrol agents against Diplodia die-back of pines.

6. CONCLUSIONS

The *Diplodia pinea sensu lato* species complex, like most species of the Botryosphaeriaceae, represents a suite of well-known pathogens causing disease symptoms such as die-back and cankers on numerous woody and non-woody hosts. These fungi have been notoriously difficult to identify accurately due to their having very similar morphological characteristics. This difficulty has been substantially alleviated since DNA-based phylogenetic studies have become available. The circumscription of members of the *D. pinea sensu lato* species complex, therefore, needs to be addressed.

Management of *Diplodia*-induced diseases is typically based on planting of resistant pine species and silvicultural practices that reduce stress on these trees. Huge economic losses, however, still occur especially in plantations of non-native pine species and in hail-prone areas such as the summer rainfall regions of South Africa. As our knowledge of the *D. pinea sensu lato* species complex increases and especially that pertaining to the distribution, virulence and genetic structure of the pathogen populations, it should be possible to refine management practices.

The exploitation of dsRNA elements that naturally occur in *D. pinea sensu lato* could potentially augment disease management strategies. This would need to follow an approach similar to that used for the chestnut blight pathogen, *C. parasitica*. Several dsRNA elements have been reported from *D. pinea sensu lato*, two of which been characterized. However, characterization of the novel dsRNA elements is needed. Furthermore, an assessment of the distribution of these dsRNA elements is needed in order to promote a deeper understanding of the genetic structure of virus populations in their fungal hosts. Although, no phenotypic effects have thus far been associated with dsRNA elements in *D. pinea sensu lato*, knowledge of their presence and of their genome organization could provide a foundation to engineer them towards inducing hypovirulence.

There is clearly much to learn regarding the biology, taxonomy and ecology of *D. pinea sensu lato* and the role these factors play in the evolution of the Botryosphaeriaceae. In the studies making up this dissertation, I will consider the taxonomy of *D. pinea sensu lato* and related fungi. Furthermore, I will characterize a novel dsRNA element more commonly associated with the B morphotype of *D. pinea* and determine the frequency and distribution of different viruses in isolates of *D. pinea sensu lato*. The longer term view is that these dsRNA elements might prove useful in promoting hypovirulence in the pathogen complex. In this way, they could contribute to an integrated biological control strategy for Diplodia die-back of pines in South Africa.

