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Preface

The forestry industry in South Africa is based predominantly on the planting of exotic tree species such as *Eucalyptus*, *Acacia* and *Pinus* species. Planting of these trees in South Africa has led to the introduction of numerous pests and pathogens of these species. Among these is the notorious fungal pathogen *Cryphonectria cubensis* (Bruner) Hodges that causes Cryphonectria canker on *Eucalyptus*. Since Cryphonectria canker causes serious losses in *Eucalyptus*, it is important to have effective management strategies to reduce losses caused by this fungus.

The first chapter of this thesis provides an overview of the literature on *Cryphonectria cubensis*, emphasising the taxonomy, hosts, symptoms, biology and the control of this fungus. Hypovirulence in fungi is also treated with the emphasis on viruses associated with *Cryphonectria*.

The discovery of double stranded (ds) RNA elements in *C. cubensis* has raised the possibility of using hypovirulence for biological control. In chapter two, I identified dsRNA elements in South African *C. cubensis* isolates. I further determined the full nucleotide sequence for these two mitoviruses and analysed the open reading frames for homologies to other viruses. Furthermore, abundance of the viruses in the fungal population, quantification of the viruses in a single *C. cubensis* isolate and transmission of the viruses to the conidia were considered. The pathogenicity of mitovirus containing isolates was also investigated.

Currently, the most effective means to reduce the impact of Cryphonectria canker is by selecting desirable planting stock with the best disease tolerance. These trees are selected for disease tolerance by artificial inoculation with one of the most virulent *C. cubensis* isolates in South Africa and monitoring disease progress. In the third chapter of this thesis, I investigated how different South African *C. cubensis* isolates respond to different *Eucalyptus* clones, which differ in disease susceptibility. The aim here was to determine whether disease screening using a single *C. cubensis* isolate provides an effective management strategy.

A dsRNA element has been previously characterised from the fungus *Diaporthe perjuncta* Niessl., which is an important pathogen of grapevine, and has been provided the name the *Diaporthe RNA virus* (DaRV). DaRV was used previously in transfection studies and

successful transfection was established in *D. ambigua*, a related pathogen of pome and stone fruit, as well as a *Phomopsis* isolate from peach. However, due to a taxonomic confusion, the original host of DaRV (*D. perjuncta*) was never transfected. In chapter four I used DaRV to transfect the original host and also attempted to establish co-infection of DaRV in a *C. parasitica* hypovirus (CHV1-EP713) containing *C. cubensis* isolate.

In a previous study a virulent *C. cubensis* isolate was transfected with the *C. parasitica hypovirus* (CHV1-EP713). In Chapter five of this thesis, I used this isolate as well as other virulent *C. cubensis* isolates in a field trial. Here, my aim was to determine whether the tranfected isolates show reduced virulence under field conditions. Secondly, I considered whether already existing cankers can be reduced in sized by the treatment of these cankers with the virus containing isolate.

All studies presented in this thesis concern the *Eucalyptus* fungal pathogen *C. cubensis*. They were, however, conducted independently and have been written as separate publishable units, developed over a period of four years. There is thus some repetition between parts of chapters and they also contain a progression of knowledge accumulated over a relatively long period of time. Nonetheless, it is my hope that they will all contribute to a better understanding of Cryphonectria canker in South Africa, and efforts to reduce its impact.

Chapter 1:

Cryphonectria cubensis in South Africa, and opportunities for biological control via hypovirulence: A review

1. INTRODUCTION

South African forestry depends almost exclusively on exotic species of *Pinus* and *Eucalyptus* to produce fiber for pulp and solid timber. The planting of these trees outside their natural habitat has made forestry in this and other countries with similar forestry programmes especially profitable. This is largely because the trees have been separated from their natural enemies (Bright 1998; Wingfield and Wingfield 1999).

Cryphonectria cubensis (Bruner) Hodges is a plant pathogenic ascomycetous fungus that has a wide geographical distribution in tropical and subtropical *Eucalyptus* growing regions of the world. The fungus causes a severe stem canker disease on *Eucalyptus* species that are susceptible to this pathogen. This disease was first observed in South Africa in 1989 (Wingfield *et al.* 1989) and it has subsequently become important to implement an effective disease management strategy to reduce its impact. There are various means to accomplish this goal. The most effective of these has been to plant disease tolerant hybrid clones of *Eucalyptus* (Alfenas *et al.* 1983; Wingfield 1990). Chemical control is also an option, but due to the low economic return of individual *Eucalyptus* trees, it is not economical (Sharma *et al.* 1985). Biological control through hypovirulence, which is linked to dsRNA (mycovirus) infections, could be an attractive control strategy in the future (van Heerden *et al.* 2001).

In this chapter I will review the literature pertaining to the taxonomy and biology of *C. cubensis*. Because much of the material treated in this thesis also deals with mycoviruses and hypovirulence, I also review that topic. In this regard emphasis is placed on the chestnut

blight pathogen *Cryphonectria parasitica* (Murr.) Barr., a tree pathogen related to *C. cubensis* and for which most knowledge pertaining to hypovirulence, is available.

2. TAXONOMY AND BIOLOGY OF *CRYPHONECTRIA CUBENSIS*

In 1916, Bruner described *Endothia havanensis* Bruner from *Eucalyptus* species in Cuba (Bruner 1916). A year later he described another fungus *Diaporthe cubensis* Bruner also from *Eucalyptus,* in the same country (Bruner 1917). Subsequent to these reports, almost 50 years passed without further mention of either of the fungi. Then in 1970, Boerboom and Maas reported *E. havanensis* from Surinam on *Eucalyptus saligna* Sm. and *Eucalyptus grandis* (Hill) Maiden (Boerboom & Maas 1970). Shortly thereafter, Hodges and Reis (1974), observed that the same fungus identified in Surinam caused a canker disease in Brazil. The causal agent for the diseases in Surinam and Brazil was reported as *D. cubensis* (Hodges & Reis 1974). Hodges (1980) compared specimens of *E. havanensis* with specimens of *D. cubensis* and observed significant morphological differences between the two fungi. He also found that *D. cubensis* was more similar to species of *Cryphonectria* and, therefore, transferred *D. cubensis* to *Cryphonectria* as *C. cubensis*.

Various differences exist between the South African form of *C. cubensis* and *C. cubensis* from other parts of the world. The most prominent difference is that *C. cubensis* in South Africa forms basal cankers compared to cankers which tend to be higher up in trees in other countries (Hodges *et al.* 1979, Sharma *et al.* 1985, Wingfield *et al.* 1989). By comparing the β-Tubulin and histone *H3* gene sequences of *C. cubensis* from South America, Asia, Australia and South Africa, it was found that the South East Asian/Australian *C. cubensis* isolates formed a closely related clade. Here the South African *C. cubensis* isolates grouped away from the other isolates of the fungus (Myburg *et al.* 2002b). However, only slight morphological differences in the pycnidia were observed between the South African isolates and the other isolates used. This study, has therefore, suggested that the South African *C. cubensis* has a different origin than the South American, Southern Asian and Australian *C. cubensis*. More recently Heath *et al*. (2002) confirmed these findings through the discovery of the South African fungus on native *Myrtaceae* and by confirming its identity using β-tubulin gene sequences.

2.1 Geographic distribution and origin:

Subsequent to the first discovery of *C. cubensis* in Cuba, this fungus was recorded in numerous other countries in tropical and sub-tropical regions of the world. These countries include Brazil and Surinam (Boerboom & Maas 1970; Hodges 1980). *Cryphonectria cubensis* has also been described from Florida, Hawaii, Puerto Rico (Hodges *et al.* 1979), Kerala in India (Sharma *et al.* 1985) and from various African countries such as Cameroon (Gibson 1981) and South Africa (Wingfield *et al.* 1989). One of the most unusual records of *C. cubensis* is that from Western Australia (Davison & Coates 1991). In this situation the fungus was isolated from roots of *Eucalyptus marginata* Donn: Smith (Davison & Coates 1991). The unusual aspect of this report is the fact that the environment in Western Australia is very different to that normally associated with *C. cubensis*. However, using isozyme comparison the authors of this report were able to confirm that the isolates grouped together with *C. cubensis* isolates originating elsewhere in the world. This was later confirmed using sequence data from the ITS1 and ITS2 regions and the 5.8S rRNA operon (Myburg *et al.* 1999) and subsequently using β-tubulin and histone *H3* gene sequences (Myburg *et al.* 2002b).

The question of the possible origin of *C. cubensis* is intriguing and has been treated in various studies. Acute die-back of clove trees *Syzygium aromaticum* (L.) Merr. and Perry, which is related to *Eucalyptus*, in the *Myrtaceae*, was observed in Zanzibar in 1952, with the causal agent *Endothia eugeniae* (Nutman and Roberts) Reid and Booth (Nutman & Roberts 1952). The similarity of the pathogen to *C. cubensis* led to detailed comparisons, based on morphology, cultural characteristics, pathogenicity as well as total protein and isozyme analyses (Alfenas *et al.* 1984; Micales & Stipes 1984; Hodges *et al.* 1986; Micales *et al.* 1987). All of these studies confirmed that *C. cubensis* and *E. eugeniae* are similar and could be reduced to conspecificity. In a more recent study based on sequence data from the ITS regions and the rRNA operon Myburg *et al*. (1999) confirmed that the isolates from clove formed a well-resolved clade within *C. cubensis*. These studies have led to the hypothesis that *C. cubensis* is native to the Indonesian Molucca Islands, where it is thought to occur as a mild pathogen on clove (Hodges *et al*. 1986). It has further been suggested that when *Eucalyptus* was brought into Indonesia these trees became infected with *C. cubensis* from native clove. However, what remains to be achieved to confirm or reject this hypothesis, is to collect *C. cubensis* from clove in the Molucca islands, and to compare these collections with those from *Eucalyptus* elsewhere in the world, using polymorphic molecular markers.

Another possible origin for *C. cubensis* might be from the Melastomatalean hosts which include *Tibouchina* spp. In 1999, *C. cubensis* was discovered in Colombia causing severe cankers on *Tibouchina urveleana* (DC.) Logn., a native species in Colombia as well as on *Tibouchina lepidota* Baill, which is native to Brazil (Wingfield *et al.* 2001). This was followed in 1999 by the discovery of *C. cubensis* on an ornamental tree, *Tibouchina granulosa* Cogn. in South Africa (Myburg *et al.* 2002a). These fungi resemble *C. cubensis* based on morphology. Comparison of DNA sequence data has also revealed that the fungus is *C. cubensis*, and that the *C. cubensis* found on *Tibouchina* in South Africa is closely related to the fungus found on *Eucalyptus* in the same country (Myburg *et al.* 2002a). This study also showed that the South American *Tibouchina* isolates are more closely related to those from *Eucalyptus* on that continent. Furthermore, *C. cubensis* found in South Africa is different to that from other parts of the world, and might have a different origin (Myburg *et al.* 2002a). These studies have thus provided further evidences that *C. cubensis* in South Africa has an origin different to that found elsewhere in the world.

2.2 Host range:

Cryphonectria cubensis has mainly been reported on *Eucalyptus*, with a wide range of *Eucalyptus* species being infected (reviewed by Conradie *et al.* 1990). In South Africa, the most susceptible of these is *E. grandis* with hybrids of *E. grandis* x *E. urophylla* S.T. Blake and *E. grandis* x *E. camaldulensis* Dehnh., being more resistant to the fungus (Wingfield, personal communication). *Cryphonectria cubensis*, has however, also been found on various species of *Syzygium* including clove (*S. aramaticum*) (Hodges *et al.* 1986), and a recent discovery in South Africa on the native *S. cordatum* Hachst. and *S. guineense* (CD.) Willd (Heath *et al*. 2002). Other hosts in the *Myrtaceae* include *Psidium guajava* L. which was shown to be susceptible using pathogenicity tests but not from natural infections (Swart *et al.* 1991). Another intriguing recent discovery has been the finding of *C. cubensis* causing a serious stem canker disease on *T. urveleana* and *T. lepidota* in Colombia (Wingfield *et al.* 2001) as well as on *T. granulosa* in South Africa (Myburg *et al.* 2002a). These *Tibouchina* spp. belong to the *Melastomataceae* which like *Eucalyptus* reside in the order Myrtales. There is also substantial evidence to show that the two families are closely related (Conti *et al.* 1997) and that the occurrence of the fungus on trees in the two families is perhaps not surprising.

2.3 Biology & Symptoms:

Cryphonectria canker is characterised by sunken elongated areas at the bases or higher up on infected trees (Fig. 1A). The tissue below the bark is typically brown and dead, with kino exudation usually observed on older cankers (Fig. 1B) (Boerboom & Maas 1970; Sharma *et al.* 1985). In South Africa, only basal cankers have been observed, which is different to the situation in other parts of the world where cankers are commonly found higher up in trees (Hodges *et al.* 1979; Sharma *et al.* 1985; Wingfield *et al.* 1989). Trees react to *C. cubensis* infection by producing callus around the site of invasion (Hodges *et al.* 1979).

Cryphonectria canker tends to be more severe on actively growing trees. Thus, the development of cankers is limited by stress factors such as drought, which results in smaller cambial lesions (Swart *et al.* 1992). This is consistent with the epidemiology of the disease that is known to occur predominantly in higher rainfall areas in South Africa and elsewhere in the world (Hodges *et al.* 1979; Sharma *et al.* 1985; Florence *et al.* 1986; Wingfield *et al.* 1989). Rainfall (2000-2400 mm/ annum) and temperatures above 23ºC are known to favour Cryphonectria canker (Sharma *et al.* 1985; Florence *et al.* 1986).

Both anamorph and teleomorph states of *C. cubensis* are known. Pycnidia of what has been recently referred to as the *Endothiella* Sacc. anamorph are cylindrical to broadly pyriform, occurring singly or in groups (Fig. 1C) (Hodges 1980; Conradie *et al.* 1990). The conidia are hyaline and one-celled, ranging from 2.5-4.0 x 1.8-2.2 μm in size (Hodges *et al*. 1979), perithecia develop during drier periods and their rounded bases are embedded in the bark and a long neck emerges in groups from the bark surface and varies in length depending on the humidity (Fig. 1D) (Hodges 1980). The asci are 25.0-33.0 x 5.0-6.5 μm in size and contain eight hyaline, two celled ascospores which are 5.8-8.2 x 2.2-3.0 μm in size (Hodges 1980).

2.4. Control strategies and factors influencing their efficacy:

Various strategies have been used to reduce the impact of Cryphonectria canker on *Eucalyptus*. Chemical control has been tested as an immediate control measure, but due to the low economic return of individual *Eucalyptus* trees, this is not a viable option (Sharma *et al.* 1985). The most effective means to reduce losses due to *C. cubensis* is to plant resistant or less susceptible species or clones of *Eucalyptus* (Alfenas *et al.* 1983). This approach has

been shown to be effective in various parts of the world (Campinhos & Ikemori 1983; Wingfield 1990).

Various methods exist to screen desirable *Eucalyptus* planting stock for tolerance to infection by *C. cubensis*. Van Zyl and Wingfield (1999) used the capacity of *Eucalyptus* clones to close wounds through callus production, to assess relative susceptibility to *C. cubensis*. In their study, they found that tolerant clones, close wounds significantly faster than susceptible trees. Another method that is currently used to hasten selection of disease tolerant planting stock, is to screen trees, using artificial inoculation (Ferreira *et al.* 1977; Alfenas *et al.* 1983; van der Westhuizen 1992). However, due to the genotype x environmental (GxE) interaction observed for disease susceptibility, it is also important to undertake disease screening in the areas where the clones will be commercially propagated (van Heerden & Wingfield 2002).

Another exciting, if longer term, prospect to reduce the impact of Cryphonectria canker is potentially via biological control through hypovirulence. Hypovirulence is a pathogen phenotype of reduced virulence, and is associated by the presence of double stranded (ds) RNA in *C. parasitica* (Day *et al.* 1977; Nuss 1992). This topic is discussed extensively later in this review.

Although *C. cubensis* was first observed in South Africa in 1989, the teleomorph is extremely rare (Wingfield, personal communication). Studies conducted by van Heerden & Wingfield (2001) confirmed these observations by inoculating branch sections with a diverse set of South African *C. cubensis* isolates. Results indicated that only the anamorph (pycnidia) is produced. This explains the fact that the South African *C. cubensis* population on *Eucalyptus* has a narrow genetic diversity (van Heerden & Wingfield 2001). This is in contrast to the situation in other parts of the world such as Brazil, Venezuela and Indonesia where the genetic diversity is much greater (van Zyl *et al.* 1998; van Heerden *et al.* 1997).

In a study conducted by van Zyl *et al*. (1998), it was shown that from a relatively large collection of Brazilian *C. cubensis* isolates, a high population diversity was displayed. Similar results were also obtained from population studies done with Indonesian and Venezuelan isolates of *C. cubensis* (van Heerden *et al.* 1997). In these countries, sexual reproduction occurs frequently, which explains the fact that the genes can continuously

recombine, giving rise to new genetic combinations and high population diversity (van Heerden *et al.* 1997; van Zyl *et al.* 1998).

Double stranded RNA viruses are known to be able to spread through a fungal population via hyphal anastomosis (Nuss 1996). This movement is favoured when isolates belong to the same vegetative compatibility group (Anagnostakis 1977; Anagnostakis & Day 1979). Thus, a biological control strategy involving hypovirulence, could be much more efficient in a country such as South Africa, where *C. cubensis* on *Eucalyptus* has a narrow genetic diversity.

3. HYPOVIRULENCE IN FUNGI

3.1 Introduction

Hypovirulence in fungi can result from many causes. These include mitochondrial DNA mutations (Mahanti *et al*. 1993; Monteirro-Vitorello *et al.* 1995), nuclear genome mutations or the presence of mycoviruses such as double stranded (ds) RNA viruses (Smart & Fulbright 1996). Double stranded RNA viruses are known to occur in various plant pathogenic fungi. Some examples include those in *Sclerotinia homoeocarpa* F. T. Bennett (Zhou & Boland 1997), *Sphaeropsis sapinea* (Fr.:Fr.) Dyko & Sutton (Preisig *et al.* 1998; Steenkamp *et al.* 1998), *Fusarium graminearum* Schwabe (Chu *et al.* 2002), *Rhizoctonia solani* Kühn (Castanho *et al.* 1978), *Diaporthe perjuncta* (Smit *et al.* 1996; Moleleki *et al.* 2002), *Leucostoma persoonii* (Nits.) Hoehn (Hammar *et al.* 1989) and *Cryphonectria parasitica* (Murr.) Barr (Day *et al.* 1977).

Most mycoviruses reside in the families *Totiviridae*, *Partitiviridae*, *Narnaviridae* and *Hypoviridae*. The viruses in the families *Totiviridae* and *Partitiviridae* form isomeric particles (20-25nm in diameter), compared to the unencapsidated viruses in the families *Hypoviridae* and *Narnaviridae* (Ghabrial 1994; Ghabrial 1998; Hillman *et al*. 2000b; Wickner *et al*. 2000). The *Totiviridae* include the genus *Totivirus*, that infects fungi and the *Leismaniavirus* and *Giardiavirus*, which infect protozoan hosts (Ghabrial 1994). The *Partitiviridae* include four genera. Of these *Partitivirus* and *Chrysovirus* infect fungi and *Alphacryptovirus* and *Betacryptovitus* infects plants (Ghabrial 1998; Ghabrial *et al.* 2000). The family *Hypoviridae* includes the single genus *Hypovirus* and the family *Narnaviridae* includes the genera *Mitovirus* and *Narnavirus* (Hillman *et al*. 2000b; Wickner *et al.* 2000).

The latter two families will be discussed extensively in this review, since they pertain to the latter chapters of this thesis.

3.2 HYPOVIRUS

3.2.1 Historical overview:

Cryphonectria hypovirus 1 and the *Cryphonectria hypovirus 2* are the only species in the genus *Hypovirus*, with two other tentative species, *Cryphonectria hypovirus 3/GH2* and the *Cryphonectria hypovirus 4/SR2* (Hillman *et al*. 2000a). These *Cryphonectria* hypoviruses all infect *C. parasitica*, the causal agent of chestnut blight. *Cryphonectria parasitica* was first reported in 1904 in North America where it has been responsible for the devastation of the American chestnut (*Castanea dentata* Borkh.) (Merkel 1906). In 1938, this disease appeared in Italy in the province of Genoa on the European chestnut *Castanea sativa* Mill. (Pavari 1949). However, Biraghi (1950) observed the spontaneous healing of cankers on sprouts growing from the stem of a chestnut tree. Studying this phenomenon led to the isolation of hypovirulent strains of *C. parasitica* (Grente 1965). Subsequently, the factor causing hypovirulence was shown to be transmissible via hyphal anastomosis. Fungal isolates harbouring this factor had the ability to heal actively growing cankers on trees after inoculation (Grente & Sauret 1969; Grente & Berthelay-Sauret 1978). Healing blighted trees were also observed in North America in 1976, which led to the isolation of a hypovirulent isolate in that country (Anagnostakis 1982a).

A detailed study of *C. parasitica* strains from both North America and Europe has shown that hypovirulence is consistently associated with dsRNA infections (Day *et al.* 1977). The molecular weights and the concentration of the dsRNA in isolates from North America and Europe differed, distinctly. The molecular weights were between 4.0 and 7.0 x 10^6 and the concentrations of dsRNA were lower in the American than the European isolates (Dodds 1980). Dot blot hybridisation has confirmed the lack of sequence homology between the ds RNA elements of the European and the American strains of *C. parasitica* (L'Hostis *et al.* 1985). Curing these hypovirulent isolates using cycloheximide, resulted in an increase in the virulence converting the effect of the dsRNA on virulence (Fulbright 1984).

3.2.2 Phenotypic changes associated with the presence of dsRNA:

Various phenotypic changes other than hypovirulence can be associated with the presence of dsRNA. These include altered colony morphology (Anagnostakis 1982a; Elliston 1985a;

Elliston 1985b), reduced or abolished sporulation, especially the formation of perithecia (Anagnostakis 1982a; Elliston 1985a), reduced pigmentation (Anagnostakis 1982a), reduced oxalate accumulation (Havir & Anagnostakis 1983) and reduced laccase production (Rigling *et al*. 1989). Puhalla and Anagnostakis (1971) showed that, when *C. parasitica* is grown in the dark, little or no pigment is produced. Moreover, studies by Hillman *et al*. (1990) demonstrated that high light intensity can relieve most of these hypovirulence-associated symptoms. Thus, they suggested that light intensity and hypovirulence-associated dsRNA, might influence gene expression by the same pathways. However, not all these phenotypic changes are consistently associated with all hypovirulent strains (Elliston 1985a). It has thus been proposed that the symptoms are not a direct result of the response of the host due to the presence of dsRNA, but rather to the gene products encoded by the dsRNA (Nuss & Koltin 1990).

Choi and Nuss (1992a), transformed a virus free isolate with a cDNA copy of ORF A generated from the dsRNA genome of the virus (CHV1-EP713), under the transcriptional control of the *C. parasitica gdp1* promotor. This resulted in traits associated with the dsRNA containing hypovirulent isolates, such as reduced pigmentation, reduced laccase accumulation and suppressed conidiation (Choi & Nuss 1992a). These studies thus, confirmed that the traits are caused by the viral coding domain and not the host response to virus infection.

Other hypovirulence-associated traits in *C. parasitica* include: reduced levels of cutinase (Varley *et al.* 1992). Some gene products can also be reduced by the presence of a hypovirus either at mRNA level or protein level. These include: Cryparin (Carpenter *et al.* 1992), Vir 1 and Vir 2 (fungal sex pheromone) (Powell & van Alfen 1987; Zhang *et al*. 1993; Kazmierczak *et al*. 1996), LAC 1 (extracellular laccase) (Larson *et al*. 1992), CBH 1 (Cellobiohydrolase) (Cell wall degrading enzyme) (Wang & Nuss 1995) and the CPG-1 (GTP Binding protein α subunit) (Choi *et al*. 1995).