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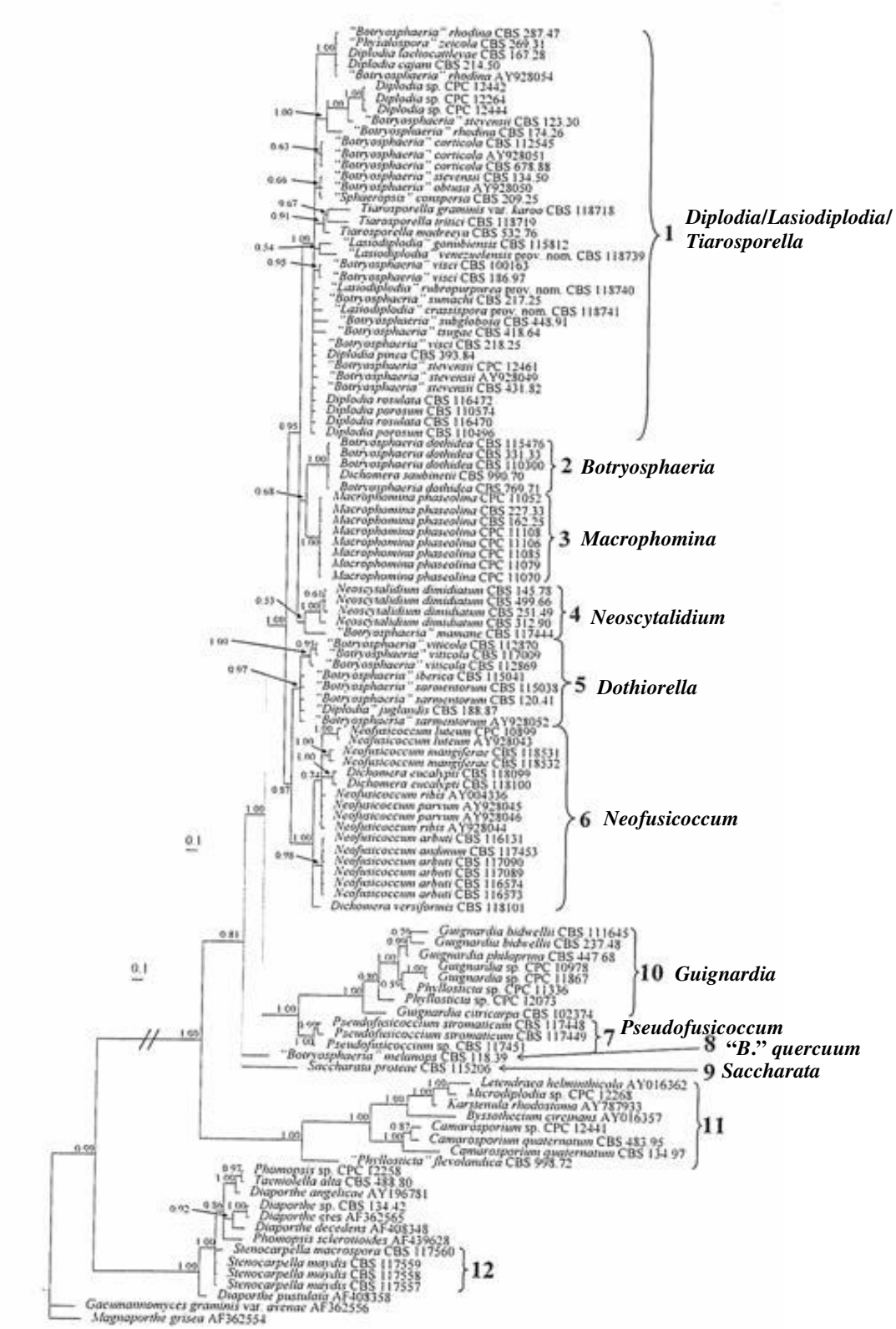
Figure 1. Ten lineages of the Botryosphaeriaceae based on sequences of the large ribosomal sub-unit as described by Crous *et al*. 2006. Clade 11 and 12 represent *Camarosporium*/*Microdiplodia* and *Stenocarpella*, respectively. They are considered to group outside the Botryosphaeriaceae.