3.2.3 Genome organisation and structure:

The nucleotide sequence of four viruses in the genus *Hypovirus*, has been determined. These include the viruses CHV1-EP713, CHV1-Euro7, CHV2-NB58 and CHV3-GH2 (Dawe & Nuss 2001). Subsequent to the discovery of dsRNA in *C. parasitica*, various studies have been undertaken to determine the genomic structure of the viruses within the genus

Hypovirus. For example, Hansen *et al*. (1985) have shown that dsRNA containing particles observed in hypovirulent isolates, lack a protective protein capsid, but are associated with membrane vesicles.

The dsRNA of CHV1-EP713 can be grouped into three size classes. The (L) large is a single band of ~12.7 kb in size, the (M) medium ranges from 8-10 kb and the (S) small dsRNA ranges from 0.6-1.7 kb in size (Shapira *et al.* 1991). Further, full sequence analysis of the L-dsRNA of strain CHV1-EP713, revealed the existence of two open reading frames (ORF), ORF A and ORF B (Shapira *et al.* 1991). Choi *et al*. (1991a) further showed that ORF A encodes two polypeptides, P29 and P40, which are generated by an autoproteolytic process governed by P29 (Fig. 2). P29 has resemblance to the potyvirus encoded protease HC-Pro (Choi *et al.* 1991b) and is known to be a determinant in viral symptoms (Craven *et al.* 1993). ORFB encodes a 48 kb polypeptide (protease) (P48) (Shapira *et al.* 1991) (Fig. 2). Other domains have also been identified which encode a RNA-dependant RNA polymerase (RdRp) and RNA helicase (Koonin *et al.* 1991) (Fig. 2).

Sequence analysis of the hypovirus species CHV2-NB58 showed a 60% nucleotide sequence identity to CHV1-EP713. The ORFA gene product of CHV2-NB58 differs from that of CHV1-EP713, in that it encodes a 50-kDa product and does not undergo autoproteolysis (Hillman *et al.* 1994). *Cryphonectria hypovirus* 3 (CHV3-GH2) differs from the other two species in this genus in that it contains only a single open reading frame, which encodes a putitative proteinase, RNA-dependant RNA polymerase and a helicase (Smart *et al.* 1999). The dsRNA of GH2 is also considerably smaller than that of CHV1-EP713, 9.8kb compared to 12.7kb (Smart *et al.* 1999). *Cryphonectria hypovirus* 3 further contains three other smaller dsRNAs, that represent satellite and defective RNAs, the importance of which is unknown (Hillman *et al.* 2000a).

3.2.4 Transmission of dsRNA:

For the effective application of mycoviruses in any biological control system, it is important to have a clear understanding regarding the transmission of dsRNA, through the fungal population. DsRNAs are known to be transmitted by two means in fungal populations. This is either vertically to a different level through spores or horizontally via hyphal anastomosis (Nuss 1996). The movement of dsRNA via hyphal anastomosis is favoured when isolates belong to the same vegetative compatibility group (Anagnostakis 1977).

Garbelotto *et al*. (1992) showed that the Italian *C. parasitica* population consists of a few vegetative compatibility groups (VCGs). Thus, by using five hypovirulent strains, they were able to convert 77% of the isolates to the hypovirulent phenotype. In North America the *C. parasitica* population has a much higher level of genetic diversity than it has in Europe, which might explain the unsuccessful dissemination of hypovirulence in North America (Anagnostakis 1982a; Anagnostakis *et al*. 1986; Anagnostakis 1987; Heiniger & Rigling 1994).

In *C. parasitica*, vegetative incompatibility is controlled by six vegetative incompatibility (*vic*) loci, each with two alleles (Anagnostakis 1982b; Cortesi & Milgroom 1998). Liu & Milgroom (1996) have further shown that a negative correlation exists between hypovirus transmission and the number of *vic* genes that differ between isolates of *C. parasitica*. Thus, the level of hypovirus transmission will decrease as the number of *vic* genes, differing between the donor and recipient isolate, increases (Liu & Milgroom 1996). Hypovirus transmission can still occur between fungal strains that are unable to form heterokaryons, due to different alleles at a single *vic* locus (Huber & Fulbright 1994; 1995). It was later concluded that the transmission of viruses in *C. parasitica* is primarily controlled by the *vic* genes (Cortesi *et al*. 2001)

The inability of dsRNA to pass through to the conidia of *C. parasitica*, has been observed and might be a reason for the ineffective spread of hypovirulence through the American chestnut plantations (Shain & Miller 1991). In contrast, Peever *et al*. (2000) have shown that the hypoviruses were transmitted to >95% of the conidia of all the European isolates tested. It is not clear whether this variation in vertical transmission is caused by the viruses or the fungal genotypes (Peever *et al.* 2000). Biological control strategies should, therefore, be focused on using hypovirus/ fungal combinations that have the least effect on sporulation of the fungal pathogen (Peever *et al.* 2000).

3.2.5 Application of biological control:

Although dsRNA can spread naturally through fungal populations, various field applications with the *Cryphonectria hypovirus* have been attempted, to reduce the impact of chestnut blight. The first of these was in France between 1966 and 1974 when Grente & Berthelay-Sauret (1978) developed a method to treat blighted trees. After identifying the predominant

VC group present in the population, they produced and distributed the appropriate mixture of hypovirulent strains to the chestnut growers. These growers in turn removed small pieces of bark around existing cankers, placing the inoculum in the wound and sealed these with masking tape, to reduce desiccation (Grente & Berthelay-Sauret 1978; Heiniger & Rigling 1994). These cankers started to heal and mortality decreased. Biological control has also been successfully applied in Italy where similar application techniques were used to those in France (Heiniger & Rigling 1994). In North America, cankers on trees have been treated successfully but no natural spread of the hypoviruses has been reported (Anagnostakis 1982a). However, the use of transgenic hypovirulent strains has shown effective transmission of the virus in field trials in North America (Nuss 2000)

In a recent study Robin *et al*. (2000), attempted to determine whether the release of the hypoviruses for biological control of *C. parasitica* in 1974 in France has led to the reduction of blight severity. They also investigated the effect on the population structure. Results showed that there was a low severity of chestnut blight in the areas under investigation. In addition, the VC group diversity was lower in the *C. parasitica* populations than in 1981 (Robin *et al*. 2000). The results of these studies reflect the successful establishment of the biological control agent in the *C. parasitica* population.

3.2.6 Genetic engineering of fungal hypoviruses

The completion of the full genome sequence of the *C. parasitica hypovirus* CHV1-EP713 (Shapira *et al.* 1991), allowed for the construction of a full-length cDNA clone of this virus (Choi & Nuss 1992b). Choi and Nuss (1992b) used this full-length cDNA for transformation into a virus-free *C. parasitica* strain. Transformants had the hypovirulence phenotype and they also contained a chromosomally integrated copy of the virus as well as a cytoplasmically replicating form (Choi & Nuss 1992b). As mentioned earlier in this review, hypoviruses are not transmitted to the ascospore progeny and the transmission into conidia does not occur consistently (Nuss 1996). The construction of an infectious cDNA copy of CHV1-713 has overcome these problems. Chen *et al*. (1993) have used repeated rounds of conidiation to show that the chromosomally integrated viral cDNA copy is stable and that the virus can be transmitted to ascospore progenies. This is a novel form of transmission, since the progenies contain a range of different VC groups due to allelic rearrangement at the *vic* loci (Nuss *et al.* 2002). The transgenenic strains, therefore, have enhanced dissemination properties and thus enhanced biological control properties. A reporter gene was also incorporated into

Cryphonectria transgenic strains. For this purpose the green fluorescent protein (GFP) gene from *Aequorea victoria* was used to track the movement of hypoviruses through hyphal anastomosis from strain to strain (Suzuki *et al.* 2000).

The transfection of spheroplasts produced from virus free *C. parasitica* isolates with the full length *in vitro* produced CHV1-EP713 transcripts, using electroporation has been successful (Chen *et al.* 1994). In this case, the success of the transfection protocol relies on hyphal anastomosis. After electroporation, the spheroplasts are plated onto a regeneration medium and the RNA present in a small number of successfully transfected speroplasts, will spread through the colony (Nuss *et al*. 2002). This transfection strategy has made it possible to expand the range of fungi that can be infected by CHV1-EP713. Three species in the genus *Cryphonectria* namely *C. cubensis*, *C. havanenesis* (Bruner) Barr and *C. radicalis* (Schw.:Fries) Barr, and one species in the genus *Endothia* namely *E. gyrosa* (Schw.: Fries) Fries have been successfully transfected with the *C. parasitca hypovirus* RNA (Chen *et al.* 1994). These transfections resulted in phenotypic changes in the recipient fungi. The phenotypic changes observed for *C. radicalis* were similar to those of hypovirulent *C. parasitica* isolates, including reduced growth rate, reduced sporulation and a suppression of the orange pigmentation (Chen *et al.* 1994). Further, Chen *et al*. (1994) showed that transfected *E. gyrosa* and *C. cubensis* isolates had increased bright orange pigment, whereas transfected *C. havanensis* had only slight morphological change. In addition, a study by Chen *et al*. (1996) showed that virus transmission to the asexual spores ranges from 0% for *C. cubensis* to 50-100% for *C. parasitica*.

Van Heerden *et al.* (2001) have recently been able to transfect a virulent South African *C. cubensis* isolate with CHV1-EP713. In this study, it was shown that a blockage of the transmission of the virus to the asexual spores occurred, and that the transfection also resulted in the production of a bright yellow-orange pigment similar to that observed by Chen *et al*. (1994). Additionally, it was shown that the virus in the transfected isolate can spread via hyphal anastomosis into nearly half of the VC groups of *C. cubensis* identified from *Eucalyptus* in South Africa (van Heerden *et al.* 2001). This study has also shown the possibility of implementing a biological control strategy for one pathogen, using the virus from a different pathogen (van Heerden *et al.* 2001).

3.3 MITOVIRUS

Cryphonectria parasitca has been shown to harbour a second type of virus other than the *Hypovirus*. This is a *Mitovirus*. This ssRNA virus has been isolated from mitochondria of *C. parasitica* and belongs to the genus *Mitovirus* in the family *Narnaviridae* (Wickner *et al.* 2000). The type species of the genus is *Cryphonectria parasitica mitovirus 1-NB631* (CpMV1-NB631) (Wickner *et al.* 2000). Mitoviruses are naked viruses that lack a capsid and the genome contains a single open reading frame (Wickner *et al.* 2000)

The *C. parasitica mitovirus* is considerably smaller (2728 bp) than the *C. parasitica hypovirus* and encodes for a RdRp (Polashock & Hillman, 1994; Wickner *et al.* 2000). The genome is very A-U rich, with an A-U content of 63.4%. Polashock & Hillman (1994) have shown that this mitovirus reduces virulence of *C. parasitca* only slightly and that it is closely related to yeast cytoplasmic T and W dsRNAs. The *C. parasitica mitovirus* can be transmitted via hyphal anastomosis, asexual (conidia) or sexual spores (ascospores) (Polashock *et al*. 1997). However, ascospore transmission of the dsRNA only occurred when the donor strain was the female in the cross (Polashock *et al*. 1997). These findings confirmed those of Milgroom and Lipari (1993) which had shown that mitochondria are maternally inherited in *C. parasitica*. This mode of transmission to the ascospores does not occur for the members of the family *Hypoviridae* (Dawe & Nuss, 2001). This gives the mitoviruses a better chance to spread through the fungal population than the hypoviruses.

Mitochondrial dsRNAs similar to the *C. parasitica mitovirus* have been found in isolates of the Dutch elm disease fungus, *Ophiostoma novo ulmi* (Rodgers *et al*. 1987). The *O. novo ulmi* isolate in that study contained twelve dsRNA segments ranging from 0.33 kb to 3.5 kb in size (Cole *et al.* 1998; Rodgers *et al.* 1986, 1987). It was shown that the dsRNA predominantly occurs in the positive single stranded RNA form and encodes for a RNA-dependant RNA polymerase (RdRp) with an A-U content of 61.9% (Hong *et al.* 1998a; Cole *et al.* 2000).

Rodgers *et al*. (1986) indicated that the transmission of dsRNA via hyphal anastomosis in *O. novo-ulmi* resulted in the transmission of all the dsRNA segments. However, some of the conidial isolates reverted back to the healthy phenotype and thus lost some of the dsRNA segments (Rodgers *et al.* 1986). The sexual cross between a dsRNA-containing isolate, which acted as the female parent, and a dsRNA free isolate which acted as the male parent

showed that of the 20 selected ascospore progeny, 19 were dsRNA-free (Rodgers *et al.* 1986). This is contrary to a finding by Polashock *et al*. (1997) for *C. parasitica* where 46% of the ascospore progeny contained the dsRNA. A possible reason for the lack of transmission of dsRNA into ascospores of *O. novo-ulmi* might be that some of the dsRNA-containing mitochondria are respiratory-deficient due to a reduction in cytochrome oxidase levels, which may lead to the selection against these mitochondria during ascospore formation (Buck & Brasier 2002).

The genetic structure of the *O. novo-ulmi mitovirus* differs from that of other dsRNA and ssRNA viruses. The 5'and 3' terminal sequences of *O. novo-ulmi mitovirus* RNA-7 are inverted complementary repeats of each other, and could cause the ssRNA to form a panhandle (Hong *et al.* 1998b). In addition, it is possible that stem-loop structures and hairpin structures are formed (Hong *et al.* 1998b). Sequence comparisons of RNAs 3a, 4, 5 and 6 of *O. novo-ulmi* suggests that these RNA can form panhandle as well as stem loop structures, it was also shown that these RNAs are the genomes of four different viruses, which replicate separately in the cell (Hong *et al.* 1999). These structures may act as recognition sites for the RdRp to initiate RNA replication starts (Buck & Brasier 2002).

The *C. parasitica mitovirus* does not have a significant effect on phenotypic or confer hypovirulent characteristics. The virus-infected isolate appear similar in culture to the wild-type virus-free isolates (Polashock & Hillman 1994). Virulence was shown to be slightly reduced, but not to the levels associated with members of the *Hypovirus* genus (Polashock & Hillman 1994; Polashock *et al.* 1997). *Ophiostoma* mitovirus containing isolates are characterised by slow growth, abnormal "ameboid" colonies, a reduction in numbers of viable asexual spores and reduced levels of mitochondrial cytochrome oxidase (Brasier 1983; Rodgers *et al.* 1987). These characteristics should be taken into careful consideration when the viruses are destined as a possible biological control agent.

4. CONCLUSION

Cryphonectria cubensis is known to cause a serious stem canker disease on *Eucalyptus* in the tropics and sub-tropics. Currently, the most effective means to reduce the impact of this disease in South Africa is to plant *Eucalyptus* hybrid clones, which have been selected for disease tolerance. It is, therefore, necessary to understand the response of various *Eucalyptus*

clones to a range of *C. cubensis* isolates, representing the larger part of the fungal population. This is the most effective means to ensure that the best *Eucalyptus* planting stock, resistant to diseases, is deployed.

Biological control of Cryphonectria canker involving dsRNA mediated hypovirulence presents an exciting, if somewhat longer term opportunity to deal with this disease. In *C. parasitica* a range of viruses have been identified. Among these, the hypoviruses have shown characteristics that would make them effective bio-control agents. Mitochondrial viruses have also been identified in this fungus, but these have not displayed any significant effect on the host. The fact that *C. parasitica* and *C. cubensis* are closely related and since no virus has been characterised in *C. cubensis* the studies presented in this thesis were undertaken to find dsRNA genetic elements in the South African *C. cubensis* population. Studies also include those concerning the biology of *C. cubensis* and that might, in the future be useful in attempting biological control of the pathogen.

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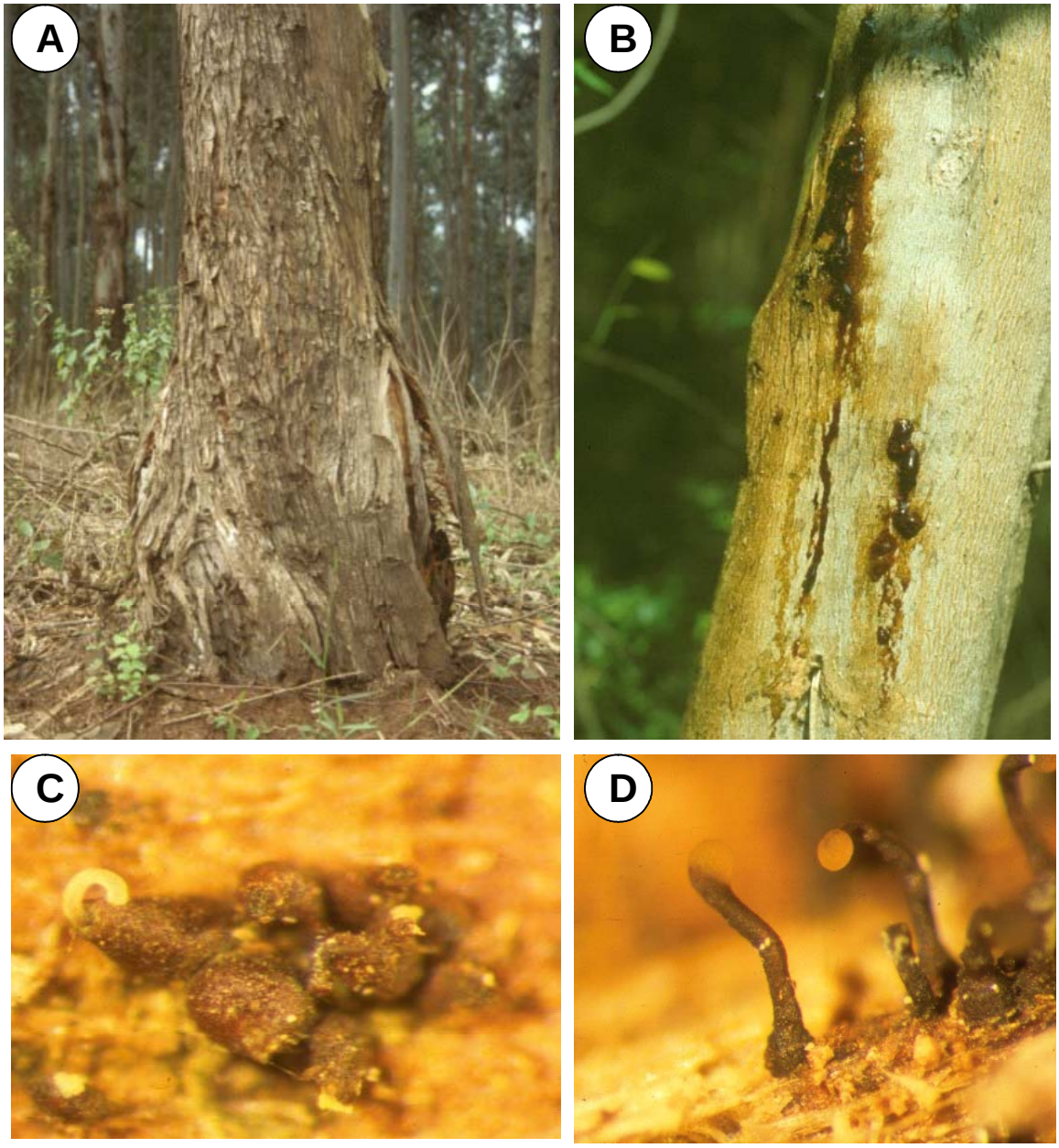

Figure 1: Symptoms and fruiting structures associated with infection by *Cryphonectria cubensis*. A. Basal canker. B. Kino exudation. C. Pycnidia. D. Perithecia.

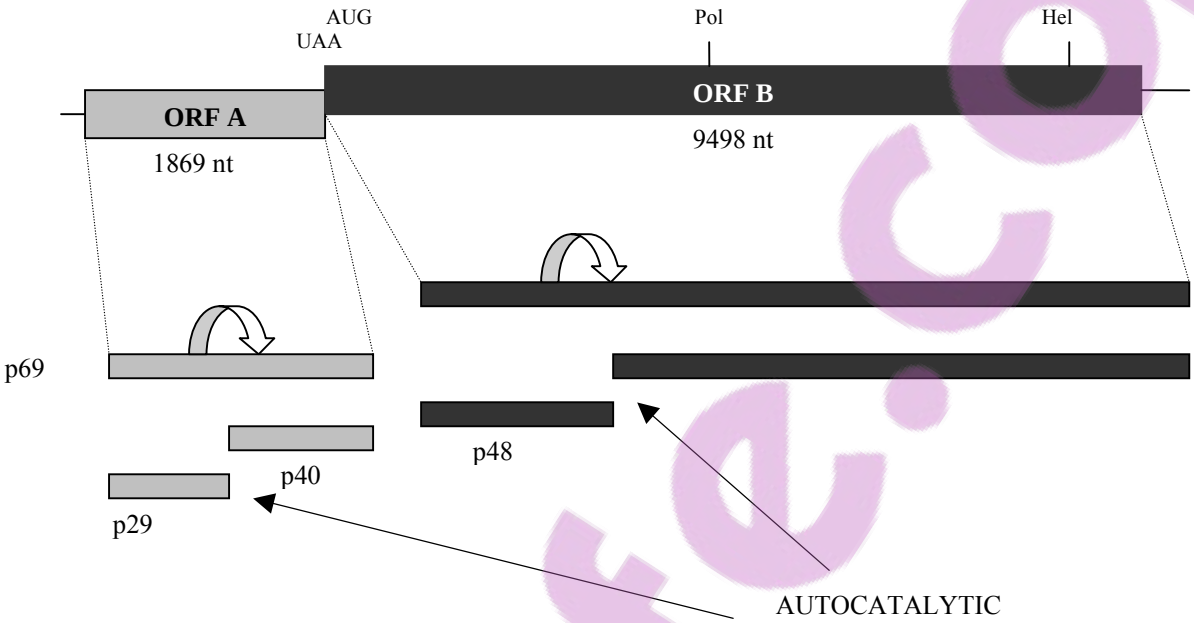

Figure 2: Genome organisation of the *Cryphonectria parasitica hypovirus* (CHV1-EP713). The genome is 12712 nucleotides in size and contain two open reading frames, ORF A and ORF B, which encode the polypeptides p29, p40 and p48. The positions of the polymerase (pol) and the helicase (hel) domain are indicated on ORF B. (The figure is according to Dawe & Nuss 2001)

Chapter 2:

Molecular characterisation of mitoviruses co-infecting South African isolates of the *Eucalyptus* canker pathogen *Cryphonectria cubensis*

ABSTRACT

Cryphonectria canker caused by *Cryphonectria cubensis* is an important disease of *Eucalyptus* trees in South Africa and other parts of the world. The importance of this disease has led to numerous studies on the biology, taxonomy and population biology of the causal agent. In this study the complete nucleotide sequence of double stranded (ds) RNA segments occurring in South African isolates of *C. cubensis* was determined. Sequence analysis led to the identification of two distinct viruses that are related to mitochondrial RNA viruses in the genus *Mitovirus*, family *Narnaviridae*. The viruses were thus provided with the names *Cryphonectria cubensis* mitovirus 1 (CcMV1) and *Cryphonectria cubensis* mitovirus 2 (CcMV2). The RNA genomes of the two viruses are 2601 bp and 2639 bp in size respectively and share a 22.6 % sequence identity at the protein level. Using the mitochondrial genetic code where UGA codes for a tryptophan, the RNA of CcMV1 encodes a putative protein of 744 amino acids (84255 Da) and CcMV2 a protein of 556 amino acids (63552 Da). These proteins probably function as RNA-dependant RNA polymerase (RdRp) since the conserved motifs for the RdRp could be identified. Northern blot hybridisations indicate that the viruses occur predominantly in the positive single stranded RNA form, rather than the dsRNA form. It was also shown that the viruses could be transmitted through the asexual spores, which is the predominant form of reproduction for the fungus in South Africa. Pathogenicity tests in this study suggest that the viruses have no significant impact on the virulence of infected isolates. This diminishes opportunities for applying these viruses for biological control of *C. cubensis.*

Genbank accession numbers of the sequences reported in this paper are: AY328476, AY328477, AY328478, AY328479, AY328480, AY328481

INTRODUCTION

Cryphonectria cubensis (Bruner) Hodges is an important pathogen of *Eucalyptus* spp. and causes substantial losses to plantation forestry. High temperatures and rainfall favour this disease, which is most serious in the tropics and sub-tropics (Hodges *et al.* 1979; Alfenas *et al.* 1983; Sharma *et al.* 1985; Florence *et al.* 1986). Cryphonectria canker was first reported in South Africa in 1989 and it has subsequently become important to implement effective control measures against this disease (Wingfield *et al.* 1989). Currently, the most effective means of reducing losses due to *C. cubensis* is through planting disease tolerant hybrid *Eucalyptus* clones (Alfenas *et al.* 1983; Wingfield 1990). It has, however, also been suggested that double stranded (ds) RNA linked to hypovirulence might form the basis of a strategy to reduce the impact of Cryphonectria canker in South Africa (van Heerden *et al.* 2001).