"Botryosphaeria" rhodina CBS 287.47
"Physalospora" zeicola CBS 269.31
Diplodia laeliocattleyae CBS 167.28
Diplodia cupani CBS 214.50
"Botryosphaeria" rhodina AY928054
Diplodia sp. CPC 12442
Diplodia sp. CPC 12264
Diplodia sp. CPC 12444
"Botryosphaeria" stevensii CBS 123.30
"Botryosphaeria" rhodina CBS 174.26
"Botryosphaeria" corticola CBS 112545
"Botryosphaeria" corticola AY928051
"Botryosphaeria" corticola CBS 678.88
"Botryosphaeria" stevensii CBS 134.50
"Botryosphaeria" obtusa AY928050
"Sphaeropsis" conspersa CBS 209.25
Tiarosporella graminis var. karoo CBS 118718
Tiarosporella triseti CBS 118719
Tiarosporella madreeya CBS 532.76
"Lasiodiplodia" gonubiensis CBS 115812
"Lasiodiplodia" venezuelensis prov. nom. CBS 118739
"Botryosphaeria" viseti CBS 100163
"Botryosphaeria" viseti CBS 186.97
"Lasiodiplodia" rubropurpurea prov. nom. CBS 118740
"Botryosphaeria" sumachi CBS 217.25
"Lasiodiplodia" crassispora prov. nom. CBS 118741
"Botryosphaeria" subglobosa CBS 443.91
"Botryosphaeria" tsugae CBS 418.64
"Botryosphaeria" viseti CBS 218.25
Diplodia pinea CBS 393.84
"Botryosphaeria" stevensii CPC 12461
"Botryosphaeria" stevensii AY928049
"Botryosphaeria" stevensii CBS 431.82
Diplodia rosulata CBS 116472
Diplodia porosum CBS 110574
Diplodia rosulata CBS 116470
Diplodia porosum CBS 110496
1 Diplodia/Lasiodiplodia/ Tiarosporella
Botryosphaeria dothidea CBS 115476
Botryosphaeria dothidea CBS 331.33
Botryosphaeria dothidea CBS 110300
Dichomera saubinetii CBS 990.70
Botryosphaeria dothidea CBS 769.71
2 Botryosphaeria
Macrophomina phaseolina CPC 11052
Macrophomina phaseolina CBS 227.33
Macrophomina phaseolina CBS 162.25
Macrophomina phaseolina CPC 11108
Macrophomina phaseolina CPC 11106
Macrophomina phaseolina CPC 11085
Macrophomina phaseolina CPC 11079
Macrophomina phaseolina CPC 11070
3 Macrophomina
Neoscytalidium dimidiatum CBS 145.78
Neoscytalidium dimidiatum CBS 499.66
Neoscytalidium dimidiatum CBS 251.49
Neoscytalidium dimidiatum CBS 312.90
"Botryosphaeria" mamane CBS 117444
4 Neoscytalidium
"Botryosphaeria" vitricola CBS 112870
"Botryosphaeria" vitricola CBS 117009
"Botryosphaeria" vitricola CBS 112869
"Botryosphaeria" iberica CBS 115041
"Botryosphaeria" sarmentorum CBS 115038
"Botryosphaeria" sarmentorum CBS 120.41
"Diplodia" juglandis CBS 188.87
"Botryosphaeria" sarmentorum AY928052
5 Dothiorella
Neofusicoccum luteum CPC 10899
Neofusicoccum luteum AY928043
Neofusicoccum mangiferae CBS 118531
Neofusicoccum mangiferae CBS 118532
Dichomera eucalypti CBS 118099
Dichomera eucalypti CBS 118100
Neofusicoccum ribis AY004336
Neofusicoccum parvum AY928045
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Neofusicoccum ribis AY928044
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Neofusicoccum australe CBS 117453
Neofusicoccum arbuti CBS 117090
Neofusicoccum arbuti CBS 117089
Neofusicoccum arbuti CBS 116574
Neofusicoccum arbuti CBS 116573
Dichomera versiformis CBS 118101
6 Neofusicoccum
0.1
Guignardia bidwellii CBS 111645
Guignardia bidwellii CBS 237.48
Guignardia philoprina CBS 447.68
Guignardia sp. CPC 10978
Guignardia sp. CPC 11867
Phyllosticta sp. CPC 11336
Phyllosticta sp. CPC 12073
10 Guignardia
Guignardia citricarpa CBS 102374
Pseudofusicoccum stromaticum CBS 117448
Pseudofusicoccum stromaticum CBS 117449
Pseudofusicoccum sp. CBS 117451
7 Pseudofusicoccum
8 "B." quercuum
"Botryosphaeria" melanops CBS 118.39
9 Saccharata
Saccharata proteae CBS 115206
Letendraea helminthicola AY016362
Microdiplodia sp. CPC 12268
Karstenula rhodostoma AY787933
Byssothecium circinans AY016357
Camarosporium sp. CPC 12441
Camarosporium quaternatum CBS 483.95
Camarosporium quaternatum CBS 134.97
"Phyllosticta" flevolanica CBS 998.72
11
Phomopsis sp. CPC 12258
Taeniolella alta CBS 483.80
Diaporthe angelicae AY196781
Diaporthe sp. CBS 134.42
Diaporthe eres AF362565
Diaporthe decedens AF408348
Phomopsis sclerotioides AF439628
Stenocarpella macrospora CBS 117560
Stenocarpella maydis CBS 117559
Stenocarpella maydis CBS 117558
Stenocarpella maydis CBS 117557
Diaporthe pustulata AF408358
12
Gaeumannomyces graminis var. avenae AF362556
Magnaporthe grisea AF362554

Figure 2. A schematic representation of the current classification system of mycoviruses according to The Universal Virus Database of the International Committee on the Taxonomy of Viruses (ICTVdB). Families, genera and types species of the mycoviruses are shown.