Double stranded RNA viruses are known to occur in many fungi. The best studied of these is the hypovirus that occurs in the chestnut blight pathogen *Cryphonectria parasitica* (Murr) Barr (Day *et al.* 1977). Day *et al.* (1977) have shown that this virus residing in the genus *Hypovirus* (*Hypoviridae*) causes a significant reduction in virulence of the fungus and could, therefore, be a suitable agent for biological control (Hillman *et al.* 2000). The elucidation of the complete nucleotide sequence of the *C. parasitica hypovirus* has enabled researchers to expand the viral host range by transfecting other fungi in the genera *Cryphonectria* and *Endothia* (Shapira *et al.* 1991; Chen *et al.* 1996). A South African *C. cubensis* isolate has also been transfected with the *C. parasitica hypovirus*, leading to effective hypovirulence in transfected strains (van Heerden *et al.* 2001).

Cryphonectria parasitica has been shown to harbour a second virus type, other than the hypovirus. This ssRNA virus occurs in the mitochondria and belongs to the genus *Mitovirus* (Mitochondrial) residing in the family *Narnaviridae* (Naked RNA virus) (Polashock & Hillman 1994; Wickner *et al.* 2000). Mitoviruses are naked viruses without capsids and with a genome containing one open reading frame (Wickner *et al.* 2000). The genome of the type species, the *Cryphonectria parasitica mitovirus 1-NB631* (CpMV1-NB631) is 2728 bp in size and encodes for a RNA-dependant RNA polymerase (RdRp) (Polashock & Hillman 1994; Wickner *et al.* 2000). This virus slightly reduces virulence in *C. parasitica*, but not to the levels observed with the hypoviruses (Polashock & Hillman 1994). This virus can be

transmitted through the fungal population via hyphal anastomosis, as well as through the asexual conidia or the sexual ascospores (Polashock *et al.* 1997). Ascospore transmission has been observed only when the donor strain was the female in a cross between isolates (Polashock *et al.* 1997). This is consistent with the maternal inheritance of the mitochondria in *C. parasitica* (Milgroom & Lipari 1993). Transmission both through asexual as well as sexual spores would enhance opportunities of the establishment of mitoviruses in a fungal population.

Double stranded RNA elements have previously been observed in the *Eucalyptus* pathogen *C. cubensis*. Van Zyl *et al*. (1999) screened a large number of Brazilian *C. cubensis* isolates for the presence of dsRNA, and discovered a 3 kilo base pair dsRNA element in some isolates. The virus infected isolates showed altered colony morphology as well as a reduction in virulence, suggesting hypovirulence (van Zyl *et al.* 1999). It is known that *C. cubensis* in Brazil reproduces sexually and that the population diversity of this fungus in that country is high (van Zyl *et al.* 1998). This would reduce the ease with which viruses could spread through the pathogen population (Anagnostakis 1977). This low level of spread is also consistent with the fact that low numbers of isolates were found to be virus infected (van Zyl *et al*. 1999). In South Africa, *C. cubensis* has a low genetic diversity and sexual reproduction is extremely rare (van Heerden & Wingfield 2001). This would enhance opportunities to utilise hypovirulence to reduce the impact of Cryphonectria canker in South Africa.

The objectives of this study were, to screen the South African *C. cubensis* population for the presence of dsRNA elements, using slow growth as a selection criterion. The complete nucleotide sequence of the genomes was then determined for the two mitoviruses that were subsequently discovered. Once these sequences have been deduced the open reading frames were analysed to ascertain homologies to other viruses. Since mitoviruses are known to be ssRNA, it was important to established whether the *C. cubensis* viral genome was single stranded. Furthermore, relative abundance of the viruses in the fungal population, quantification of the viruses in a single *C. cubensis* isolate, transmission of the viruses to asexual spores and the pathogenicity of mitovirus – containing isolates were considered.

MATERIALS AND METHODS

Isolate selection and growth studies

One hundred bark samples from trees infected with *C. cubensis* were randomly collected in KwaZulu-Natal, South Africa. The fungus was induced to sporulate and isolations were made using the method previously described by van Heerden & Wingfield (2001). All isolates used in this study are maintained in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria.

One hundred *C. cubensis* isolates were inoculated onto 2% Malt extract agar (MEA) and incubated at 25ºC for 7 days at normal illumination cycles (12 hours light and 12 hours darkness). A 5 mm diam. mycelial plug from each of these isolates was inoculated in the middle of two 9 cm diam. Petri dishes containing 2% MEA. The plates were incubated at 25ºC. Colony diameters were measured four days after inoculation. Seven slow growing (CMW11325, CMW11332, CMW11341, CMW11353, CMW11354, CMW11342 and CMW11329) and two rapidly growing (CMW11355 and CMW11351) South African *C. cubensis* isolates were selected for dsRNA extraction (Table 1). These isolates were grown in Erlenmeyer flasks containing 2% Malt extract broth at 25°C. The mycelium was harvested after sufficient growth, freeze-dried and stored at -20°C before use.

Extraction and purification of dsRNA

Double stranded (ds) RNA was extracted using the method of Valverde *et al.* (1990) with modifications outlined by Preisig *et al.* (1998). One gram lyophilised ground mycelium was transferred to 40 ml centrifuge tubes into which 10 ml 2 x STE (0.2 M NaCl, 0.1 M Tris HCl, 2 mM EDTA pH 8, pH 6.8) and 1% SDS was added. Samples were mixed using a vortex mixer and subsequently incubated at 60°C for 10 min. After incubation, 10 ml Phenol (pH 7.5) was added. The solution was shaken at room temperature for 30 min and centrifuged in a Beckman JA25.50 rotor for 30 min at 15000 rpm. The aqueous phase was transferred to clean centrifuge tubes and 10 ml of chloroform was added. The samples were mixed with a vortex mixer before being centrifuged at 10000 rpm for 15 min. This step was repeated until the inter phase was free of protein. After the last extraction step, the aqueous phase was transferred to a clean centrifuge tube and 16% absolute ethanol was added and centrifuged at 5000 rpm for 5 min to remove the chromosomal DNA.

The supernatant was passed through CF11 cellulose columns, to isolate the dsRNA. The columns were prepared by packing 2 ml syringes (Promega) with 0.5 g CF11 cellulose (Whatman). The columns were washed with 10 ml 2 x STE containing 16% ethanol after which the dsRNA-containing samples were passed through. The cellulose bound dsRNA was washed with 10 ml 2 x STE containing 16% ethanol. The dsRNA was eluted with 8 ml 2 X STE in eight fractions collected in 1.5 ml Eppendorf tubes. The dsRNA was precipitated, washed with 70% ethanol, dried and resuspended in 50 µl 0.1% (v/v) diethyl pyrocarbonate (DEPC) treated double deionised water. The dsRNA found in isolates CMW11329 and CMW11332 was then separated using an agarose gel in the presence of ethidium bromide and visualised using UV light. The dsRNA segments were excised from the gel and purified using an RNaid kit with spin (BIO101 Inc., QBiogene Inc.). The purified dsRNA was stored at -20ºC until further use.

cDNA synthesis and RT-PCR

Purified dsRNA was used to determine the complete nucleotide sequence of the genomes of virus infected isolate CMW11329. The dsRNA was initially denatured for 10 min at 99ºC. cDNA was produced using the cDNA synthesis system (Roche Diagnostics). cDNA fragments were cloned in the vector pGEM®-3Zf(+) and transformed into *E. coli* JM 109 high efficiency competent cells (Promega).

Once a small number of cDNA clones had been sequenced, the remainder of the viral genome sequences were obtained by reverse transcription polymerase chain reaction (RT-PCR) experiments, using sequence specific 20 to 22-mer primers (MWG-Biotech, Inqaba Biotec) produced from the cDNA fragments. RT-PCR was done using the Titan one tube RT-PCR system (Roche Diagnostics). The reverse transcription was performed for 1 hour at 50ºC followed by the PCR reaction with the amplification conditions of a single cycle at 99ºC for 2 min, 10 cycles at 94ºC for 30 s, 60ºC for 30 s and 68ºC for 2 min. This was followed by another 35 cycles of 94ºC for 30 s, 60ºC for 30 s and 68ºC for 2 min with a cycle elongation of 5 s per cycle. A final elongation step of 10 min at 68ºC was included.

Determination of the distal ends of the viral genomes

The sequences of the distal ends of the dsRNA elements were determined by the RACE approach (Frohman 1994) using a 5'/3' RACE kit (Roche Diagnostics). cDNA was initially synthesised from the dsRNA at 55ºC for 1 hour with 1 U AMV reverse transcriptase. The

cDNA was purified using the High Pure PCR Product Purification Kit (Roche Diagnostics). A poly (A) tail was enzymatically added to the purified cDNA by the addition of 2 mM dATP in the presence of terminal transferase. The tailed cDNA was then used in a PCR amplification with the mixture containing a nested internal sequence specific primer and an oligo dT-anchor primer using the Expand High Fidelity PCR system (Roche Diagnostics). Amplification conditions for the PCR were 1 cycle at 94ºC for 2 min, 10 cycles at 94ºC for 20 s, 60ºC for 45 s and 72ºC for 40 s. This was followed by 25 cycles at 94ºC for 20 s, 60ºC for 45 s and 72ºC for 40 s with a time increase of 5 s per cycle. A final elongation step at 72ºC for 7 min was included. In some cases a secondary PCR was performed.

Cloning and sequencing of cDNA

The PCR products from both the RT-PCR and RACE-PCR protocols were gel-purified from the agarose gel using the PCR product purification kit (Roche Diagnostics). The purified products were ligated into the pGEM®-T Easy Vector (Promega), and incubated overnight at 4ºC. Two µl of the reaction mixture was transformed in 25 µl of JM 109 *E. coli* high efficiency competent cells (Promega). Positive clones were selected on LB medium (10 g/l Bacto®-Tryptone, 5 g/l Bacto®-Yeast extract, 5 g/l NaCl, 15 g/l Biolab agar) containing 0.5 mM IPTG, 80 µg/ml X-Gal and 120 µg/ml ampicillin. White colonies were selected and used to inoculate 2 ml LB medium containing 100 µg/ml ampicillin. The plasmids were isolated from the cells using alkaline lysis (Sambrook & Russell 2001). The insert size was determined by cutting the plasmids with the restriction enzyme *Eco*RI (Roche Diagnostics). The inserts were sequenced using universal primers SP6 (5' TATTTAGGTGACACTATAG 3') and T7 (5' TAATACGACTCACTATAGGG 3') to obtain both strands. The products were sequenced using the ABI PRISM Big Dye Terminator cycle sequencing ready reaction kit (Perkin Elmer). The sequenced products were analysed using an ABI 377 and ABI 3100 automated DNA sequencers (Applied Biosystems). The sequences were analysed using Sequence Navigator version 1.0.1 (Applied Biosystems) and CLUSTAL X multiple sequence alignment programs (Thompson *et al.* 1994). Analyses were also done with programs available on the NCBI (http://www.ncbi.nlm.nih.gov/BLAST) and ExPASY (http://www.expasy.ch) web pages. These included: BLAST, Translate, Protein machine and SIM + LALNVIEW. Genbank/EMBL and the Swiss-Prot databases were used for the homology searches.

The conserved domains of the aligned sequence were analysed using Phylogenetic Analysis Using Parsimony (PAUP) software (Swofford 1998). The heuristic search option based on parsimony with random stepwise addition and tree bisection reconnection was used. Gaps were treated as fifth character and confidence intervals using 1000 bootstrap replicates were calculated. *Saccharomyces cerevisiae Narnavirus* sequence (Rodriguez-Cousino *et al.* 1991) was used as an outgroup taxon.

Preparation of DIG labeled strand specific RNA probes

Plasmids containing cDNA, specific for the *C. cubensis* dsRNA elements were selected as templates to generate digoxigenin (DIG) labeled probes. These plasmids were linearised with *Spe*I and *Nco*I respectively, in the multiple cloning sites, before being subjected to a phenol/chloroform purification step to eradicate the RNase in the mixture. The *in vitro* production of positive and negative strand specific DIG labeled RNA probes was achieved by preparing a reaction mixture containing 200 ng of the linearised plasmid DNA, 1 X DIG RNA labeling mix (10 mM dATP, 10 mM dGTP, 10 mM dCTP, 3.5 mM DIG-11-UTP), 1 X transcription buffer, 0.5 U T7 RNA polymerase or SP6 RNA polymerase with a total reaction volume of 20 µl. The *in vitro* transcription reaction was performed for 2 hours at 30ºC. The probes were stored at -20ºC.

Northern blot hybridisations and colorimetric detection

Northern blot hybridisations were performed to determine whether *C. cubensis* mitovirus genomes are maintained in an ssRNA or dsRNA form. Total nucleic acid was extracted from the virus infected isolate (CMW11329) and a Colombian isolate (CMW11348). The positive and negative stranded RNA was produced in *vitro* with either SP6 RNA polymerase or T7 RNA polymerase as described previously. The isolated total nucleic acids and the *in vitro* produced RNA was separated on a 1% Agarose gel in 1 x TAE (0.04 M Tris Base, 0.001 M EDTA, 1.14ml/l Acetic acid) buffer at a current of 80 V for 45 min. The agarose gel was denatured for 30 min in a 50 mM NaOH / 150 mM NaCl solution before being neutralised twice for 15 min in a 1 M Tris-HCl (pH 7.5). The agarose gel was placed on a tray and overlaid with a positively charged nylon membrane (Roche Diagnostics) and covered with several Whatman filter papers (Whatman). The procedure was allowed to proceed for 3 hours to ensure the complete transfer of the nucleic acids to the membrane. Hybridisation with the DIG labeled strand specific RNA probes was carried out according to manufacturer's guidelines in DIG Easy Hyb buffer at 68ºC. Detection was achieved using

the DIG High Prime Labeling and Detection Kit I (Roche Diagnostics). Colorimetric-detection was done by adding freshly prepared colour solution (5 µl NBT/BCIP (18.75 mg/ml Blue tetrazolium chloride, 9.4 mg/ml 5-Bromo-4-Chloro-3-Indolyl Phosphate) added to 10 ml detection buffer) to the membrane and incubated in the dark until colour formation. The reaction was terminated by washing the membrane in 50 ml strerilised distilled H_2O.

Abundance of the mitoviruses

To determine whether the two *C. cubensis* mitoviral genomes occur singly or together in infected *C. cubensis* isolates and to determine their relative occurrence in a population of isolates, two sequence specific primer pairs were designed for the two viruses. These were ccmv62 (5' GCACCGATGTCTCGTAAGAG 3') and ccmv68 (5' GCCCCACTTGTCAATCATCC 3') for viral genome 1 (amplification product of 215 bp) and ccmv46 (5' CGTTGGGTCGCTAGCCCGTG 3') and ccmv59 (5' GACTCTTCAAGTCACTTGAC 3') for genome 2 (amplification product of 126 bp). Twenty *C. cubensis* isolates were randomly selected for this experiment. These isolates included 18 from South Africa and two from Colombia (Table 2).

Total RNA was extracted from lyophilised mycelium of all isolates using the High Pure RNA Isolation Kit (Roche Diagnostics). The RNA was stored at -70ºC until use. For detection of the viruses, a one step RT-PCR reaction was performed using a LightCycler (Roche Diagnostics) with the LightCycler- RNA Amplification Kit SYBR Green I (Roche Diagnostics). A reaction mixture was prepared that contained 0.75 ng total RNA, 0.5 µM primer ccmv 62 or ccmv 46, 0.5 µM primer ccmv 68 or ccmv 59, 1 x LightCycler-RT-PCR reaction mix SYBR green I, 6 mM $MgCl_2$, 0.4 µl LightCycler-RT-PCR enzyme mix and PCR grade sterile H_2O to a total volume of 20 µl. The cycle conditions were a reverse transcription reaction at 50ºC for 10 min, followed by a single cycle at 95ºC for 10 sec, 35 cycles at 95ºC for 0 sec, 62ºC for 4 sec and 72ºC for 16 sec. At the end of each PCR cycle, the amount of the amplified product was monitored by a single fluorescence reading of the ds DNA binding dye, SYBR Green I. A melting curve and cooling program was also added. The data were analysed based on the melting curves of the PCR products, which would indicate whether sequence specific products were obtained. The RT-PCR products are indicated by a sharp peak that is centered at the Tm of the product. Non-specific amplification products such as dimers tend to melt at lower temperatures over a broader

range. The final PCR products were also separated on a 1.5% agarose gel stained with ethidium bromide and visualised under UV light.

Quantification of the two mitoviruses in a single isolate

Quantification of the two mitoviral genomes in a single virus-infected *C. cubensis* isolate (CMW11329) was achieved by using a LightCycler (Roche Diagnostics). The same primer pairs as those discussed above were used. Purified dsRNA of isolate CMW11329 was used to produce cDNA using the cDNA Synthesis System (Roche Diagnostics) with AMV Reverse Transcriptase. For quantification, the LightCycler – FastStart DNA Master SYBR Green I kit was used (Roche Diagnostics). For the quantification reaction, a dilution series was prepared with the cDNA (1:1, 1:10, 1:100 and 1:1000 dilutions). A reaction mixture was prepared containing 5 mM $MgCl_2$, 0.5 µM primer ccmv 46 or ccmv 62, 0.5 µM primer ccmv 59 or ccmv 68 and 2 µl LightCycler – FastStart DNA Master SYBR Green I. Four µl template was added to this mixture, with the total volume being adjusted to 20 µl with sterile PCR grade water. The templates used were the 1:1 cDNA dilution, 1:10 cDNA dilution, 1:100 cDNA dilution and a 1:1000 cDNA dilution. The cycle conditions were 10 min at 95ºC followed by 35 cycles at 95ºC for 10 sec, 60ºC for 5 sec and 72ºC for 16 sec with the amount of product being determined after each cycle based on SYBR green I fluorescence. A melting and cooling program followed. Quantification of the difference in concentration between the two viruses was analysed using the LightCycler software (Roche Diagnostics). This was based on a standard amplification curve using the second derivative maximum analysis method. The standards used in this experiment were the dilution series of CcMV2. The relative concentration of the viral amplicons could then be determined based on the standard curve. The experiment was repeated with independently extracted dsRNA and new cDNA synthesis.

Mitovirus transfer to conidia

To determine whether the mitoviruses were present in the asexual spores of the virus-infected *C. cubensis* isolate (CMW11329), it was first necessary to produce asexual fruiting structures. To achieve this, twenty freshly cut stem sections (15 cm in length) of a *Eucalyptus grandis* clone (ZG14) were inoculated with the fungus as described by van Heerden & Wingfield (2001). Mass conidial cultures were made and transferred to Erlenmeyer flasks containing 100 ml 2% ME broth and incubated at 25ºC for 1 week. After sufficient growth, the mycelium was harvested and lyophilised. Total RNA was isolated

from the different isolates using the High Pure RNA Isolation Kit (Roche Diagnostics). The presence of the viruses in the isolates was detected using specific primers for CcMV1 and CcMV2 in a RT-PCR amplification using the LightCycler (Roche Diagnostics). Amplification conditions were the same as those described above. The products were separated on a 2% agarose gel stained with ethidium bromide and fragments visualised using UV light.

Pathogenicity tests

A greenhouse inoculation trial was conducted to determine whether the mitoviruses present in isolates of *C. cubensis* have an influence on pathogenicity of the fungus. *Eucalyptus grandis* clone ZG14, which is known to be highly susceptible to *C. cubensis* infection (van Heerden & Wingfield 2001) was used for the inoculations. The trees were planted in 2 litre planting bags and maintained under shade nets until they were approximately 1 m high. The plants were moved to a greenhouse and allowed to acclimatise prior to inoculation. Eleven South African *C. cubensis* isolates including those known to harbour mitoviruses or to be free of these mitoviruses were used for the inoculation, and sterile 2% MEA disks were used for the control (Fig. 2). The *C. cubensis* isolates were grown on 2% MEA plates.

Trees were inoculated by removing a cambial disc from the main stem with an 8 mm diam. cork borer. Similar sized discs from the actively growing margins of the fungus were placed mycelium side facing towards the wounds and covered with Parafilm to reduce desiccation. Ten trees were inoculated with each of the isolates and an additional ten trees were inoculated with a sterile MEA disc to serve as controls. After 4 weeks, the Parafilm covering the wounds was removed and the lesion lengths were measured. The differences in lesion length associated with the test isolates were analysed using a one way ANOVA. A Bonferroni pairwise comparison was used to determine differences between the isolates.

RESULTS

Growth studies

The average colony diameter after 4 days for all of the 100 isolates of *C. cubensis* initially tested in this study was 35 mm (± 0.85 SEM). Of these, seven isolates (CMW11325, CMW11332, CMW11341, CMW11353, CMW11354, CMW11342 and CMW11329) with the slowest mean growth and two isolates (CMW11355 and CMW11351) that grew most

rapidly, were selected for dsRNA isolation (Table 1). Two (CMW11329 and CMW11332) of these seven isolates contained dsRNA elements and reached a mean diameter of 16 mm and 24 mm in 4 days respectively (Table 1). No dsRNA was detected in the remaining seven isolates (CMW11325, CMW11355, CMW11341, CMW11353, CMW11354, CMW11342 and CMW11351), which in 4 days, attained a mean colony diameter of 22, 49, 22, 22, 23, 18 and 51 mm respectively.

Determination of the complete nucleotide sequence and sequence analysis of the genomes

The dsRNA element from the South African *C. cubensis* isolate (CMW11329) was approximately 2600 base pairs in size (Fig. 1). Using the cDNA synthesis system, random cDNA fragments of the viral dsRNA were produced. Cloning of the cDNA fragments yielded plasmids with inserts ranging from 100 -500 bp in size. A BLAST search showed homology of these fragments to the *Cryphonectria parasitica mitovirus* and other mitoviral genomes.