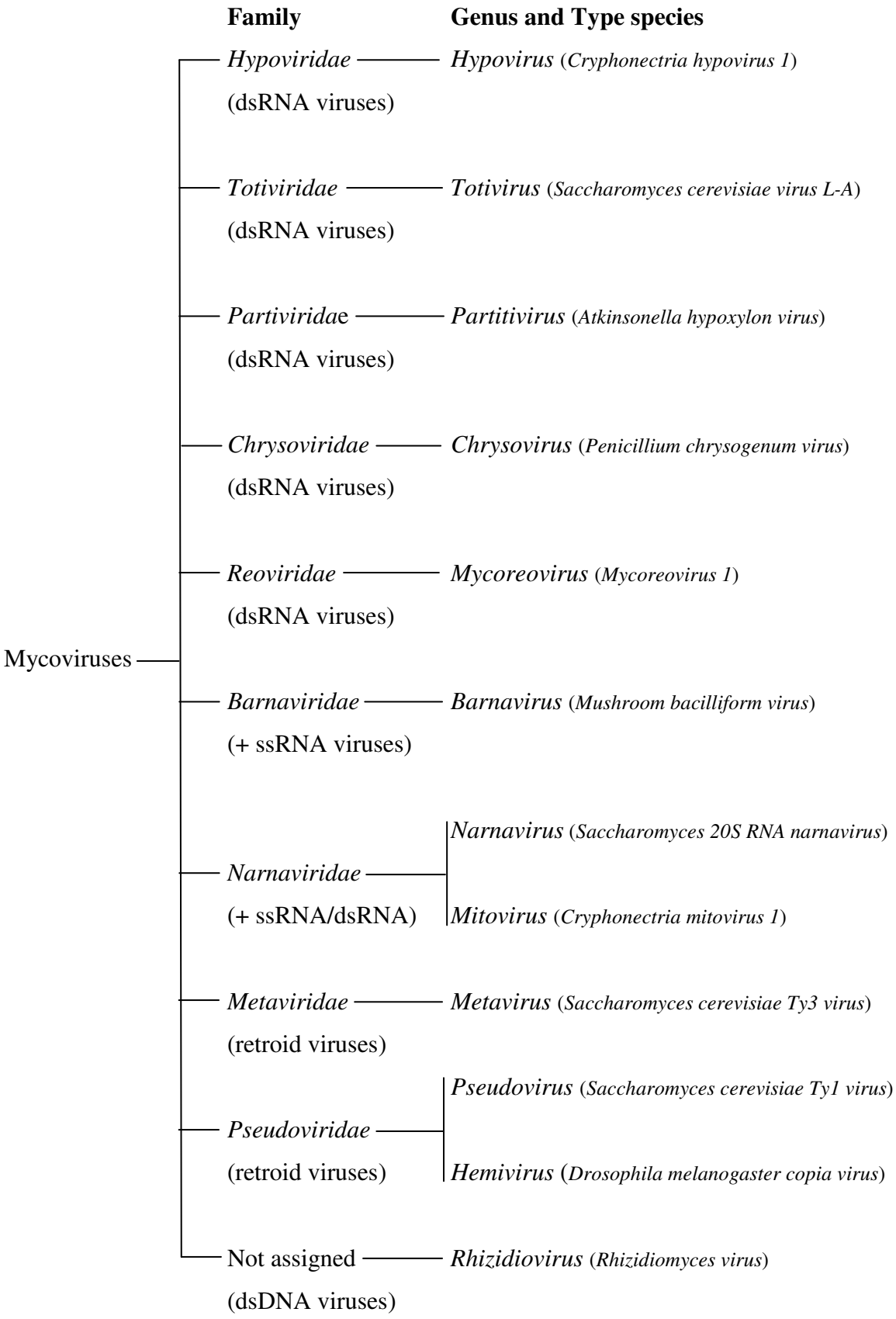
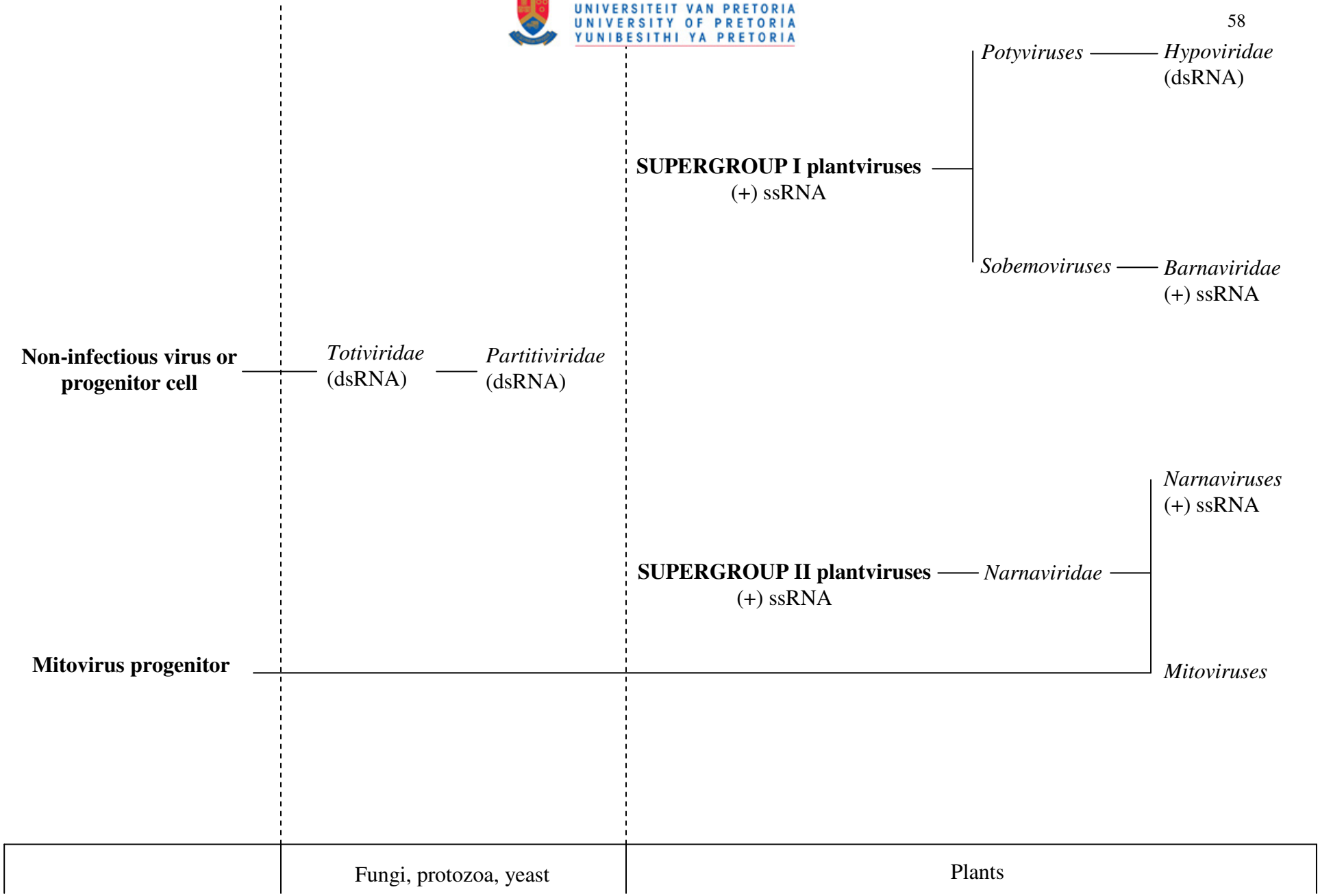