The translation of the nucleotide sequence to an amino acid sequence and subsequent alignment to the *C. parasitica* mitoviral RdRp indicated the probable positions and orientation of the cDNA fragments in the genome. The entire genome sequence was obtained and two distinct mitoviral genomes were detected within the single *C. cubensis* isolate. These have been given the names CcMV1 and CcMV2 (*Cryphonectria cubensis* mitovirus) respectively.

The terminal 5' and 3' end sequences of the genomes were determined by the 5'/3' RACE approach. The sequence data showed a poly-A-tail at the 3' end and a poly-U-tail at the 5' end for both the genome sequences (Appendix 1 & 2). It was further shown that different possible ends exist for both mitoviruses (Appendix 1 & 2). This led to the establishment of the *C. cubensis* mitovirus (CcMV) genomes namely CcMV1a (2555 nucleotides, AY328476), CcMV1b (2601 nucleotides, AY328477), CcMV1c (2501 nucleotides, AY328478), CcMV2a (2639 nucleotides, AY328479), CcMV2b (2257 nucleotides, AY328480) and CcMV2c (2419 nucleotides, AY328481). For further analysis, the largest fragment for each of these two viruses (CcMV1b and CcMV2a) were selected. These are, hereafter, referred to as CcMV1 and CcMV2. These virus genomes are A-U rich with, CcMV2 having a A-U content of 62.4% and CcMV1 having a A-U content of 51.8%.

The homology of the *C. cubensis* mitoviruses to the *C. parasitica* mitoviral RdRp suggests that the viruses are located in the mitochondria. The genetic code for the mitochondria was used when the nucleotide sequence was translated to the amino acid sequence. In the mitochondrial genetic code UGA codes for a tryptophan. Since there are various possible initiation codons for mitochondrial open reading frames, we used the first methionine after the stop codons in the amino acid sequence to locate the start codon. The data showed a single large ORF for CcMV1 and CcMV2. The ORF of CcMV1 has the potential to encode a protein of 744 amino acids (Molecular mass: 84255 Da) (Appendix 3, position 184 to 2418 in Appendix 1) and CcMV2 has the potential to encode a protein of 556 amino acids (Molecular mass: 63552 Da) respectively (Appendix 4, position 893 to 2563 in Appendix 2). Complete amino acid sequence identity comparisons using the SIM alignment tool (www.expasy.ch) indicated that CcMV1 and CcMV2 are 22.5% identical with CcMV2 having a 40.5% identity to the *C. parasitica mitovirus* (CpMV1-NB63) (Table 3). The alignment of the amino acids showed conserved domains as described by Hong *et al.* (1999) (Fig. 3). These are typical of RdRp, suggesting that the ORF probably encodes for a functional RdRp (Poch *et al.* 1989). Parsimony analysis using amino acid sequence within the conserved area of the viruses in the *Mitovirus* genus showed that CcMV2 and CpMV1-NB63 grouped together in one clade. CcMV1 grouped with the *O. novo-ulmi* mitoviral segment (OnuMV3A), but away from CcMV2. All these viruses grouped within the genus, *Mitovirus* with the *Narnavirus* forming an outgroup (Fig. 4).

Northern blot hybridisations

Hybridisation with the probe for the positive-stranded RNA of CcMV2 resulted in an intense signal in the lane where the total nucleic acid for the virus infected (CMW11329) isolate was loaded (Fig. 5 A). A fainter signal was observed in the lane where the dsRNA was loaded (Fig. 5 A). This dsRNA was isolated using CF11 cellulose column chromatography. The negative strand specific probe resulted only in a signal in the lane where the purified dsRNA was loaded (Fig. 5 B). No signal was detected in the lane where the total nucleic acid of the virus infected isolate was loaded (Fig. 5 B). No hybridisation occurred in the lanes where the virus-free isolate was loaded, for either probe. For CcMV1 the positive strand specific probe gave a signal for the lane where the total nucleic acid of the virus infected isolate was loaded (Fig 5 C). No signal was observed for the negative strand specific probe in the lane where the total nucleic acids of the virus infected isolate was loaded (Fig. 5 D). Both the probes gave a signal in the lanes where the purified dsRNA was loaded but this was less intense

where the negative stranded specific probe was used for CcMV1. No signal was observed in the lanes where the virus-free isolate was loaded. These results showed that the positive stranded RNA molecules are present in a much higher concentration than the dsRNA, in the virus-infected isolate. This was true for both the *C. cubensis* mitoviruses, CcMV1 and CcMV2.

Abundance of the mitoviruses

Separation of the PCR products using the viral specific primers for the two viral genomes on an agarose gel confirmed the presence of either a 215 bp amplicon for CcMV1 or a 126 bp amplicon for CcMV2 (Fig. 6). Of the 20 isolates tested, 9 were shown to contain CcMV1 and 11 isolates contained CcMV2 (Table 2). Some isolates had smaller peaks on the melting curve as well as different intensity of the amplicons on the gel, suggesting that the viruses occur in different concentrations in the different isolates (Fig. 6).

Quantification of the mitoviruses in a single C. cubensis isolate

cDNA was successfully produced from the viral RNA of isolate CMW11329. The Tm of the CcMV1 amplicon produced using the primers ccmv 62 and ccmv 68 was 84.5ºC while the Tm of the CcMV2 amplicon using primers ccmv 46 and ccmv 59 was 87ºC. From the profiles on the real time PCR, it was possible to distinguish the presence of the different viral amplicons as well as the relative abundance of these products in the fungus (Fig. 7). The average ratio of the relative concentration for CcMV2 vs. CcMV1 was 1**:**4.9 (Table 4). This experiment was repeated and the ratio was 1**:**3.9. The overall average ratio was thus 1:4.4.

Mitovirus transfer to conidia

Six weeks after inoculation of the *E. grandis* stem pieces, pycnidia of *C. cubensis* were evident on the bark. RT-PCR reactions showed that viruses were present in most of the mass conidial cultures derived from them (Fig. 8). This established that the viruses are transmitted to the asexual spores of the fungus.

Pathogenicity tests

All trees inoculated with the *C. cubensis* isolates developed distinct lesions 4 weeks after inoculation. No lesions were associated with the control inoculations. Lesion lengths differed significantly between the different isolates inoculated ($F=17.62$; $df=11$; $p<0.001$).

The lesion lengths of the virus-infected isolates (CMW11329, CMW11332 and CMW11326) did not differ significantly from the other isolates used in the inoculation study (Fig 2).

DISCUSSION

In this study we have established that fungal viruses commonly occur in South African isolates of *C. cubensis*. The complete nucleotide sequences produced from the dsRNA elements have shown that these are mitochondrial viruses in the genus *Mitovirus*, family *Narnaviridae*. Northern blot hybridisation showed that the genomes of these viruses occur predominantly as positive single stranded RNA viruses. The names *Cryphonectria cubensis* mitovirus 1 (CcMV1) and *Cryphonectria cubensis* mitovirus 2 (CcMV2) have been provided for these two viruses.

Mitoviruses such as those found in *C. cubensis* have previously been found in other fungi such as *C. parasitica* and *O. novo-ulmi* (Polashock & Hillman 1994; Rodgers *et al.* 1986, 1987). Our discovery of two different mitoviruses in a single isolate of *C. cubensis* is also not unusual because 12 elements of virus-like dsRNA have been identified in *O. novo-ulmi* (Rodgers *et al.* 1986, 1987; Cole *et al.* 1998). In this study it was shown that in some isolates the *C. cubensis* mitoviruses do not occur together. For the *O. novo-ulmi* dsRNA elements, it has been shown that some do not cause the degenerate disease in the *O. novo-ulmi* isolate alone, but that they require other dsRNA elements to do so (Rodgers *et al.* 1986; Cole *et al.* 1998).

The *C. cubensis* mitovirus (CcMV) genomes range in size from 2257-2639 bases. This is in comparison to the *C. parasitica mitovirus* genome (Polashock & Hillman 1994) which is 2728 bases, and is similar in size to the CcMV's. In *O. novo-ulmi*, the virus like dsRNAs range from 330 bases - 3500 bases in size (Rodgers *et al.* 1986, 1987; Cole *et al.* 1998). The mitoviruses identified in this study are, therefore, similar in size to some other known mitoviruses and especially to that in *C. parasitica*.

The CcMV2 genome was found to have an A-U content of 62.4%. This is consistent with findings for other mitoviruses such as the *O. novo ulmi* mitoviruses which all have an A-U % of higher than 60% (Hong *et al.* 1998a; Hong *et al.* 1999). The *C. parasitica mitovirus* has a A-U % of 63.4% (Polashock & Hillman 1994). In contrast, CcMV1 has a lower A-U % of

51.8%, which was unexpected and is similar to the A-U % of the *Rhizoctonia solani* dsRNA virus which is 56.8 % (Hong *et al.* 1998a; Jian *et al*. 1997). The high A-U content of the mitoviruses is comparable with the plant mitochondrial genomes (Unseld *et al.* 1997) and confirms the mitochondrial localisation of the mitoviruses.

Using the mitochondrial genetic code where UGA codes for tryptophan, it was possible to identify the open reading frames of the two mitoviruses discovered in this study. We could thus show that CcMV1 possibly encodes for a single protein of 744 amino acids in size. CcMV2 apparently encodes for a single protein of 556 amino acids in size. The alignment of these amino acid sequences with protein sequences encoded by other mitoviruses showed the presence of six conserved RNA dependant RNA polymerase (RdRp) motifs (motifs I-VI). This is similar to motifs described by Poch *et al.* (1989). Similar motifs II, III and IV have also been identified by Habili & Symons (1989) for positive stranded RNA plant viruses. Motif VI is also similar to motif 7 described by Bruenn (1993). The motifs (II-VI) identified in this study are all similar to those that have been identified for the *O. novo-ulmi* mitoviruses (Hong *et al.* 1998a, 1999). Motif I is not found in all the RdRp's of other RNA viruses, but it is characteristic of the RdRp, which is encoded by the mitochondrial viruses (Hong *et al.* 1998a, 1999). This provides further evidence that the *C. cubensis* viral genomes are of mitochondrial origin and have a single ORF that encodes for a RNA dependant RNA polymerase.

The *C. cubensis* mitovirus genome contains a poly-A tail at the 3' end and a poly-U tail at the 5' end. This could promote secondary structure development that would protect the naked single stranded RNA from degradation. It has been shown that the *O. novo-ulmi mitovirus* 7 can form a very stable stem loop structure since the 5' terminal sequence and the 3' sequence contain complementary sequences, consequently a pan handle can also form (Hong *et al.* 1998b). These complementary 5' and 3' terminal sequences are a characteristic of negative stranded RNA viruses such as the *Myxoviridae* (Desselberger *et al.* 1980), but stem loop structures are known to occur for the positive single stranded RNA viruses (Buck 1996).

Sequence alignment and parsimony analysis of the ORF showed that CcMV2 groups together with the *C. parasitica mitovirus*. CcMV1 grouped most closely with the *O. novo-ulmi* mitovirus 3. Interestingly, CcMV1 did not group with CcMV2 from the same fungus but still falls within the group accommodating mitoviruses. This low amino acid identity (22.6%) of

the two *C. cubensis* mitoviruses suggests that they might have different origins. Polashock & Hillman (1994) showed that the *C. parasitica mitovirus* is ancestrally related to the T (23S) and the W (20S) *Saccharomyces cerevisiae* RNA replicons as well as to positive stranded RNA bacteriophages of the *Leviviridae*. Hong *et al.* (1998a) used a large set of isolates to show that mitochondrial viruses formed a well defined clade within this set of isolates based on the relationship between the viral RdRp sequence. They could further distinguish between two lineages in the RNA viruses based on RdRp sequence. One of these lineages includes the mitoviruses, yeast RNA replicons and the *Leviviridae* and the other consists of all the other viruses (Hong *et al.* 1998a).

Both of the *C. cubensis* mitoviruses could be transmitted via asexual spores. The majority of the mass conidial cultures tested in this study contained the viral genomes. Transmission via asexual spores suggests a reasonably easy route of transfer, which is consistent with our finding that viral genomes were present in approximately half the isolates screened. Mitoviruses have previously been reported to also be transmitted to ascospores of *C. parasitica* (Pollashock *et al.* 1997) and of *O. novo-ulmi* (Rodgers *et al.* 1986). As the South African *C. cubensis* population has a narrow genetic diversity and apparently only reproduces asexually (van Heerden & Wingfield 2001), it was not possible to test whether transmission to ascospores is possible for CcMV1 and CcMV2.

Greenhouse inoculations showed that isolates of *C. cubensis* infected with the *C. cubensis* mitoviruses are equally pathogenic as virus-free isolates. This is in contrast to the *C. parasitica* mitovirus that reduces levels of virulence slightly although its impact is not equivalent to that of the *C. parasitica hypovirus* (Polashock & Hillman 1994; Polashock *et al.* 1997). The *C. cubensis* mitoviruses will, therefore, not be suitable for biological control of Cryphonectria canker in South Africa

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Table 1: Eight slow growing and two fast growing South African *C. cubensis* isolates that were selected for dsRNA extraction.

Isolate number	Mean colony diam (mm)[a]
CMW11325	21.85
CMW11332	23.92
CMW11355	48.79
CMW11341	21.78
CMW11353	21.61
CMW11354	23.36
CMW11342	17.67
CMW11351	50.99
CMW11329	16.41

[a] Mean colony diameter calculated from two replicate plates 4 days after incubation at 25°C.

Table 2: Isolates of *C. cubensis* screened for the presence of CcMV1 and CcMV2.

Consecutive number[a]	Isolate number[b]	CcMV1[c]	CcMV2[c]
1	CMW11317[b]	N	N
2	CMW11348[b]	N	N
3	CMW11340	N	N
4	CMW11342	N	N
5	CMW11324	N	**Y**
6	CMW11329	**Y**	**Y**
7	CMW11337	N	N
8	CMW11338	N	N
9	CMW11345	N	**Y**
10	CMW11323	**Y**	**Y**
11	CMW11332	**Y**	**Y**
12	CMW11328	N	N
13	CMW11326	**Y**	**Y**
14	CMW11350	N	N
15	CMW11334	**Y**	**Y**
16	CMW11325	**Y**	**Y**
17	CMW11336	N	N
18	CMW11320	**Y**	**Y**
19	CMW11343	**Y**	**Y**
20	CMW11321	**Y**	**Y**

[a] Consecutive numbers which refers to the lanes in Figure 6

[b] Colombian *C. cubensis* isolates

[c] The presence of the viruses in the isolates are indicated with a Y (yes) for present and a N (no) for absent

Table 3: Comparison of amino acid sequence identities (%) of some known mitochondrial viruses[a]

	CcMV2	CpMV1-NB63	OnuMV3A	OnuMV4-LD	OnuMV5-LD	OnuMV6-LD	ScNV-20S
CcMV1	22.6	21.7	25.3	25.1	23	23.9	19.2
CcMV2	100	40.5	25	26.7	26.2	24.7	17.8

[a] *Ophiostoma novo-ulmi*, OnuMV3A (Hong *et al.* 1998a), OnuMV4-LD, OnuMV5-LD, OnuMV6-LD (Hong *et al.* 1999), *Cryphonectria parasitica* CpMV1-613 (Polashock & Hillman 1994), *Saccharomyces cerevisiae* ScNV (Rodriguez-Cousino *et al.* 1991)

Table 4: Relative concentrations and ratios of concentration between CcMV2 and CcMV1 in isolate CMW11329.

Experiment number	Dilution	Relative concentration CcMV2 (Units)	Relative concentration CcMV1 (Units)	Ratio CcMV2:CcMV1
1	1:10	100.6	311.9	1:3.1
1	1:100	9.9	49.4	1:4.9
1	1:1000	1	6.8	1:6.8
2	1:10	60.9	201	1:3.3
2	1:100	10	34.3	1:3.4
2	1:1000	1	5.1	1:5.1

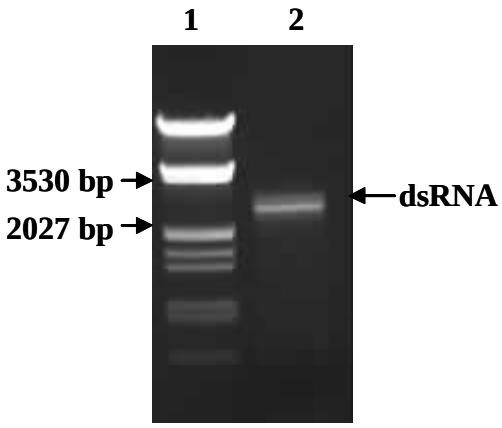

Figure 1: Double stranded RNA isolated from a South African *Cryphonectria cubensis* isolate (CMW11329). Lane 1: Lambda DNA marker cut with *Hin*dIII was used as an approximate size marker. Lane 2: dsRNA element isolated with a size of 2600 bp based on a DNA size marker. A 1% agarose gel stained with ethidium bromide was used. The fragments are visualised with UV light.

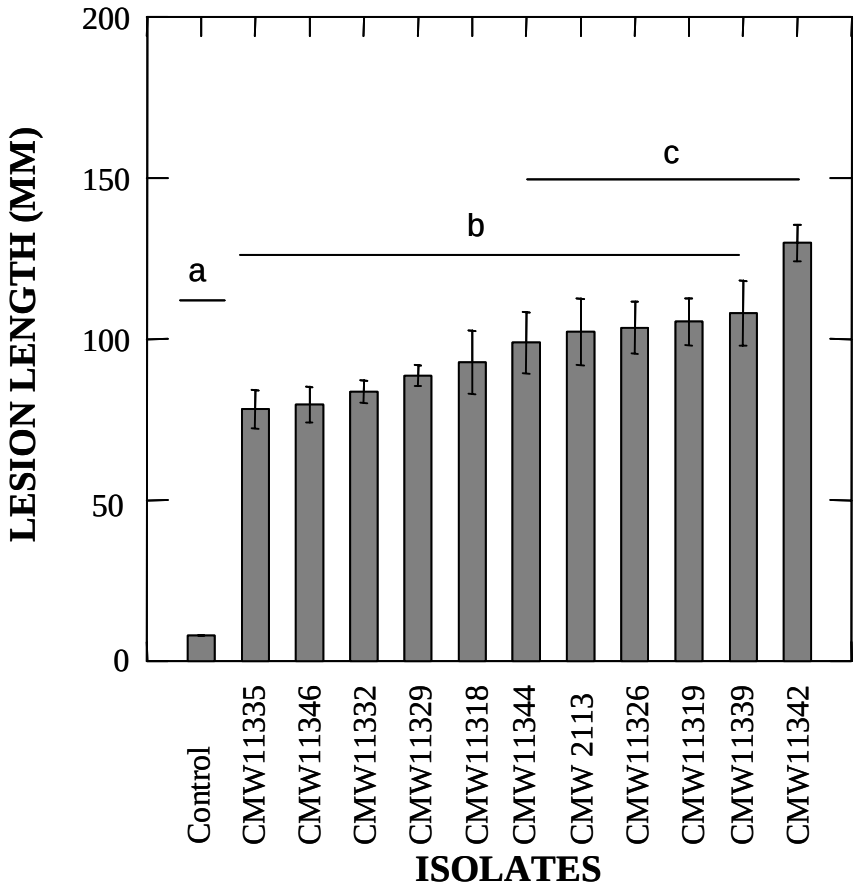

Figure 2: Mean lesion length (mm) (±SEM), 4 weeks after inoculation on *Eucalyptus grandis* clone (ZG14) with different South African *Cryphonectria cubensis* isolates. Isolate CMW11329, CMW11332 and CMW11326 are co-infected with CcMV1 and CcMV2 and the remainder of the isolates was free of virus. Columns marked with the same letter do not differ significantly from each other

Figure 3: Aligned amino acid sequences of the RdRp-like proteins encoded by coding domains of CcMV1, CcMV2 (this study) and that of the mitochondrial viruses from *Ophiostoma novo-ulmi*, OnuMV3A (Hong *et al.* 1998a), OnuMV4-LD, OnuMV5-LD, OnuMV6-LD (Hong *et al.* 1999), *Cryphonectria parasitica* CpMV1-613 (Polashock & Hillman 1994). CLUSTAL X was used to align the amino acid sequences. Symbols below the aligned sequence: (*) identical amino acids in all the sequences, (:) and (.) indicate higher and lower chemically similar residues. Conserved motifs are indicated in red and marked with I-VI (Hong *et al.* 1999).

```
CLUSTAL X (1.81) multiple sequence alignment


OnuMV4-LD       ------------------------------------------------------------
OnuMV5-LD       ------------------------------------------------------------
CcMV2           MTTQVTWQSXCTNPSQSTLPSQVRCAFGYNGTFHSDSRRVXWDNIKIRIARASDPTXXXI
CpMV1-NB631     ----------------------------MAMIIHDPVYKLWYAX---RAKSNLPGLAPH
OnuMV6-LD       ------------------------------------------------------------
OnuMV3A         ------------------------------------------------------------
CcMV1           ------------------------------------------------------------


OnuMV4-LD       ------------------------------------------------------------
OnuMV5-LD       ------------------------------------------------------------
CcMV2           TPNCIVKHLHXLINTKTQQHIQNYTTNLMQNWVXXEVTIFTCYFYXNHSSSSTKVLMSNV
CpMV1-NB631     KTESITTVLS-LTNVKNKQATN----SILLNKITDLVLGLKAFSIKSKGVRTTLRVNKHW
OnuMV6-LD       ------------------------------------------------MKLKQL
OnuMV3A         ------------------------------------MKRLTLSQNKSNQLTNNDLSNV
CcMV1           -------MTKADHYSSASSDPPRWTDCCHNTRQHQPTFAMKWIMTKTMKRNTNKTYMHT


OnuMV4-LD       MKRNNLQIIIIKRLILHIFKINLS----VEIDKFLGFINHLRKSNGLLYTIKYMKAAKLH
OnuMV5-LD       MKKIN---KTIKILLSIYFNRKYS----SYGIRWIVTVERMRKINGLKFTIKYMKAVKLH
CcMV2           MVRAGSPKRRFQNLSSWFVGVPVHSPIKDTLWRCRMDLSLSEMTTATHSSLSILKRXCVL
CpMV1-NB631     ITPKEFP--RFVKLVVWCTRTQEH---EDSFMKIIGKCDHIWQTAGPNFLFKYLKEVMRL
OnuMV6-LD       KLMKNKTYQIIRILLIVFFPSIKR--QTVILNIFMSKINKMIKNNGTLFTVKYLKELRLH
OnuMV3A         GYITKQLFPHWIRLLVWSLQLSPAP-----YKKFGSRIAILWKANGVSFTVQYLKECTRI
CcMV1           NTLRDIGAMKLSTVWFTHWSHTETGMASRTVSSAVARFQTTATTRGRNAAMSEFKASRTA
                             :                                   .    .. :*

OnuMV4-LD       ITRYMCGKPLYSNN-ENVALDKTGFP--LRFWYLKRLVN----DNPRALLTLLTYTRRIV
OnuMV5-LD       ITKYIANERLLSISGSRVSVDKDGFP--TKFNYIKHIIDSGDIDGIRFVMTLLTYTRAIN
CcMV2           QXEDWRMXTLFTVKKIFVKLNKYRFPAIIPTEICKDTSWFPG--RFHVLSRKVMATTTVI
CpMV1-NB631     SVRRIANIELEPSKKIFVKLNKFRFPNIIPLPICDQIIRDQNDQVLWASKRLIICLLTIL
OnuMV6-LD       ITKYISGEP-YRNSLNRVSVDKDGFP---TLCKELKVLVNGTYLEKRFVLTIITLSKLLI
OnuMV3A         VQHFVSGHPVFVTDVMPIGLAG-GLPTIIPGTLRTLLRSKDSSTIRGVLSTLAVYRIMKM
CcMV1           FTRWTCGRP--TSGKVGAPMTKAGTPKVIPREARTLLTRERPTYLVKAVMTVTSIGRYFK
                  .               :     *

OnuMV4-LD       PNKSES-KARIVKLSTITDPYKG-KVYTIPKWFILDFISKYNLSSTKP------IYTDND
OnuMV5-LD       PTKKEY-LKIFPDYSTITNEFTGSNKIAIPNKIIKEFVDYYKLANPNNNNNPELQWSRDD
CcMV2           SIFRVLPTKVLPDYTTISKPHSGTIETCSSESIALACKNTEIKRVDKVMKTRLKGSTKAG
CpMV1-NB631     SVHRVLPTKVVPDYSTIVDPFTGVSKTIDQKLLRKAIHLLNIKRV-KQLKLKITGSMKAG
OnuMV6-LD       PQKSES---IPFSTKSITDHWSGIDNISNEELDKSCSELNISTREVQWDVKNFKLLTKAG
OnuMV3A         P--------CVLKLESITDPFKGISDTLPKSEIINGLASLG-FEIPKGRSKHLLTLSNPI
CcMV1           GGN-------PVKWENITKPSTPTTPKDGEITFGLEKLNIDVGQFEPTDWKFRWVVTAGP
                            .  .* .  .
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```
OnuMV4-LD       HYLSIKGSPNGKASMSS---LYSIIS--FNSSNIRYLFNIVG--DYQLVLNKFYQDLSQF
OnuMV5-LD       HYLSFKSSPNGQSTLHSSYGLFSMIF--VGHTILENILKIVGEKQYHEIIGNHIKKLYHD
CcMV2           PNGKMSLTTSLLDATAFWSDPLRVIH--FIWFNIRCYGYFWGTMWSMWLIFIMIISLPYY
CpMV1-NB631     PNGKISLLTSSVDALSFITQPTKIFT--YLDFSVRVY-KFRGLLLWMWMMCILLITLPYA
OnuMV6-LD       PHGPQSLTWYHTIKLYDFNQWLGIIG--ILPKSVLDLFTETLSYASKLVLPEIKSNIKS-
OnuMV3A         IYLLSAGPNHSISMMGIWKDIYAWYVSPLFPTLLSFIGRMNRGNVLIDLLRAEVSYWEAT
CcMV1           NGPSMSSCLQDTPKFNG------------TFRSQVEVITPETTPIIDTTLTWEKSFKTS

                                                I
OnuMV4-LD       YTKYIN-RDKLGLGKLSIVHDPELKERVIAMVDYTTQFALRPIHNILLNNLSKLPCDRTF
OnuMV5-LD       HRLFIPGKIDYLFGKISIVKDPELKMRVIAMVDYHSQFVLKKIHNSLFNKLKLIKSDRTF
CcMV2           TMALCTGARAPVMGQLATVYDQAGKARIVASTNSWIQCSTFGLHNKIFSILRSIPQDGTF
CpMV1-NB631     IVSFMLGALIPIMGKLSVVYDQAGKARIVAITNSWIQTAFYSLHLHVFKLLKNIDQDGTF
OnuMV6-LD       ---------TKLIRRLSIVHDPECKERVIAIFDYGSQMVLKPIADVLFDLLRNIPSDRTF
OnuMV3A         GVKPSVSPLDLKLGKLAIKEEAAGKARVFAMADSITQSVMAPLNSWVFSKLKDLPMDGTF
CcMV1           TTMGLAGFKDDSTRKMAIKDDREGKSRPFAMFDYWSQTVLSPTHDWAYATTRSIPQDCTF
                              :::   :   * * .*  :   *               :  * **

                                        II
OnuMV4-LD       TQDP-------FHKWNDDHKERYHSLDLSAATDRFPIFLQQKLISLIFNDYEFGKNWRNL
OnuMV5-LD       TQDP-------IFTT-PTMGHRFWSMDLSAATDRFPIDLQERLLSYLYG-SEISSAWKQL
CcMV2           DQNKPF---DLLLEST-QPGYMLYGFDTSAATDRTPIAFQKDITNHLG---YPGGPWRRL
CpMV1-NB631     DQERPF---KLLIKWLNEPTQKFYGFDLTAATDRLPIDLQVDILNIIFK-NSPGSSWRSL
OnuMV6-LD       TQSP------FFTHTDLDNKSKFWSIDLSSATDRFPIVFQKRVLQKILG-KQMTDSWERI
OnuMV3A         NQQAPLNRLVQLYQDGLLHDVEFYSYDLSSATDRLPMAFQKQIISVLFG-SKFAKDWATL
CcMV1           NQAEG-----TSKVTARPSQKYFYSYDTEAATDRFPMQFQKKVTSTIFN-TTYAQAWAEM
                 *                       . *  :**** *: :*  : . :         *  :

                                        III                             IV
OnuMV4-LD       LVDRNYDY--QGISYRYSVGQPMGAYTSWAAFTLTHHLVVHWAAELAGLKN-FKDYIILG
OnuMV5-LD       LIDRTYKTP-EGDELHYKVGQPMGAYSSWAAFTLTHHLVVFYSARMAGIKD-FTNYILLG
CcMV2           TGIKYNSP---CGFISYAVGQPMGAYSSFAMTATTHHVLVQVAAQKAGFSDRFTDYCITG
CpMV1-NB631     LRIKYKSP---QGFLTYAVGQPMGAYSSFAMLALTHHVIVQVAALNSGFTTRFTDYCILG
OnuMV6-LD       MIGSKFLAP-DGDTVSYNCGQPMGAQSSWPMFTLAHHVIVRVAANRCGLSN-FDKYIILG
OnuMV3A         LVGRDWYL--KDIPYRYSVGQPMGALSSWAMLALSHHVIVQIAAMRVGKLP-FTNYALLG
CcMV1           MTQEPFRVKGLSDPLRWGAGQPTGAKSSWAIFTLCHHTVVHMAAVRTNSDP--YYVMTG
                              :  *** ** :*:.  :  ** :*  :*   .       * : *

                                        V               VI
OnuMV4-LD       DDIVIKNNKVAQIYINLMTKWG-VDISLSKTHVSYDTYEFAKRWIK-NGKEISGISLKGI
OnuMV5-LD       DDIVINNDKVAKYYIRTMKRLG-VELSMNKTHVSKNTYEFAKRWFK-NKKEITGLPLRGI
CcMV2           DDIVMANSLVAEAYKSLIFDLG-LEISESKSVISGTFTEFAKKLRG-PTMDISPIGAGLM
CpMV1-NB631     DDIVIAHDTVASEYLKLMETLG-LSISSGKSVISSEFTEFAKKLKGRNNFDIFYRSWFSI
OnuMV6-LD       DDIVINNDNVALKYMEIMNDFK-VEISRNKTHVSNDTYEFAKRWIK-NKMEFFPLPIRGI
OnuMV3A         DDIVIADKAVATSYHMIMTQILGVEINLSKSLVSNNSFEFAKRLVT-MDGEVSAVGAKNL
CcMV1           DDMVTRGSRTATVYKRMMSETG-VSMSETKSHVSKDTFEFAKMWMHQGRNASGFPVVGTA
                **:*   . .*  *   :     :.:.  *: :*    ****

OnuMV4-LD       LTNIRHIHVVYMNIFTYLQR--IPSLN-VDILTCVGKLYGYLLIRNRIKS-PNTIKRSLY
OnuMV5-LD       LNNLNNYGIVFQELFKFHYK--YPHLTNVKLTDIMFIIFKGLKIKGRIIT-NSQLRFNLM
CcMV2           LYSLRNKYYICVLVFEITER--GLCMWYDVYPQLTSLLP---KIYRRYFK-TCDWFIATH
CpMV1-NB631     IHFEKQILHLCT-VFELLRR--GVCELYDLYPQYINKLP---KIYLRYNL-LIDWVVVAF
OnuMV6-LD       VDNINNKYIIFNILYSFFVEKGNTFLNKDTLLVCVSKFIQLHSLTLKKPIGLNKVKGILY
OnuMV3A         LVALKSRWGISSVILDLYNK--GLALSEQDLRQRFSSIPTVSKQFGVDKLLWLVLGPFGF
CcMV1           ETTRKPTEMAATFVFETPAKGYPVTITPRTVSQYFTTVARYNTIPPRTAVWTADKVVWYY
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OnuMV4-LD       DFHHSIRYSFGLLNYEEIRNYLHNKFPF--DNYYAWPERLVHSKLNEIFKLEMVESAKSF
OnuMV5-LD       KINFLLRYINKLVNFDETRLFYTKFIKS--EDISMVNEHNFLDFTRGMLKLGLTQKIENS
CcMV2           LRRREHTGDQDHEILNPRIAYFNVFLNK--EKIISLTEIMWNSTVRDWFRLWNSIKYTTN
CpMV1-NB631     TN-QILIGDRPRADG---IRLFDYFVGL--EVIPPLLRIMLHTIKKDWNGLWNSIKYTLN
OnuMV6-LD       PFNFMLRYRQNLCTNEEIRIFLGSSTCKRDDYMLPISAKDVSLELTRVISAALVGMAYNA
OnuMV3A         IPSKDGLSAFMKLNRSLSLVDMHILLSCVDEAKFDLDKKTWEANIQETVHTLLRFGMLSE
CcMV1           SFLSWTATRDDGWAKYIAQSASTLVSPNTAHDTTMKAVRDKWAKQTDKSTMDFQDFGFDM
                                                .

OnuMV4-LD       SKDFMN-----QSTMLINTVTDNEIMVQWPLYKGFMNHIEKLKDYIKSKQNQH-DIDLLD
OnuMV5-LD       VKELKTFYDDVLKNSFISNIENKNDLQYEPLINGLYNKMLIMRNSIDRIVRNK-DFDIID
CcMV2           KGTFMS--------QARVGTPDWSELIFFPLLPXTYIMIMSYATSTNDISKAFGNWWTTN
CpMV1-NB631     KGFVVS--------QVRVGLPDWTELFLLPILPSTYIIIRDYCRSFNDLTKLFGEWWLLR
OnuMV6-LD       EKSLKN---------IYFDLDKLSPWIGDGFKTGKHPKVMIQS--IYNSVKSLSDFGLKM
OnuMV3A         PAGFEV----------FSDFTSSPLYSFIRGQFGNKLSALVQDKPVRRLIFDGPLLHFNF
CcMV1           FDKVKT---------LPPFKPTWDPEAWPGRLSASGIEFNPAPRKVPMFAATQEEGEIKY
                   .

OnuMV4-LD       LMQNLRFQNLDSIVKKLRNSYTNLIMLDKFWKSAFNREYRDLERESILTIEKQESNMMSR
OnuMV5-LD       AMNDMRLDNPEAYLEKIKNSNKPLSNLNDMFN---------TAKKRIKEINEYNSEYFQD
CcMV2           S-TEKDQINIFDVIAMMERESMTDTDMNDKKK--------------VKLSLDNTYKLNS
CpMV1-NB631     FESESYQVSILDVIDRLAHTSIPNLDIHDKKK--------------VKLTLDNLYKLSL
OnuMV6-LD       AQNKLTLSAAMDSLLLVDLDSISSSERIKYIQ--------------MKQNICLSQKVRK
OnuMV3A         YTEGWCDGLMEHLTKKIQSDSQETVSPSNPFK--------------------DDKVILPL
CcMV1           SDYTQQKLEMTDDQTTFEEMESLKTPPRPQTKG--------------FTPKRTREYVRT
                              .              :                        .

OnuMV4-LD       IWDMALSYRTSPMSYSTLTFETDSEFYMMPSIWDMASSSTTGPKPFTTATFKTDFVGLST
OnuMV5-LD       IYDFDN-------------------------------------------------FSNFRP
CcMV2           MINRTG-------------------------------------------------AGTEI
CpMV1-NB631     IVNIPS-------------------------------------------------GGARR
OnuMV6-LD       ELRFDP-------------------------------------------------------
OnuMV3A         RGNIKG-------------------------------------------------------
CcMV1           TNTISHG------------------------------------------------------


OnuMV4-LD       FDDKLLKDLEN--LKIDITLRTGKYTNKTQPLESKIHQHPIECIEDNKVPNFNNIK
OnuMV5-LD       YESYYRAELST--QIDNLDMIRGAYWRDPQKETEMLQYW-----------------
CcMV2           FMDRWRKTSFR--LMTYKSTTASSTSDMPFSITSFDENGKL---------------
CpMV1-NB631     YIEFLRFNGLKSPLIVERYIKDGIRIEKPLTLQGLHRSGDIQLGFKIS--------
OnuMV6-LD       -------------LQMEQKARAMMLVKHMKDLEG----------------------
OnuMV3A         --------------IFFKHVLALMAERDPATVMRWM--------------------
CcMV1           ---------------LNRDTRAQCMGTRPDVYNMKD--------------------
```

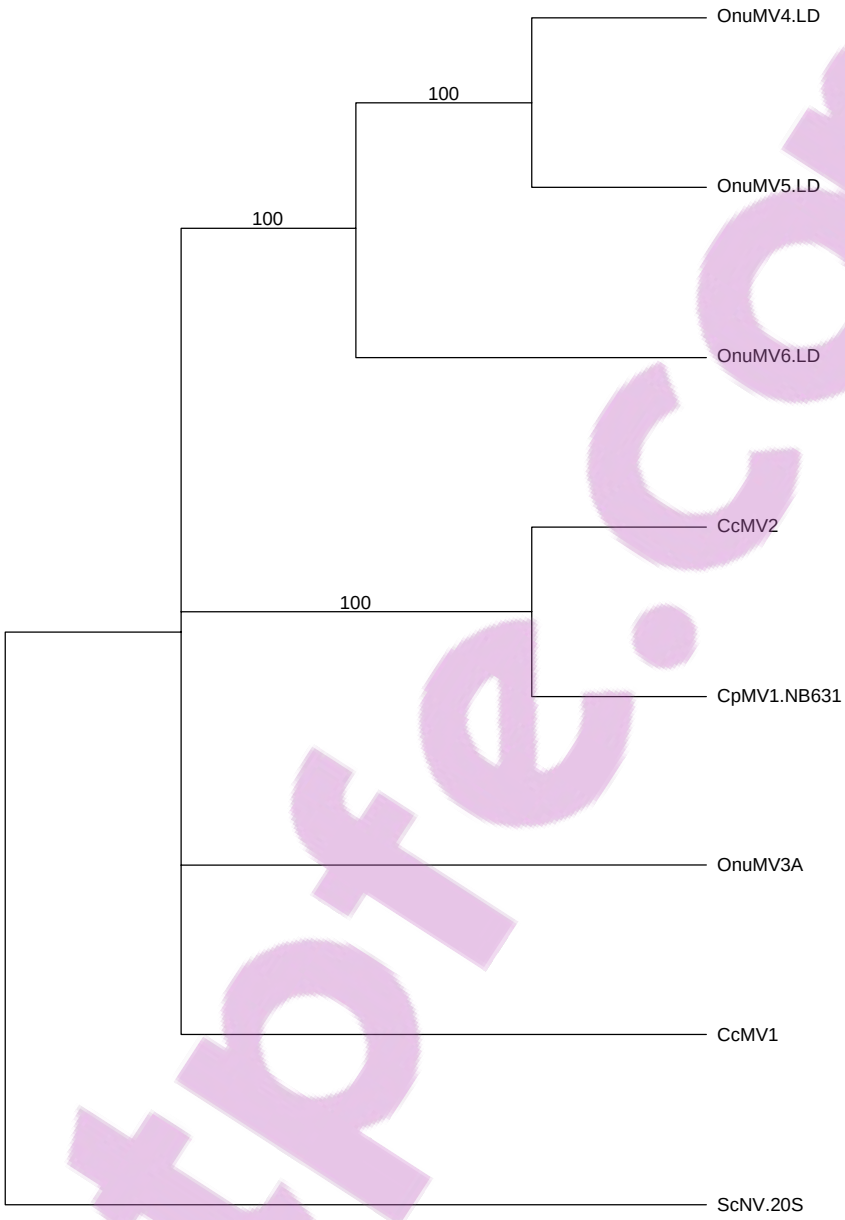


Figure 4: Phylogenetic relationships inferred from the conserved area of the aligned amino acid sequences of the viruses in the genus *Mitovirus*. These viruses are: *Ophiostoma novo-ulmi mitoviruses*, OnuMV3A (Hong *et al.* 1998a), OnuMV4-LD, OnuMV5-LD, OnuMV6-LD (Hong *et al.* 1999), *Cryphonectria parasitica mitovirus* CpMV1-613 (Polashock & Hillman 1994) as well as the *C. cubensis* mitoviruses CcMV1 and CcMV2 (current study). Bootstrap values are indicated above the branches (1000 replicates). The *Saccharomyces cerevisiae Narnavirus* (Rodriguez-Cousino *et al.* 1991) is used as the outgroup taxon.

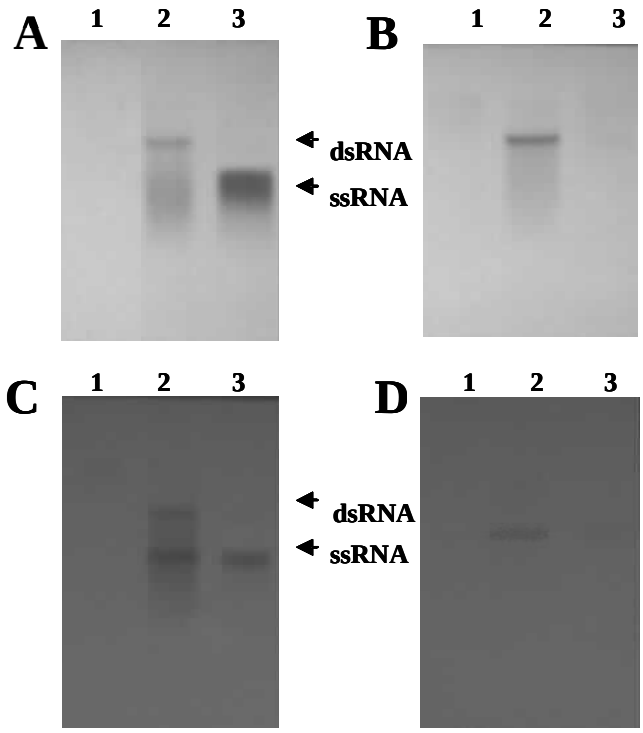


Figure 5: Detection of dsRNA and ssRNA of CcMV1 (C & D) and CcMV2 (A & B) using Northern Blot hybridisation with DIG labeled strand specific RNA probes. A & C were hybridised with positive-strand specific probes. B & D were hybridised with the negative-strand specific probes. The following samples were loaded and separated on a 1% agarose gel before blotting onto a positively charged nylon membrane and hybridised: Lane 1: Total nucleic acid isolated from a Colombian isolate (CMW11348), Lane 2: CF11 cellulose purified dsRNA from virus infected isolate (CMW11329), Lane 3: Total nucleic acid from the virus infected isolate (CMW11329). All the blots were developed for 2 hours.

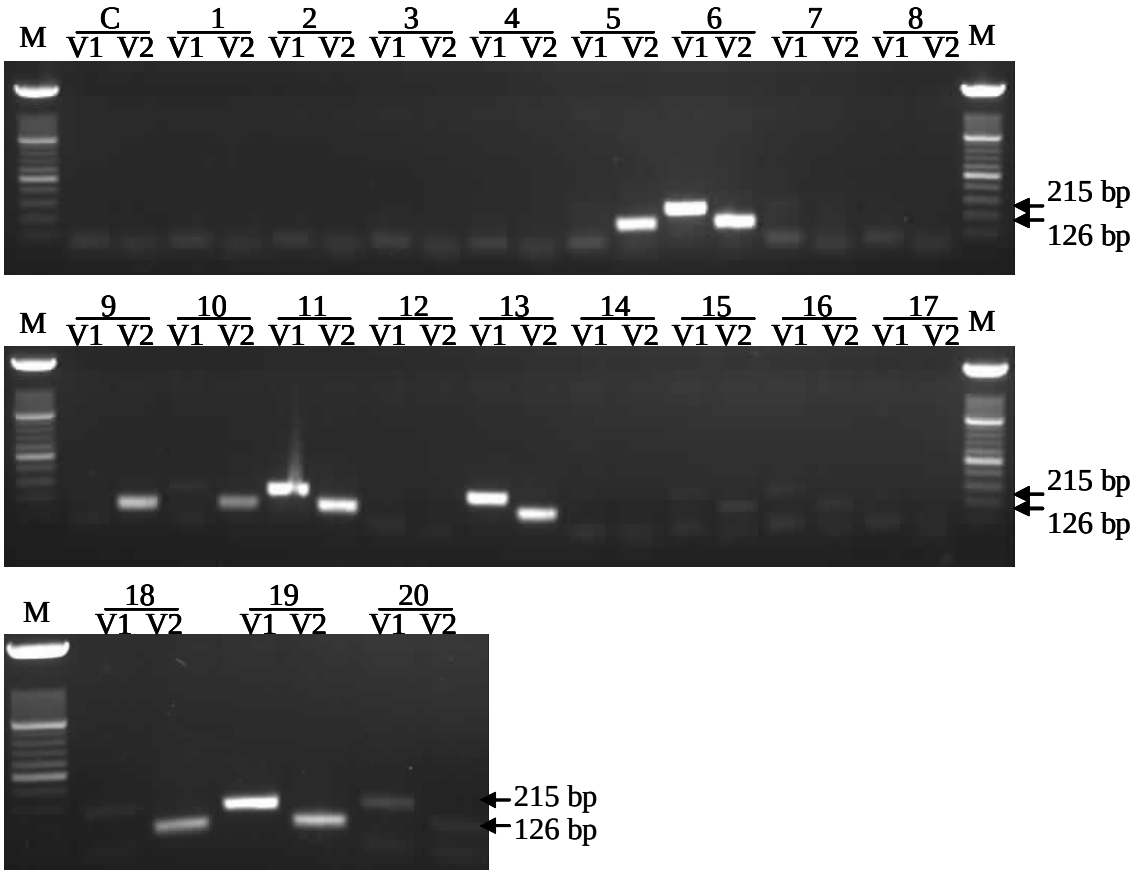
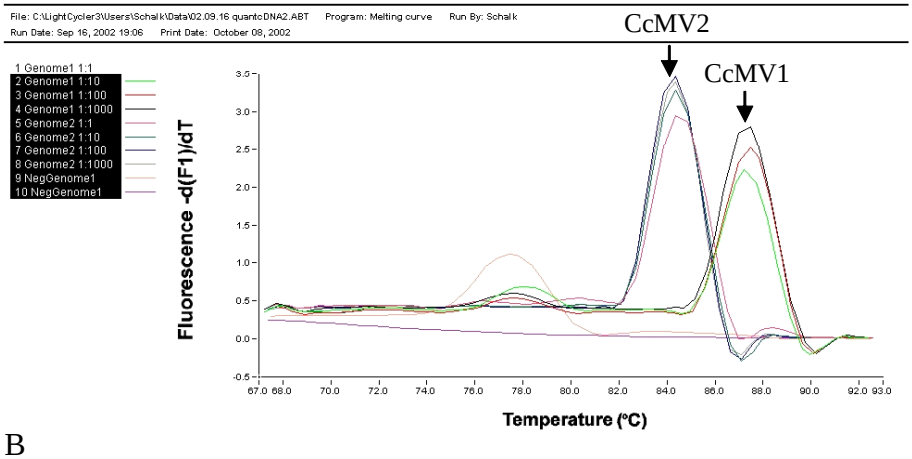
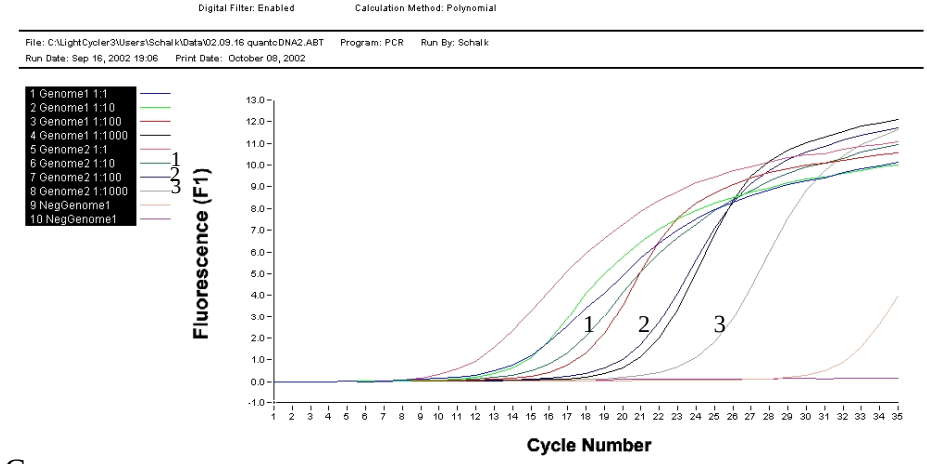


Figure 6: Confirmation of the presence of the two viruses CcMV1 (V1) and CcMV2 (V2) in 20 *C. cubensis* isolates. The RT-PCR products were separated on a 1.5% agarose gel stained with ethidium bromide and visualised under UV light. Lanes M: 100 bp molecular weight marker. Lane C: Negative control. Lanes 1-20: Isolates used in this study as indicated in Table 2. The presence of the viruses is indicated by a 215 bp amplicon for CcMV1 and a 126 bp amplicon for CcMV2. Different band intensities indicate varying concentrations of the virus present.