Family
Genus and Type species
Mycoviruses
Hypoviridae (dsRNA viruses)
Hypovirus (Cryphonectria hypovirus 1)
Totiviridae (dsRNA viruses)
Totivirus (Saccharomyces cerevisiae virus L-A)
Partiviridae (dsRNA viruses)
Partitivirus (Atkinsonella hypoxylon virus)
Chrysoviridae (dsRNA viruses)
Chrysovirus (Penicillium chrysogenum virus)
Reoviridae (dsRNA viruses)
Mycoreovirus (Mycoreovirus 1)
Barnaviridae (+ ssRNA viruses)
Barnavirus (Mushroom bacilliform virus)
Narnaviridae (+ ssRNA/dsRNA)
Narnavirus (Saccharomyces 20S RNA narnavirus)
Mitovirus (Cryphonectria mitovirus 1)
Metaviridae (retroid viruses)
Metavirus (Saccharomyces cerevisiae Ty3 virus)
Pseudoviridae (retroid viruses)
Pseudovirus (Saccharomyces cerevisiae Ty1 virus)
Hemivirus (Drosophila melanogaster copia virus)
Not assigned (dsDNA viruses)
Rhizidiovirus (Rhizidiomyces virus)

Figure 3. A schematic representation of the evolutionary pathways of mycoviruses and their closest relatives among the plant viruses.


Potyviruses
Hypoviridae
(dsRNA)
SUPERGROUP I plantviruses
(+) ssRNA
Sobemoviruses
Barnaviridae
(+) ssRNA
Non-infectious virus or progenitor cell
Totiviridae
(dsRNA)
Partitiviridae
(dsRNA)
Narnaviruses
(+) ssRNA
SUPERGROUP II plantviruses
(+) ssRNA
Narnaviridae
Mitovirus progenitor
Mitoviruses
Fungi, protozoa, yeast
Plants