A



B



C

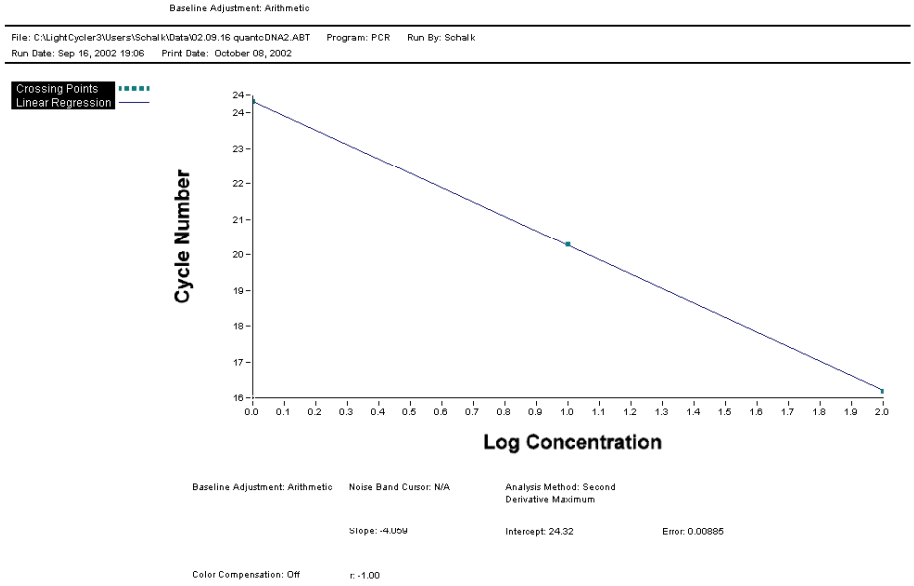


Figure 7: Relative quantification of the *Cryphonectria cubensis* mitovirus *1* (CcMV1) in relation to CcMV2. A: Melting peaks shown as the negative derivative of fluorescence with respect to temperature. B: Baseline adjustment showing the amplification profiles with 1-3 being the selected standards. C: Standard curve with the three standards indicated

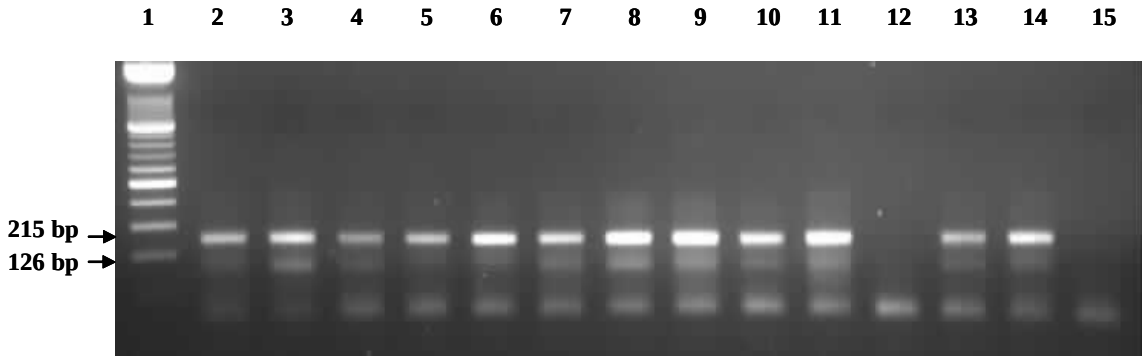


Figure 8: Confirmation of the transmission of the mitoviruses to the conidia using RT-PCR. The PCR products were separated on a 1.5% agarose gel stained with ethidium bromide. Lane 1: 100 bp molecular weight marker. Lanes 2-13: Mass conidial cultures from isolate CMW11329. Lane 14: Wild type CRYCMW11329 isolate included as positive control. Lane 15: Negative control. The presence of the viruses is indicated by a 215 bp amplicon for CcMV1 and a 126 bp amplicon for CcMV2.

Appendix 1: Aligned RNA sequence of the *Cryphonectria cubensis* mitovirus *1* using the CLUSTAL X program. The direction is 5' to 3'. The virus has different sequences at the 5' end. Symbols below the sequence alignment: (*) indicates identical nucleotides

```
CLUSUAL X (1.81) multiple sequence alignment

                1                                                           60
CcMV1b          CUUUUUUUUUUUUUUUUUCUUUCUUCUUUUUUUUUUUUUUCUUUUUUUUCCCCCCUUGCC
CcMV1c          ------------------------------------------------------------
CcMV1a          ---------------------------------------------CUUAGCCCCUUGCC

                                                                           120
CcMV1b          CCGGUUUCCCGGGGAAGGCAACGGAGAAGGGGUGACAGGACCCGAAAGGCCCCACUUGUC
CcMV1c          ------------------------------------CUUUUCCUUUUUCUUUUUUU
CcMV1a          CCGGUUUCCCGGGGAAGGCAACGGAGAAGGGGUGACAGGACCCGAAAGGCCCCACUUGUC
                                                    *           *  ** *
                                                                           180
CcMV1b          AAUCAUCCGUUCCGUGUCCACUAGCUGCUGACUGUUACUGACUAGCCUUGUAACAACAAA
CcMV1c          UUUUAUCCGUUCCGUGUCCACUAGCUGCUGACUGUUACUGACUAGCCUUGUAACAACAAA
CcMV1a          AAUCAUCCGUUCCGUGUCCACUAGCUGCUGACUGUUACUGACUAGCCUUGUAACAACAAA
                  * ************************************************************
                                                                           240
CcMV1b          GGGAUAACCAAAGCUGACCACUACUCAAGUGCGAGCAGCGACCCGCCAAGGUGAACCGAU
CcMV1c          GGGAUAACCAAAGCUGACCACUACUCAAGUGCGAGCAGCGACCCGCCAAGGUGAACCGAU
CcMV1a          GGGAUAACCAAAGCUGACCACUACUCAAGUGCGAGCAGCGACCCGCCAAGGUGAACCGAU
                ************************************************************
                                                                           300
CcMV1b          UGCUGUCAUAAUACUAGGCAGCACCAGCCCACGUUUGCUAUAAAAUGAAUUAUAACAAAA
CcMV1c          UGCUGUCAUAAUACUAGGCAGCACCAGCCCACGUUUGCUAUAAAAUGAAUUAUAACAAAA
CcMV1a          UGCUGUCAUAAUACUAGGCAGCACCAGCCCACGUUUGCUAUAAAAUGAAUUAUAACAAAA
                ************************************************************
                                                                           360
CcMV1b          ACCAUGAAAAGAAAUCUCAAUAAACUCUAUAUACACCUAAAUCUCUUACGAGACAUCGGU
CcMV1c          ACCAUGAAAAGAAAUCUCAAUAAACUCUAUAUACACCUAAAUCUCUUACGAGACAUCGGU
CcMV1a          ACCAUGAAAAGAAAUCUCAAUAAACUCUAUAUACACCUAAAUCUCUUACGAGACAUCGGU
                ************************************************************
                                                                           420
CcMV1b          GCAAUAAAAUUGAGUCUAGUGUGAUUCCUUCACUGAUCCCACCUUGAGCUAGGUAUAGCG
CcMV1c          GCAAUAAAAUUGAGUCUAGUGUGAUUCCUUCACUGAUCCCACCUUGAGCUAGGUAUAGCG
CcMV1a          GCAAUAAAAUUGAGUCUAGUGUGAUUCCUUCACUGAUCCCACCUUGAGCUAGGUAUAGCG
                ************************************************************
                                                                           480
CcMV1b          UCACGUCUCGUCUCUUCCGCGGUAGCCAGAUUUCAACUUCUGGCCACCACGAGAGGGCGG
CcMV1c          UCACGUCUCGUCUCUUCCGCGGUAGCCAGAUUUCAACUUCUGGCCACCACGAGAGGGCGG
CcMV1a          UCACGUCUCGUCUCUUCCGCGGUAGCCAGAUUUCAACUUCUGGCCACCACGAGAGGGCGG
                ************************************************************
                                                                           540
CcMV1b          AACGCCGCUAUAUCCGAGUUUAAGGCGUCGCGGCUAGCGUUUACACGAUGACUCUGUGGG
CcMV1c          AACGCCGCUAUAUCCGAGUUUAAGGCGUCGCGGCUAGCGUUUACACGAUGACUCUGUGGG
CcMV1a          AACGCCGCUAUAUCCGAGUUUAAGGCGUCGCGGCUAGCGUUUACACGAUGACUCUGUGGG
                ************************************************************
                                                                           600
CcMV1b          CGGCCGCUCUCAGGUAAAGUUGGUGCCCCGAUAACAAAAGCUGGGCUUCCUAAGGUAAUC
CcMV1c          CGGCCGCUCUCAGGUAAAGUUGGUGCCCCGAUAACAAAAGCUGGGCUUCCUAAGGUAAUC
CcMV1a          CGGCCGCUCUCAGGUAAAGUUGGUGCCCCGAUAACAAAAGCUGGGCUUCCUAAGGUAAUC
                ************************************************************
                                                                           660
CcMV1b          CCUAGGGAGGCCAGACUUUUGUUGCUUCGGGAACGCCCUCUUUACUUGGUAAAGGCCGUC
CcMV1c          CCUAGGGAGGCCAGACUUUUGUUGCUUCGGGAACGCCCUCUUUACUUGGUAAAGGCCGUC
CcMV1a          CCUAGGGAGGCCAGACUUUUGUUGCUUCGGGAACGCCCUCUUUACUUGGUAAAGGCCGUC
                ************************************************************
```

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                                                                      720
CcMV1b          AUAACGGUGCUAUCGAUCGGGCGUUAUUUUAAGGGUGGAAACCCUGUAAAGUGGGAGAAU
CcMV1c          AUAACGGUGCUAUCGAUCGGGCGUUAUUUUAAGGGUGGAAACCCUGUAAAGUGGGAGAAU
CcMV1a          AUAACGGUGCUAUCGAUCGGGCGUUAUUUUAAGGGUGGAAACCCUGUAAAGUGGGAGAAU
                ************************************************************
                                                                      780
CcMV1b          AUUACGAAGCCGUCAACUCCUACCCUCCCAAAAGAUGGAGAAAUCCUCUUUGGGUUGGAG
CcMV1c          AUUACGAAGCCGUCAACUCCUACCCUCCCAAAAGAUGGAGAAAUCCUCUUUGGGUUGGAG
CcMV1a          AUUACGAAGCCGUCAACUCCUACCCUCCCAAAAGAUGGAGAAAUCCUCUUUGGGUUGGAG
                ************************************************************
                                                                      840
CcMV1b          AAGUUGAACAUUGACGUCGGGCAGUUCGAGCCAACGGAUUGGAAAUUCCGUUGAGUCGUG
CcMV1c          AAGUUGAACAUUGACGUCGGGCAGUUCGAGCCAACGGAUUGGAAAUUCCGUUGAGUCGUG
CcMV1a          AAGUUGAACAUUGACGUCGGGCAGUUCGAGCCAACGGAUUGGAAAUUCCGUUGAGUCGUG
                ************************************************************
                                                                      900
CcMV1b          ACAGCCGGGCCAAAUGGGCCAAGUAUAUCUUCCUGCUUACAGGACCUCCCGAAAUUUAAC
CcMV1c          ACAGCCGGGCCAAAUGGGCCAAGUAUAUCUUCCUGCUUACAGGACCUCCCGAAAUUUAAC
CcMV1a          ACAGCCGGGCCAAAUGGGCCAAGUAUAUCUUCCUGCUUACAGGACCUCCCGAAAUUUAAC
                ************************************************************
                                                                      960
CcMV1b          GGUCUCUUCAGGUCCCAGGUAGAAGUCAUUCUACCUGAGCUCCUUCCCAUCAUUGAUACC
CcMV1c          GGUCUCUUCAGGUCCCAGGUAGAAGUCAUUCUACCUGAGCUCCUUCCCAUCAUUGAUACC
CcMV1a          GGUCUCUUCAGGUCCCAGGUAGAAGUCAUUCUACCUGAGCUCCUUCCCAUCAUUGAUACC
                ************************************************************
                                                                     1020
CcMV1b          CUAUUGACCUGGGAGAAAAGCUUUAAGCUGUCAACCCUUAUGGGAUUGGCAGGCUUUAAA
CcMV1c          CUAUUGACCUGGGAGAAAAGCUUUAAGCUGUCAACCCUUAUGGGAUUGGCAGGCUUUAAA
CcMV1a          CUAUUGACCUGGGAGAAAAGCUUUAAGCUGUCAACCCUUAUGGGAUUGGCAGGCUUUAAA
                ************************************************************
                                                                     1080
CcMV1b          GACGAUUCCCUCCGGAAAAUAGCUAUCAAGGAUGAUAGGGAGGGUAAGAGUAGACCUUUU
CcMV1c          GACGAUUCCCUCCGGAAAAUAGCUAUCAAGGAUGAUAGGGAGGGUAAGAGUAGACCUUUU
CcMV1a          GACGAUUCCCUCCGGAAAAUAGCUAUCAAGGAUGAUAGGGAGGGUAAGAGUAGACCUUUU
                ************************************************************
                                                                     1140
CcMV1b          GCGAUAUUCGAUUACUGAUCCCAGACAGUUUUAUCACCUCUGCAUGACUGAGCGUACGCG
CcMV1c          GCGAUAUUCGAUUACUGAUCCCAGACAGUUUUAUCACCUCUGCAUGACUGAGCGUACGCG
CcMV1a          GCGAUAUUCGAUUACUGAUCCCAGACAGUUUUAUCACCUCUGCAUGACUGAGCGUACGCG
                ************************************************************
                                                                     1200
CcMV1b          ACCCUGAGGUCAAUUCCUCAGGAUUGCACGUUCAACCAGGCAGAGGGACUGUCGAAGGUC
CcMV1c          ACCCUGAGGUCAAUUCCUCAGGAUUGCACGUUCAACCAGGCAGAGGGACUGUCGAAGGUC
CcMV1a          ACCCUGAGGUCAAUUCCUCAGGAUUGCACGUUCAACCAGGCAGAGGGACUGUCGAAGGUC
                ************************************************************
                                                                     1260
CcMV1b          ACAGCUCGGCCAUCGCAAAAGUAUUUCUAUUCUUACGACCUUGAAGCGGCAACAGACCGU
CcMV1c          ACAGCUCGGCCAUCGCAAAAGUAUUUCUAUUCUUACGACCUUGAAGCGGCAACAGACCGU
CcMV1a          ACAGCUCGGCCAUCGCAAAAGUAUUUCUAUUCUUACGACCUUGAAGCGGCAACAGACCGU
                ************************************************************
                                                                     1320
CcMV1b          UUUCCGAUACAAUUUCAGAAAAAGGUUCUGUCCCUGAUCUUUAACACUACUUAUGCCCAG
CcMV1c          UUUCCGAUACAAUUUCAGAAAAAGGUUCUGUCCCUGAUCUUUAACACUACUUAUGCCCAG
CcMV1a          UUUCCGAUACAAUUUCAGAAAAAGGUUCUGUCCCUGAUCUUUAACACUACUUAUGCCCAG
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```

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                                                                    1380
CcMV1b          GCGUGAGCUGAGAUAAUGACUCAAGAGCCUUUUAGAGUCAAGGGAUUGUCCGACCCCUUA
CcMV1c          GCGUGAGCUGAGAUAAUGACUCAAGAGCCUUUUAGAGUCAAGGGAUUGUCCGACCCCUUA
CcMV1a          GCGUGAGCUGAGAUAAUGACUCAAGAGCCUUUUAGAGUCAAGGGAUUGUCCGACCCCUUA
                ************************************************************
                                                                    1440
CcMV1b          AGAUGAGGAGCCGGGCAGCCUCUUGGAGCUAAAAGUUCCUGAGCCAUUUUCACAUUAUGC
CcMV1c          AGAUGAGGAGCCGGGCAGCCUCUUGGAGCUAAAAGUUCCUGAGCCAUUUUCACAUUAUGC
CcMV1a          AGAUGAGGAGCCGGGCAGCCUCUUGGAGCUAAAAGUUCCUGAGCCAUUUUCACAUUAUGC
                ************************************************************
                                                                    1500
CcMV1b          CACCACCUAGUAGUUCAUAUAGCAGCGGUACGGACUAACUCCGACCCCUACUACGUGAUA
CcMV1c          CACCACCUAGUAGUUCAUAUAGCAGCGGUACGGACUAACUCCGACCCCUACUACGUGAUA
CcMV1a          CACCACCUAGUAGUUCAUAUAGCAGCGGUACGGACUAACUCCGACCCCUACUACGUGAUA
                ************************************************************
                                                                    1560
CcMV1b          CUAGGCGAUGACAUAGUGCUCCGUGGCUCACGGCUGGCGACAGUGUACAAACGGAUAAUG
CcMV1c          CUAGGCGAUGACAUAGUGCUCCGUGGCUCACGGCUGGCGACAGUGUACAAACGGAUAAUG
CcMV1a          CUAGGCGAUGACAUAGUGCUCCGUGGCUCACGGCUGGCGACAGUGUACAAACGGAUAAUG
                ************************************************************
                                                                    1620
CcMV1b          UCCGAACUUGGAGUAUCCAUAUCCGAAACGAAAUCGCACGUGUCAAAAGACACGUUCGAA
CcMV1c          UCCGAACUUGGAGUAUCCAUAUCCGAAACGAAAUCGCACGUGUCAAAAGACACGUUCGAA
CcMV1a          UCCGAACUUGGAGUAUCCAUAUCCGAAACGAAAUCGCACGUGUCAAAAGACACGUUCGAA
                ************************************************************
                                                                    1680
CcMV1b          UUCGCUAAGAUGUGAAUGCACCAAGGUAGGAACGCGAGUGGGUUCCCUGUAGUGGGACUA
CcMV1c          UUCGCUAAGAUGUGAAUGCACCAAGGUAGGAACGCGAGUGGGUUCCCUGUAGUGGGACUA
CcMV1a          UUCGCUAAGAUGUGAAUGCACCAAGGUAGGAACGCGAGUGGGUUCCCUGUAGUGGGACUA
                ************************************************************
                                                                    1740
CcMV1b          GCUGAGACACUCAGAAAGCCACUAGAAAUGGCGGCUCUCUUUGUGUUUGAGCUCCCUGCU
CcMV1c          GCUGAGACACUCAGAAAGCCACUAGAAAUGGCGGCUCUCUUUGUGUUUGAGCUCCCUGCU
CcMV1a          GCUGAGACACUCAGAAAGCCACUAGAAAUGGCGGCUCUCUUUGUGUUUGAGCUCCCUGCU
                ************************************************************
                                                                    1800
CcMV1b          AAAGGGUAUCCAGUCACUAUUACUCCGCGCACCGUGUCGCAGUACUUCCUUCUAGUAGCA
CcMV1c          AAAGGGUAUCCAGUCACUAUUACUCCGCGCACCGUGUCGCAGUACUUCCUUCUAGUAGCA
CcMV1a          AAAGGGUAUCCAGUCACUAUUACUCCGCGCACCGUGUCGCAGUACUUCCUUCUAGUAGCA
                ************************************************************
                                                                    1860
CcMV1b          CGUUAUAAUACCAUUCCACCUCGUCUGGCGGUAUGAACUGCCGACAAAGUGGUAUGGUAC
CcMV1c          CGUUAUAAUACCAUUCCACCUCGUCUGGCGGUAUGAACUGCCGACAAAGUGGUAUGGUAC
CcMV1a          CGUUAUAAUACCAUUCCACCUCGUCUGGCGGUAUGAACUGCCGACAAAGUGGUAUGGUAC
                ************************************************************
                                                                    1920
CcMV1b          UAUAGUUUCUUGUCAUGACUGGCCACCCGGGAUGACGGAUGGGCGAAAUAUAUCGCCCAG
CcMV1c          UAUAGUUUCUUGUCAUGACUGGCCACCCGGGAUGACGGAUGGGCGAAAUAUAUCGCCCAG
CcMV1a          UAUAGUUUCUUGUCAUGACUGGCCACCCGGGAUGACGGAUGGGCGAAAUAUAUCGCCCAG
                ************************************************************
                                                                    1980
CcMV1b          UCGGCCUCCCUAUUGGUGAGCCCCAAUACCGCCCACGACCUCCUUAUGAAGGCCGUGAGA
CcMV1c          UCGGCCUCCCUAUUGGUGAGCCCCAAUACCGCCCACGACCUCCUUAUGAAGGCCGUGAGA
CcMV1a          UCGGCCUCCCUAUUGGUGAGCCCCAAUACCGCCCACGACCUCCUUAUGAAGGCCGUGAGA
                ************************************************************
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                                                                    2040
CcMV1b          GAUAAAUGGGCUAAACAGCUUGACAAGUCUCUAAUGGAUUUCCAGGAUUUUGGAUUUGAU
CcMV1c          GAUAAAUGGGCUAAACAGCUUGACAAGUCUCUAAUGGAUUUCCAGGAUUUUGGAUUUGAU
CcMV1a          GAUAAAUGGGCUAAACAGCUUGACAAGUCUCUAAUGGAUUUCCAGGAUUUUGGAUUUGAU
                ************************************************************
                                                                    2100
CcMV1b          AUGUUCGACAAAGUGAAGACCUUGCCGCCAUUCAAGCCUACCUGGGAUCCAGAAGCGUGA
CcMV1c          AUGUUCGACAAAGUGAAGACCUUGCCGCCAUUCAAGCCUACCUGGGAUCCAGAAGCGUGA
CcMV1a          AUGUUCGACAAAGUGAAGACCUUGCCGCCAUUCAAGCCUACCUGGGAUCCAGAAGCGUGA
                ************************************************************
                                                                    2160
CcMV1b          CCUGGUCGGUUGUCUGCGAGUGGAAUCGAAUUCAAUCCAGCCCCUAGGAAGGUACCAAUA
CcMV1c          CCUGGUCGGUUGUCUGCGAGUGGAAUCGAAUUCAAUCCAGCCCCUAGGAAGGUACCAAUA
CcMV1a          CCUGGUCGGUUGUCUGCGAGUGGAAUCGAAUUCAAUCCAGCCCCUAGGAAGGUACCAAUA
                ************************************************************
                                                                    2220
CcMV1b          UUUGCUGCUCUCCAAGAAGAAGGAGAGAUCAAGUAUUCCGAUUAUCUCCAACAGAAGUUG
CcMV1c          UUUGCUGCUCUCCAAGAAGAAGGAGAGAUCAAGUAUUCCGAUUAUCUCCAACAGAAGUUG
CcMV1a          UUUGCUGCUCUCCAAGAAGAAGGAGAGAUCAAGUAUUCCGAUUAUCUCCAACAGAAGUUG
                ************************************************************
                                                                    2280
CcMV1b          GAGAUGACCGACGAUCAGCUGACGUUCGAGGAGAUAGAAUCUUUGAAACUACCUCCUCGU
CcMV1c          GAGAUGACCGACGAUCAGCUGACGUUCGAGGAGAUAGAAUCUUUGAAACUACCUCCUCGU
CcMV1a          GAGAUGACCGACGAUCAGCUGACGUUCGAGGAGAUAGAAUCUUUGAAACUACCUCCUCGU
                ************************************************************
                                                                    2340
CcMV1b          CCGCAACUGAAAGGGUUCCUUCCGAAAAGGACUAGAGAGUAUGUUCGUACACUUAACCUA
CcMV1c          CCGCAACUGAAAGGGUUCCUUCCGAAAAGGACUAGAGAGUAUGUUCGUACACUUAACCUA
CcMV1a          CCGCAACUGAAAGGGUUCCUUCCGAAAAGGACUAGAGAGUAUGUUCGUACACUUAACCUA
                ************************************************************
                                                                    2400
CcMV1b          AUUAGUCACGGCUUAAACCGGGAUCUAAGGGCUCAGUGCAUAGGAACCAGACCAGACGUA
CcMV1c          AUUAGUCACGGCUUAAACCGGGAUCUAAGGGCUCAGUGCAUAGGAACCAGACCAGACGUA
CcMV1a          AUUAGUCACGGCUUAAACCGGGAUCUAAGGGCUCAGUGCAUAGGAACCAGACCAGACGUA
                ************************************************************
                                                                    2460
CcMV1b          UACAAUAUGAAAGACUAAGAGGUUCUCCCUCCCACCUUGAGAAAGUGGUGAAAGCUCUCC
CcMV1c          UACAAUAUGAAAGACUAAGAGGUUCUCCCUCCCACCUUGAGAAAGUGGUGAAAGCUCUCC
CcMV1a          UACAAUAUGAAAGACUAAGAGGUUCUCCCUCCCACCUUGAGAAAGUGGUGAAAGCUCUCC
                ************************************************************
                                                                    2520
CcMV1b          UUAGCCCCUACACAUUGUGUACCCCCAUUCCCCUUUCGGGGUAGGCAGAAGUCAACUGCU
CcMV1c          UUAGCCCCUACACAUUGUGUACCCCCAUUCCCCUUUCGGGGUAGGCAGAAGUCAACUGCU
CcMV1a          UUAGCCCCUACACAUUGUGUACCCCCAUUCCCCUUUCGGGGUAGGCAGAAGUCAACUGCU
                ************************************************************
                                                                    2580
CcMV1b          UGCUCCGGCAGGGAUGCCGGAUGGGAAGUCGACAGCGCUGGGGCAAAAAAAAAAAAAAAA
CcMV1c          UGCUCCGGCAGGGAUGCCGGAUGGGAAGUCGACAGCGCUGGGGCAAAAAAAAAAAAAAAA
CcMV1a          UGCUCCGGCAGGGAUGCCGGAUGGGAAGUCGACAGCGCUGGGGCAAAAAAAAAAAAAAAA
                ************************************************************
                                  2601
CcMV1b          AAAAGAAAAAAAAAAGAAAAA
CcMV1c          AAAAGAAAAAAAAAAGAAAAA
CcMV1a          AAAAGAAAAAAAAAAGAAAAA
                *********************
```

Appendix 2: Aligned RNA sequence of the *Cryphonectria cubensis* mitovirus *2* using the CLUSTAL X program. The direction is 5' to 3'. The virus has different ends at the 3' end. Symbol below the sequence alignment where (*) indicates identical nucleotides.

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CLUSTAL X (1.81) multiple sequence alignment

                1                                                           60
CcMV2b          CUUUUUUUGUUUUUUUUUUCUUUUCUUUGGGGUAAUGACUCUUCAAGUCACUUGACAGUC
CcMV2a          CUUUUUUUGUUUUUUUUUUCUUUUCUUUGGGGUAAUGACUCUUCAAGUCACUUGACAGUC
CcMV2c          CUUUUUUUGUUUUUUUUUUCUUUUCUUUGGGGUAAUGACUCUUCAAGUCACUUGACAGUC
                ************************************************************
                                                                           120
CcMV2b          AUAAUGCCUAAACCCCAGUCAGUCUCUCUUGCCUUCACAGGUGAGGUGUGCUUUCGGCUA
CcMV2a          AUAAUGCCUAAACCCCAGUCAGUCUCUCUUGCCUUCACAGGUGAGGUGUGCUUUCGGCUA
CcMV2c          AUAAUGCCUAAACCCCAGUCAGUCUCUCUUGCCUUCACAGGUGAGGUGUGCUUUCGGCUA
                ************************************************************
                                                                           180
CcMV2b          UAAUGGUACUUUCCAUUCGGACAGUAGACGAGUGUAAUGAGAUAACAUCAAAAUUCGAAU
CcMV2a          UAAUGGUACUUUCCAUUCGGACAGUAGACGAGUGUAAUGAGAUAACAUCAAAAUUCGAAU
CcMV2c          UAAUGGUACUUUCCAUUCGGACAGUAGACGAGUGUAAUGAGAUAACAUCAAAAUUCGAAU
                ************************************************************
                                                                           240
CcMV2b          UGCACGGGCUAGCGACCCAACGUAAUAGUAAAUCACUCCAAACUGUAUCGUGAAACAUUU
CcMV2a          UGCACGGGCUAGCGACCCAACGUAAUAGUAAAUCACUCCAAACUGUAUCGUGAAACAUUU
CcMV2c          UGCACGGGCUAGCGACCCAACGUAAUAGUAAAUCACUCCAAACUGUAUCGUGAAACAUUU
                ************************************************************
                                                                           300
CcMV2b          GCAUUAAUUAAUUAACACAAAGCUACAACAACACAUUCAAAAUUAUACUCUAAACUUAAU
CcMV2a          GCAUUAAUUAAUUAACACAAAGCUACAACAACACAUUCAAAAUUAUACUCUAAACUUAAU
CcMV2c          GCAUUAAUUAAUUAACACAAAGCUACAACAACACAUUCAAAAUUAUACUCUAAACUUAAU
                ************************************************************
                                                                           360
CcMV2b          GCAAAAUUGAGUCUAAUAAGAAGUAACUAUUUUUCUUUGUUACUUUUAUUAAAAUCAUUC
CcMV2a          GCAAAAUUGAGUCUAAUAAGAAGUAACUAUUUUUCUUUGUUACUUUUAUUAAAAUCAUUC
CcMV2c          GCAAAAUUGAGUCUAAUAAGAAGUAACUAUUUUUCUUUGUUACUUUUAUUAAAAUCAUUC
                ************************************************************
                                                                           420
CcMV2b          UUCAUCAAGCCUAAAAGUGUUAAUGUCAAACGUGAUGGUAAGAGCUGGAUCUCCCAAAAG
CcMV2a          UUCAUCAAGCCUAAAAGUGUUAAUGUCAAACGUGAUGGUAAGAGCUGGAUCUCCCAAAAG
CcMV2c          UUCAUCAAGCCUAAAAGUGUUAAUGUCAAACGUGAUGGUAAGAGCUGGAUCUCCCAAAAG
                ************************************************************
                                                                           480
CcMV2b          GAGAUUCCAAAAUUUAUCAUCAUGGUUUGUUGGUGUACCGGUACACAGUCCUAUCAAAGA
CcMV2a          GAGAUUCCAAAAUUUAUCAUCAUGGUUUGUUGGUGUACCGGUACACAGUCCUAUCAAAGA
CcMV2c          GAGAUUCCAAAAUUUAUCAUCAUGGUUUGUUGGUGUACCGGUACACAGUCCUAUCAAAGA
                ************************************************************
                                                                           540
CcMV2b          UCUUUUAUGAAGAUGCAGGAUAGAUUUGUCUUUAUCUGAAAUACUGCUGGCUCUACAUUC
CcMV2a          UCUUUUAUGAAGAUGCAGGAUAGAUUUGUCUUUAUCUGAAAUACUGCUGGCUCUACAUUC
CcMV2c          UCUUUUAUGAAGAUGCAGGAUAGAUUUGUCUUUAUCUGAAAUACUGCUGGCUCUACAUUC
                ************************************************************
                                                                           600
CcMV2b          CUCUUUAAGUAUCUUAAAGAGGUAAUGCGUCUUACAGUAAGAAGAUUGGCGAAUAUAGAC
CcMV2a          CUCUUUAAGUAUCUUAAAGAGGUAAUGCGUCUUACAGUAAGAAGAUUGGCGAAUAUAGAC
CcMV2c          CUCUUUAAGUAUCUUAAAGAGGUAAUGCGUCUUACAGUAAGAAGAUUGGCGAAUAUAGAC
                ************************************************************
                                                                           660
CcMV2b          CUUAUUCCUAGUAAAGAAAAUCUUUGUUAAAUUAAACAAAUAUAGAUUUCCUGCUAUUAU
CcMV2a          CUUAUUCCUAGUAAAGAAAAUCUUUGUUAAAUUAAACAAAUAUAGAUUUCCUGCUAUUAU
CcMV2c          CUUAUUCCUAGUAAAGAAAAUCUUUGUUAAAUUAAACAAAUAUAGAUUUCCUGCUAUUAU
                ************************************************************
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                                                                      720
CcMV2b          UCCUCUAGAGAUUUGCAAGGAUCUUUCCUGGUUUCCAGGGAGAUUCCAUGUUUUGUCUCG
CcMV2a          UCCUCUAGAGAUUUGCAAGGAUCUUUCCUGGUUUCCAGGGAGAUUCCAUGUUUUGUCUCG
CcMV2c          UCCUCUAGAGAUUUGCAAGGAUCUUUCCUGGUUUCCAGGGAGAUUCCAUGUUUUGUCUCG
                ************************************************************
                                                                      780
CcMV2b          AAAGGUAAUAGCUCUCCUUACCGUUAUUUCGAUUUUCAGGGUUUUACCUACUAAGGUAUU
CcMV2a          AAAGGUAAUAGCUCUCCUUACCGUUAUUUCGAUUUUCAGGGUUUUACCUACUAAGGUAUU
CcMV2c          AAAGGUAAUAGCUCUCCUUACCGUUAUUUCGAUUUUCAGGGUUUUACCUACUAAGGUAUU
                ************************************************************
                                                                      840
CcMV2b          GCCUGAUUACACGACUAUCAGUAAGCCCCAUUCGGGACUUAUUGAAACGUGUUCAUCAGA
CcMV2a          GCCUGAUUACACGACUAUCAGUAAGCCCCAUUCGGGACUUAUUGAAACGUGUUCAUCAGA
CcMV2c          GCCUGAUUACACGACUAUCAGUAAGCCCCAUUCGGGACUUAUUGAAACGUGUUCAUCAGA
                ************************************************************
                                                                      900
CcMV2b          GUCAAUUGCUUUAGCCUGUAAAAAUCUUGAGAUCAAAAGAGUUGAUAAGGUGAUGAAGCU
CcMV2a          GUCAAUUGCUUUAGCCUGUAAAAAUCUUGAGAUCAAAAGAGUUGAUAAGGUGAUGAAGCU
CcMV2c          GUCAAUUGCUUUAGCCUGUAAAAAUCUUGAGAUCAAAAGAGUUGAUAAGGUGAUGAAGCU
                ************************************************************
                                                                      960
CcMV2b          AAGAUUGAAAGGUUCUCUAAAAGCCGGACCGAAUGGUAAAAUAUCAUUACUUACUUCGUU
CcMV2a          AAGAUUGAAAGGUUCUCUAAAAGCCGGACCGAAUGGUAAAAUAUCAUUACUUACUUCGUU
CcMV2c          AAGAUUGAAAGGUUCUCUAAAAGCCGGACCGAAUGGUAAAAUAUCAUUACUUACUUCGUU
                ************************************************************
                                                                     1020
CcMV2b          AUUAGAUGCUCUGGCUUUUUGGUCAGAUCCUUUAAGGGUAAUCCACUUCAUCUGAUUUAA
CcMV2a          AUUAGAUGCUCUGGCUUUUUGGUCAGAUCCUUUAAGGGUAAUCCACUUCAUCUGAUUUAA
CcMV2c          AUUAGAUGCUCUGGCUUUUUGGUCAGAUCCUUUAAGGGUAAUCCACUUCAUCUGAUUUAA
                ************************************************************
                                                                     1080
CcMV2b          UAUCAGGUGUUAUGGUUACUUUUGGGGACUAAUAUGAAGUAUGUGAUUGAUUUUUAUCAU
CcMV2a          UAUCAGGUGUUAUGGUUACUUUUGGGGACUAAUAUGAAGUAUGUGAUUGAUUUUUAUCAU
CcMV2c          UAUCAGGUGUUAUGGUUACUUUUGGGGACUAAUAUGAAGUAUGUGAUUGAUUUUUAUCAU
                ************************************************************
                                                                     1140
CcMV2b          GAUCAUUUCCUUACCGUAUUACCUUAUAGCAUUGUGUCUUGGUGCGAGAGCCCCAGUAAU
CcMV2a          GAUCAUUUCCUUACCGUAUUACCUUAUAGCAUUGUGUCUUGGUGCGAGAGCCCCAGUAAU
CcMV2c          GAUCAUUUCCUUACCGUAUUACCUUAUAGCAUUGUGUCUUGGUGCGAGAGCCCCAGUAAU
                ************************************************************
                                                                     1200
CcMV2b          GGGUCAAUUGGCAACUGUUUAUGAUCAAGCUGGAAAAGCGAGAAUUGUAGCUUCUACAAA
CcMV2a          GGGUCAAUUGGCAACUGUUUAUGAUCAAGCUGGAAAAGCGAGAAUUGUAGCUUCUACAAA
CcMV2c          GGGUCAAUUGGCAACUGUUUAUGAUCAAGCUGGAAAAGCGAGAAUUGUAGCUUCUACAAA
                ************************************************************
                                                                     1260
CcMV2b          CUCGUGGAUUCAGUGUUCUCUCUUUGGUUUACACAAUAAGAUUUUUUCUAUCUUACGGAG
CcMV2a          CUCGUGGAUUCAGUGUUCUCUCUUUGGUUUACACAAUAAGAUUUUUUCUAUCUUACGGAG
CcMV2c          CUCGUGGAUUCAGUGUUCUCUCUUUGGUUUACACAAUAAGAUUUUUUCUAUCUUACGGAG
                ************************************************************
                                                                     1320
CcMV2b          UAUUCCUCAAGAUGGAACUUUUGAUCAAAACAAGCCUUUUGAUUUAUUAUUGGAGUCUCU
CcMV2a          UAUUCCUCAAGAUGGAACUUUUGAUCAAAACAAGCCUUUUGAUUUAUUAUUGGAGUCUCU
CcMV2c          UAUUCCUCAAGAUGGAACUUUUGAUCAAAACAAGCCUUUUGAUUUAUUAUUGGAGUCUCU
                ************************************************************
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```
                                                                    1380
CcMV2b          UCAGCCAGGGUACAUGUUAUAUGGUUUCGACCUGAGUGCAGCGACAGAUAGACUUCCUAU
CcMV2a          UCAGCCAGGGUACAUGUUAUAUGGUUUCGACCUGAGUGCAGCGACAGAUAGACUUCCUAU
CcMV2c          UCAGCCAGGGUACAUGUUAUAUGGUUUCGACCUGAGUGCAGCGACAGAUAGACUUCCUAU
                ************************************************************
                                                                    1440
CcMV2b          UGCAUUCCAAAAGGAUAUCCUAAAUCAUUUAGGGUAUCCCGGAGGUCCUUGAAGAAGGUU
CcMV2a          UGCAUUCCAAAAGGAUAUCCUAAAUCAUUUAGGGUAUCCCGGAGGUCCUUGAAGAAGGUU
CcMV2c          UGCAUUCCAAAAGGAUAUCCUAAAUCAUUUAGGGUAUCCCGGAGGUCCUUGAAGAAGGUU
                ************************************************************
                                                                    1500
CcMV2b          ACUGGGUAUCAAAUAUAAUUCACCUUGUGGAUUUAUUUCUUACGCAGUUGGCCAACCAAU
CcMV2a          ACUGGGUAUCAAAUAUAAUUCACCUUGUGGAUUUAUUUCUUACGCAGUUGGCCAACCAAU
CcMV2c          ACUGGGUAUCAAAUAUAAUUCACCUUGUGGAUUUAUUUCUUACGCAGUUGGCCAACCAAU
                ************************************************************
                                                                    1560
CcMV2b          GGGUGCAUAUUCUUCCUUUGCAAUGCUUGCUCUUACACAUCACGUGUUGGUUCAAGUAGC
CcMV2a          GGGUGCAUAUUCUUCCUUUGCAAUGCUUGCUCUUACACAUCACGUGUUGGUUCAAGUAGC
CcMV2c          GGGUGCAUAUUCUUCCUUUGCAAUGCUUGCUCUUACACAUCACGUGUUGGUUCAAGUAGC
                ************************************************************
                                                                    1620
CcMV2b          CGCCCAAAAGGCGGGUUUCUCAGACCGUUUCACGGACUACUGUAUUCUUGGUGACGAUAU
CcMV2a          CGCCCAAAAGGCGGGUUUCUCAGACCGUUUCACGGACUACUGUAUUCUUGGUGACGAUAU
CcMV2c          CGCCCAAAAGGCGGGUUUCUCAGACCGUUUCACGGACUACUGUAUUCUUGGUGACGAUAU
                ************************************************************
                                                                    1680
CcMV2b          CGUCAUAGCCAACUCUUUAGUUGCUGAAGCUUAUAAGUCCUUAAUCUUUGAUUUAGGCUU
CcMV2a          CGUCAUAGCCAACUCUUUAGUUGCUGAAGCUUAUAAGUCCUUAAUCUUUGAUUUAGGCUU
CcMV2c          CGUCAUAGCCAACUCUUUAGUUGCUGAAGCUUAUAAGUCCUUAAUCUUUGAUUUAGGCUU
                ************************************************************
                                                                    1740
CcMV2b          AGAAAUUUCAGAAUCUAAGAGUGUUAUUUCCGGAACAUUUACCGAAUUCGCAAAGAAGUU
CcMV2a          AGAAAUUUCAGAAUCUAAGAGUGUUAUUUCCGGAACAUUUACCGAAUUCGCAAAGAAGUU
CcMV2c          AGAAAUUUCAGAAUCUAAGAGUGUUAUUUCCGGAACAUUUACCGAAUUCGCAAAGAAGUU
                ************************************************************
                                                                    1800
CcMV2b          GAGAGGUCCACUUAUGGAUAUCUCACCUAUCGGAGCGGGUUUGAUAUUAUAUUCCUUACG
CcMV2a          GAGAGGUCCACUUAUGGAUAUCUCACCUAUCGGAGCGGGUUUGAUAUUAUAUUCCUUACG
CcMV2c          GAGAGGUCCACUUAUGGAUAUCUCACCUAUCGGAGCGGGUUUGAUAUUAUAUUCCUUACG
                ************************************************************
                                                                    1860
CcMV2b          UAACAAGUACUACAUCUGUGUGUUGGUUUUUGAGAUCCUGGAAAGGGGAUUAUGCAUGUG
CcMV2a          UAACAAGUACUACAUCUGUGUGUUGGUUUUUGAGAUCCUGGAAAGGGGAUUAUGCAUGUG
CcMV2c          UAACAAGUACUACAUCUGUGUGUUGGUUUUUGAGAUCCUGGAAAGGGGAUUAUGCAUGUG
                ************************************************************
                                                                    1920
CcMV2b          GUAUGACGUCUACCCCCAAUUACUCAGCUUGUUACCUAAGAUUUAUCGUAGGUAUUUCAA
CcMV2a          GUAUGACGUCUACCCCCAAUUACUCAGCUUGUUACCUAAGAUUUAUCGUAGGUAUUUCAA
CcMV2c          GUAUGACGUCUACCCCCAAUUACUCAGCUUGUUACCUAAGAUUUAUCGUAGGUAUUUCAA
                ************************************************************
                                                                    1980
CcMV2b          GCUUUGUGAUUGGUUUAUUGCGCUUCACUUGCGUCGGAGAGAGCAUCUGGGUGACCAAGA
CcMV2a          GCUUUGUGAUUGGUUUAUUGCGCUUCACUUGCGUCGGAGAGAGCAUCUGGGUGACCAAGA
CcMV2c          GCUUUGUGAUUGGUUUAUUGCGCUUCACUUGCGUCGGAGAGAGCAUCUGGGUGACCAAGA
                ************************************************************
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                                                                      2040
CcMV2b          UCAUGAGAUCUUGAAUCCUAGAAUUGCUUACUUCAACGUAUUCUUAAAUAAAGAGAAGAU
CcMV2a          UCAUGAGAUCUUGAAUCCUAGAAUUGCUUACUUCAACGUAUUCUUAAAUAAAGAGAAGAU
CcMV2c          UCAUGAGAUCUUGAAUCCUAGAAUUGCUUACUUCAACGUAUUCUUAAAUAAAGAGAAGAU
                ************************************************************
                                                                      2100
CcMV2b          UAUCUCUUUACUUGAGAUUAUGUGAAACUCUACUGUAAGGGAUUGGUUCCGAUUAUGGAA
CcMV2a          UAUCUCUUUACUUGAGAUUAUGUGAAACUCUACUGUAAGGGAUUGGUUCCGAUUAUGGAA
CcMV2c          UAUCUCUUUACUUGAGAUUAUGUGAAACUCUACUGUAAGGGAUUGGUUCCGAUUAUGGAA
                ************************************************************
                                                                      2160
CcMV2b          UUCGAUUAAGUAUACUCUAAAUAAGGGUCUAUUUAUAUCUCAAGCCAGAGUCGGUCUUCC
CcMV2a          UUCGAUUAAGUAUACUCUAAAUAAGGGUCUAUUUAUAUCUCAAGCCAGAGUCGGUCUUCC
CcMV2c          UUCGAUUAAGUAUACUCUAAAUAAGGGUCUAUUUAUAUCUCAAGCCAGAGUCGGUCUUCC
                ************************************************************
                                                                      2220
CcMV2b          GGACUGAAGUGAAUUGAUCUUCUCAAAAAAAAAAA----AAAAAAAGAGAAAAGGAAAAA
CcMV2a          GGACUGAAGUGAAUUGAUCUUCUUUCCUUUGUUACCCUCNACUUAUAUUAUGAUCAUGUC
CcMV2c          GGACUGAAGUGAAUUGAUCUUCUUUCCUUUGUUACCCUCNACUUAUAUUAUGAUCAUGUC
                ************************          *      *   *    *  *  *
                                                                      2280
CcMV2b          AAAAGGAAAGAAAAAAAAAGACAAAAAAAAAAAAAACUAAAA------------------
CcMV2a          CUACGCAACGUCCCUGAAUGACAUUUCCAAAGCUUUUGGAAAUUGAUGACUUCUCAAUUC
CcMV2c          CUACGCAACGUCCCUGAAUGACAUUUCCAAAGCUUUUGGAAAUUGAUGACUUCUCAAUUC
                  * * ** *      ** ****     ***     *  **
                                                                      2340
CcMV2b          ------------------------------------------------------------
CcMV2a          UCUCGAGAAGGACCAGAUUAACAUCUUCGAUGUAAUCGCCAUGAUGGAACGUGAGUCAAU
CcMV2c          UCUCGAGAAGGACCAGAUUAACAUCUUCGAUGUAAUCGCCAUGAUGGAACGUGAGUCAAU

                                                                      2400
CcMV2b          ------------------------------------------------------------
CcMV2a          ACUUGAUCUUGAUAUAAAUGAUAAGAAGAAGGUUAAAUUAUCUUUGGAUAAUCUAUAUAA
CcMV2c          ACUUGAUCUUGAUAUAAAUGAUAAGAAGAAGGUUAAAUUAUCUUUGGAUAAUCUAUAAAA

                                                                      2460
CcMV2b          ------------------------------------------------------------
CcMV2a          GUUAAAUUCUAUAAUCAAUAGGACUGGAGCAGGGCUGGAAAUCUUUAUAGAUCGUUGGCG
CcMV2c          AAAACAAAAAAAAAAGAGA----------------------------------------

                                                                      2520
CcMV2b          ------------------------------------------------------------
CcMV2a          GAAAACUUCAUUCCGUUUAAUGACCUAUAAAAGCCUAACAGCUUCUUCUCUGUCCGAUAU
CcMV2c          ------------------------------------------------------------

                                                                      2580
CcMV2b          ------------------------------------------------------------
CcMV2a          ACCCUUUAGCAUUCUUAGUUUUGACGAGAAUGGCAAAUUGUAAUAGAAUCUAACCAACAU
CcMV2c          ------------------------------------------------------------

                                                                      2640
CcMV2b          ------------------------------------------------------------
CcMV2a          GGGAUCGAGGUCUACACACUUCAGUGUGCCUUGUGUACCUGAGCUGAUGUUGCUUAGGA
CcMV2c          ------------------------------------------------------------
```

Appendix 3: Amino acid sequence generated from CcMV1b using the mitochondrial genetic code for yeast mitochondria. The program, protein machine, was used available at http://www.ebi.ac.uk/translate/. The open reading frame is indicated from first methionine to the first stop codon (black letters). The amino acid sequence is derived from Frame 1

TFFFFFTSSFFFFSFFSPTAPVSRGRQRRRGDRTRKAPTVNHPFRVH*TTTVTD*PCNNKGMTK
ADHYSSASSDPPRWTDCCHNTRQHQPTFAMKWIMTKTMKRNTNKTYMHTNTLRDIGAMKL
STVWFTHWSHTETGMASRTVSSAVARFQTTATTRGRNAAMSEFKASRTAFTRWTCGRPTSG
KVGAPMTKAGTPKVIPREARTLLTRERPTYLVKAVMTVTSIGRYFKGGNPVKWENITKPSTP
TTPKDGEITFGLEKLNIDVGQFEPTDWKFRWVVTAGPNGPSMSSCLQDTPKFNGTFRSQVEVI
TPETTPIIDTTLTWEKSFKTSTTMGLAGFKDDSTRKMAIKDDREGKSRPFAMFDYWSQTVLSP
THDWAYATTRSIPQDCTFNQAEGTSKVTARPSQKYFYSYDTEAATDRFPMQFQKKVTSTIFN
TTYAQAWAEMMTQEPFRVKGLSDPLRWGAGQPTGAKSSWAIFTLCHHTVVHMAAVRTNS
DPYYVMTGDDMVTRGSRTATVYKRMMSETGVSMSETKSHVSKDTFEFAKMWMHQGRNAS
GFPVVGTAETTRKPTEMAATFVFETPAKGYPVTITPRTVSQYFTTVARYNTIPPRTAVWTADK
VVWYYSFLSWTATRDDGWAKYIAQSASTLVSPNTAHDTTMKAVRDKWAKQTDKSTMDFQ
DFGFDMFDKVKTLPPFKPTWDPEAWPGRLSASGIEFNPAPRKVPMFAATQEEGEIKYSDYTQ
QKLEMTDDQTTFEEMESLKTPPRPQTKGFTPKRTREYVRTTNTISHGLNRDTRAQCMGTRPD
VYNMKD*EVTPPTLRKWWKTSLAPTHCVPPFPFRGRQKSTACSGRDAGWEVDSAGAKKKK
KKRKKKEK

Appendix 4: Amino acid sequence generated from CcMV2a using the mitochondrial genetic code for yeast mitochondria. The coding region is indicated form the first methionine. The program, protein machine, was used available at http://www.ebi.ac.uk/translate/. The open reading frame is indicated from first methionine after the stop codons to the first stop codon (black letters). The amino acid sequence is derived from Frame 2

FFCFFFTFFGVMTTQVTWQS*CTNPSQSTLPSQVRCAFGYNGTFHSDSRRV*WDNIKIRIARAS
DPT***ITPNCIVKHLH*LINTKTQQHIQNYTTNLMQNWV**EVTIFTCYFY*NHSSSSTKVLMS
NVMVRAGSPKRRFQNLSSWFVGVPVHSPIKDTLWRCRMDLSLSEMTTATHSSLSILKR*CVL
Q*EDWRM*TLFTVKKIFVKLNKYRFPAIIPTEICKDTSWFPGRFHVLSRKVMATTTVISIFRVLP
TKVLPDYTTISKPHSGTIETCSSESIALACKNTEIKRVDKVMKTRLKGSTKAGPNGKMSLTTSL
LDATAFWSDPLRVIHFIWFNIRCYGYFWGTMWSMWLIFIMIISLPYYTMALCTGARAPVMGQ
LATVYDQAGKARIVASTNSWIQCSTFGLHNKIFSILRSIPQDGTFDQNKPFDLLLESTQPGYML
YGFDTSAATDRTPIAFQKDITNHLGYPGGPWRRLTGIKYNSPCGFISYAVGQPMGAYSSFAM
TATTHHVLVQVAAQKAGFSDRFTDYCITGDDIVMANSLVAEAYKSLIFDLGLEISESKSVISG
TFTEFAKKLRGPTMDISPIGAGLMLYSLRNKYYICVLVFEITERGLCMWYDVYPQLTSLLPKI
YRRYFKTCDWFIATHLRRREHTGDQDHEILNPRIAYFNVFLNKEKIISLTEIMWNSTVRDWFR
LWNSIKYTTNKGTFMSQARVGTPDWSELIFFPLLPXTYIMIMSYATSTNDISKAFGNWWTTNS
TEKDQINIFDVIAMMERESMTDTDMNDKKKVKLSLDNTYKLNSMINRTGAGTEIFMDRWRK
TSFRLMTYKSTTASSTSDMPFSITSFDENGKL**NTTNMGSRSTHFSVPCVPETMLTRX

Chapter 3:

Relative pathogenicity of *Cryphonectria cubensis* on *Eucalyptus* clones differing in their tolerance to *C. cubensis*

ABSTRACT

Cryphonectria cubensis causes a destructive canker disease of *Eucalyptus*. Management of this disease is primarily through breeding and selection of disease tolerant trees. One means of selecting such trees is by artificial inoculation with the pathogen. In routine screening trials in South Africa, a highly pathogenic isolate of *C. cubensis* is used for such inoculations. Although the most tolerant clones under natural conditions are the same as those detected in inoculation trials, a question has arisen whether all clones respond similarly to different *C. cubensis* isolates. Thus, a trial consisting of five clones, known to differ in susceptibility to infection by *C. cubensis* was established. These trees were inoculated with nine South African *C. cubensis* isolates previously shown to differ in pathogenicity. Inoculations showed a significant isolate x clone interaction as well as evidence for vertical resistance. Based on these results disease screening should not be done with a single isolate.

INTRODUCTION

Cryphonectria canker caused by *Cryphonectria cubensis* (Bruner) Hodges causes a serious canker disease on *Eucalyptus* in many tropical and sub-tropical areas of the world (Boerboom & Maas 1970; Hodges & Reis 1974; Hodges *et al.* 1979; Gibson 1981; Florence *et al.* 1986). Cryphonectria canker was first reported in South Africa in 1989 (Wingfield *et al.* 1989). This disease is characterised by swollen basal cankers in South Africa and is also favoured by high rainfall (2000-2400 mm/ annum) and temperatures above 23ºC (Sharma *et al.* 1985; Florence *et al.* 1986; Wingfield *et al.* 1989). Since *Eucalyptus* is one of the major plantation trees in the country, it has been important to develop effective management to ensure minimal losses due to Cryphonectria canker.

Various options exist to reduce the impact of Cryphonectria canker. Chemical control has been considered but due to the low value of individual *Eucalyptus* trees this is not economically viable (Sharma *et al.* 1985). Biological control using hypovirulent strains of the pathogen is also attractive but more a longer term option (van Heerden *et al.* 2001). However, the most commonly used approach is to breed and select disease tolerant *Eucalyptus* trees (Alfenas *et al.* 1983; Wingfield 1990).

Deployment of naturally selected disease tolerant *Eucalyptus* spp. has reduced losses in plantations due to *C. cubensis* (Campinhos & Ikemori 1983; Conradie *et al.* 1992). Monoclonal plantations are attractive to forestry companies because of the uniformity of selected clones and their higher productivity over shorter periods of time. However, the combination of favorable environmental conditions and the genetic uniformity of these plantations might lead to substantial losses due to *C. cubensis*, if clones with poor tolerance are inadvertently planted. Virtually nothing is known regarding the genetics of susceptibility of *Eucalyptus* spp. to infection by *C. cubensis*. It has, however, been assumed that resistance to this pathogen is a quantitative trait. This is due to the broad range of susceptibility in inoculation trials on progeny resulting from a cross between a resistant and susceptible *Eucalyptus* clone (van Heerden unpublished). One means to screen trees for disease tolerance is through artificial inoculation, which reduces the confusion relating to disease escape in natural infection trials. Thus artificial inoculation has been effective in screening *Eucalyptus* trees for tolerance to Cryphonectria canker (Alfenas *et al.* 1983; van Heerden & Wingfield 2002).

Routine screening of *Eucalyptus grandis* clones and hybrids with *C. cubensis* to identify disease tolerant planting stock has been conducted in South Africa for several years. Associated trials such as those assessing the capacity of *Eucalyptus* clones to heal wounds after mechanical damage have shown a positive correlation with tolerance to disease caused by *C. cubensis* (van Zyl *et al.* 1999). Likewise, a strong genotype by environmental effect has been shown using inoculation trials with the fungus in different areas of South Africa (van Heerden & Wingfield 2002). These trials have all been conducted using a single genotype of *C. cubensis*, which was selected from a large collection of isolates to represent an isolate with a high level of pathogenicity. However, the question has arisen as to whether different *Eucalyptus* clones might show differential tolerance to infection by different isolates of *C. cubensis*. The aim of this study was thus to resolve this question by inoculating a selection of clones with a suite of isolates chosen to have different levels of pathogenicity.

MATERIALS AND METHODS

Isolates

Nine South African *C. cubensis* isolates were selected for this study. In a previous trial (van Heerden & Wingfield 2001) eight of these isolates were shown to differ in pathogenicity. Four isolates with low levels of pathogenicity and four highly pathogenic isolates were specifically selected. All isolates had also previously been shown to belong to different vegetative compatibility groups (VCGs) of *C. cubensis* (van Heerden & Wingfield 2001). As a positive control, *C. cubensis* isolate CMW 2113, which has been shown to be highly pathogenic (van Heerden & Wingfield 2001), and has been used in annual disease screening trials was included. All the isolates used in this study are stored in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa.

Eucalyptus clones

Five *Eucalyptus* clones were selected for this experiment and these were planted in a specifically designed field trial. These clones were selected based on differences in their level of disease tolerance when challenged with the *C. cubensis* isolate, CMW2113 (Table 1), as determined in a previous inoculation trial (Wingfield, unpublished data). Each of the *Eucalyptus* clones was vegetatively propagated by making cuttings from parent hedge plants.

These cuttings were rooted and hardened before being planted in a fully randomised block design.

The trail was established in the Canewoods plantation, Kwambonambi area, Kwazulu-Natal, South Africa (28º38' S; 32º06' E) (1998). The trial consisted of 20 rows of trees each with five blocks made up of ten trees. The five clones were planted in the five blocks and they were randomised between the rows. Thus, a total of 200 trees were planted per clone. The trees were planted with a spacing of 3 x 2.5m and the trial was surrounded by buffer rows of *E. grandis* trees and allowed to grow for 24 months before treatment.

Inoculation procedure and evaluation

The inoculum was prepared by growing each of the nine *C. cubensis* isolates on 90 mm diam. Petri dishes containing 2% Malt Extract Agar (MEA) (Biolab). The plates were incubated at 25 °C for seven days prior to inoculation. The trees were inoculated by removing a cambial disc about 140 cm from the ground with a 20 mm diam. cork borer. A similar sized disc taken from an actively growing fungal culture (one of the nine isolates) was placed in the wound with the mycelium side facing the cambium. The wounds were sealed with masking tape to reduce desiccation. All the trees were inoculated on the shadow side of the tree. The trial was inoculated in such a way that there were 20 replicates for each of the nine isolates on all five clones. Twenty trees were also inoculated in a similar manner on the five clones, with a sterile MEA plug, which acted as the negative control. Thus, a total of 1000 trees were tested in this experiment.

Lesion length, width and the circumference of the tree at the point of inoculation were measured six months after inoculation. Differences in lesion width among tree genotypes and the isolates were analysed using a two way ANOVA (Systat version 7.0), with lesion width as the dependant and the clones and isolates as the trial factors. The tree circumference was included as a covariate. A simple effects analysis was done with data for the nine different isolates to determine the individual effects of the isolates on the clones. The data were also re-analysed with the exclusion of the most disease intolerant *E. grandis* clone, ZG14. This was done to ensure that the interactions observed were not unjustifiably influenced by one clone.

RESULTS

Six months after inoculation most of the inoculated trees had developed obvious cankers in the cambium (Fig. 1). No lesion development was associated with the control inoculations, which were grown over by callus tissue. This was indicated by a lesion width of 20 mm and thus the same as that of the cork borer. The trees used in this inoculation study tended to have relatively small lesions (mean lesion width 60.8 mm) and thus exhibited a high level of tolerance to infection. Lesion widths differed significantly between the isolates ($F=13.6$; $df=9$; $p<0.001$) and between the clones ($F=96.2$; $df=4$; $p<0.001$) (Table 2a). Since the clones used in this study were known to differ in tolerance to *C. cubensis* and the isolates to differ in pathogenicity, this result was expected. There was also a significant Isolate x Clone interaction (Table 2a) indicating that not all the clones responded in the same way, to all the isolates. There was also a significant difference observed for the tree circumference which was used as a co-variat in the analysis of variance (Table 2a).

Simple effects analysis on the inoculation data for *C. cubensis* isolate CMW2113 showed that clone ZG14 was the least tolerant and lesions on this clone were significantly larger than those on the other clones (Table 3; Fig. 2). Inoculations with isolate CMW2113 also showed that clone TAG 5 had obviously larger lesions than those on clones GT529, GC121 and GU21, although they were not significantly different from each other (Table 3; Fig. 2)

In all the inoculations clone ZG14 was the most diseased. Typically followed by clone TAG5 while clones GT529, GC121 and GU21 for disease severity fluctuated slightly from isolate to isolate (Fig. 2). The only significant difference, which existed, was between clone ZG14 and the other clones. This was true for the inoculation with all the isolates except isolate CMW11346 where clones ZG14 and TAG5 did not differ significantly from each other (Table 3; Fig. 2).

The exclusion of ZG14 from the data and the subsequent analysis of variance also indicated that lesion widths differed significantly between the isolates ($F=9.4$; $df=9$; $p<0.001$) and between the clones ($F=11.5$; $df=3$; $p<0.001$) (Table 2b). However, the difference between the previous analysis and this was that there was a significant Isolate x Clone interaction at the 95% confidence interval and not at the 99% confidence interval in the analysis with ZG14

(Table 2b). There was also a significant difference observed for the circumference which was used as a co-variat in the analysis of variance as previously observed (Table 2b).

DISCUSSION

The current breeding strategy for *Eucalyptus* in South Africa involves disease resistance screening of possible planting stock in the field. Up until the present time, all the inoculations for disease screening have been done with a single highly pathogenic *C. cubensis* isolate. Results of this study have indicated that inoculation data for this isolate are not similar to that of the other *C. cubensis* isolates. Results have also shown a significant clone x isolate interaction as well as a case of clone GC121 showing immunity to isolate CMW11335. It can, therefore, be concluded that the disease screening protocol for disease resistance in *Eucalyptus* should not be done using a single isolate, and that more than one genotype of the fungus should be taken into consideration.

Van der Plank (1984) has indicated that virulence and vertical resistance are indicated by an interaction in the analysis of virulence. He further suggested that aggressiveness and horizontal resistance are indicated by main effects between pathogen isolates and host varieties. The overall conclusion here was that both these types of host pathogen response can be present in a host-pathogen system (Van der Plank 1984). Results of the present study have shown a significant isolate by clone interaction. Based on these results we can, therefore, speculate that disease resistance to Cryphonectria canker of *Eucalyptus* in South Africa follows a probable vertical resistance model. This also provides further evidence that a single isolate is not sufficient for appropriate disease screening, in the case of this pathogen.

It is suggested that polygenic disease resistance (Horizontal resistance) is a durable resistance and a gene-for-gene resistance (vertical resistance) represents temporary resistance (Robinson 1996). The inheritance of disease resistance in forest trees has mostly been explained by polygenic models (von Weissenberg 1990). However, a possible gene for gene model has also been proposed for resistance to fusiform rust caused by *Cronartium quercuum* (Berk.) Miyabe ex. Shirai f. sp. *fusiforme* in *Pinus teada* L. (Loblolly pine) (Kinloch & Walkinshaw 1991). Further studies have indicated that long term resistance to fusiform rust could be obtained from a single qualitative resistance gene in Loblolly pine (Wilcox *et al.* 1996). This indicated that resistance in this system was not exclusively polygenic. It is, therefore,

important in any breeding model to take the host-pathogen interaction pertaining disease resistance into consideration.

Results of this study provide further support for the reliability of the artificial inoculation protocol used to screen for trees tolerant towards Cryphonectria canker. Van Zyl *et al.* (1999) have shown that the capacity of *Eucalyptus* clones to heal wounds, caused by mechanical damage, can be directly correlated with the susceptibility of the trees. Molecular markers might also be used in the near future to select disease tolerant trees. However, for an effective disease screening strategy, which will select clones of *Eucalyptus* that will have a durable resistance, it is important to understand the genetics of plant-pathogen interactions. Our preliminary data suggesting a vertical resistance will need to be confirmed using a detailed genetic analysis of the host pathogen interaction of *Eucalyptus*.

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Table 1: *Eucalyptus* clones selected from previous field inoculations with *C. cubensis* isolate (CMW2113) with the difference in disease susceptibility indicated. These clones were used in the current study.

Clone number	Clone	Disease susceptibility
ZG14	*E. grandis*	Highly susceptible
TAG5	*E. grandis*	Moderately susceptible
GU21	*E. grandis* x *E. urophylla*	Tolerant
GC121	*E. grandis* x *E. camaldulensis*	Tolerant
GT529	*E. grandis* x *E teretericornis*	Moderately susceptible

Table 2: Two way analysis of variance table with all the clones (a) and without ZG14 (b).

A

	SS	df	Mean Square	F-Ratio	P
Isolates	464056.3	9	51561.8	13.6	0.0001
Clones	1457067.3	4	364266.8	96.2	0.0001
Isolate x Clone	397320.9	36	11036.7	2.9	0.0001
Circumference	53726.9	1	53726.9	14.2	0.0001
Error	3453295.6	912	3786.5		

B

	SS	df	Mean Square	F-Ratio	P
Isolates	158820.1	9	17645	9.4	0.0001
Clones	64825.3	3	21608	11.5	0.0001
Isolate x Clone	84650.7	27	3135	1.7	0.018
Circumference	23756.7	1	23756	12.7	0.0001
Error	1370493	732	1872		

Table 3: Mean lesion width (mm) ± SEM caused by South African *Cryphonectria cubensis* isolates after inoculation on five different *Eucalyptus* clones. Significant differences within the single isolates and within the single clones were determined by the Tukey's test (p= 0.05).

	5 *Eucalyptus* clones															
		w	x		w	x		w	x		w	x		w	x	
Isolates	**GC121**			**GT529**			**GU21**			**TAG5**			**ZG14**			[y]**Mean within isolates**
CONTROL	20 ± 0		a	20 ± 0		a	20 ± 0		a	20 ± 0		a	20 ± 0		a	**20**
CMW2113	34.2 ± 6.7	ab	a	23.9 ± 3.9	ab	a	24.5 ± 2.3	a	a	72.9 ± 11.4	ab	a	144.7 ± 30.5	abc	b	**60.04**
CMW11345	58.9 ± 10.6	b	a	61.3 ± 7	b	a	52.8 ± 15.0	a	a	90.5 ± 10.2	b	a	228.9 ± 27.4	c	b	**98.48**
CMW11344	31.9 ± 6.2	ab	a	23.3 ± 3.2	ab	a	35 ± 13.9	a	a	37 ± 9.3	a	a	144.7 ± 28.9	abc	b	**54.38**
CMW11319	37.5 ± 9.1	ab	a	48.3 ± 16.1	ab	a	61.8 ± 18.6	a	a	107.6 ± 17.9	b	a	210.3 ± 23.7	bc	b	**93.10**
CMW11326	28.2 ± 5.3	a	a	21.0 ± 1.0	a	a	38.2 ± 11.9	a	a	37.2 ±8.1	a	a	125.8 ± 29.0	ab	b	**50.08**
CMW11339	21 ± 1.0	a	a	23.5 ± 3.5	ab	a	38 ± 13.0	a	a	36.6 ± 7.9	a	a	94 ± 22.8	a	b	**42.62**
CMW11335	20 ± 0	a	a	38.4 ± 18.4	ab	a	54.2 ± 20.3	a	a	30.5 ± 5.3	a	a	151.3 ± 25.7	abc	b	**58.88**
CMW11346	35.5 ±3.1	ab	a	37.3 ± 4.4	ab	a	45.5 ± 13.7	a	a	71.7 ± 10.1	ab	ab	119.3 ± 22.9	abc	b	**61.86**
CMW11318	41.8 ± 5.8	ab	a	32.9 ± 5.1	ab	a	36.1 ± 13.1	a	a	75.5 ± 10.8	ab	a	185.3 ± 25.8	abc	b	**74.32**
[z]**Mean within clone**	**32.90**			**32.99**			**40.61**			**57.95**			**142.43**			

[w] Significant differences between the different *C. cubensis* isolates within a single *Eucalyptus* clone. Isolates with the same letter do not differ significantly from each other

[x] Significant differences between the different *Eucalyptus* clones within a single *C. cubensis* isolate. Clones with the same letter do not differ significantly from each other

[y] Mean lesion width (mm) within the isolates

[z] Mean lesion width within the clones

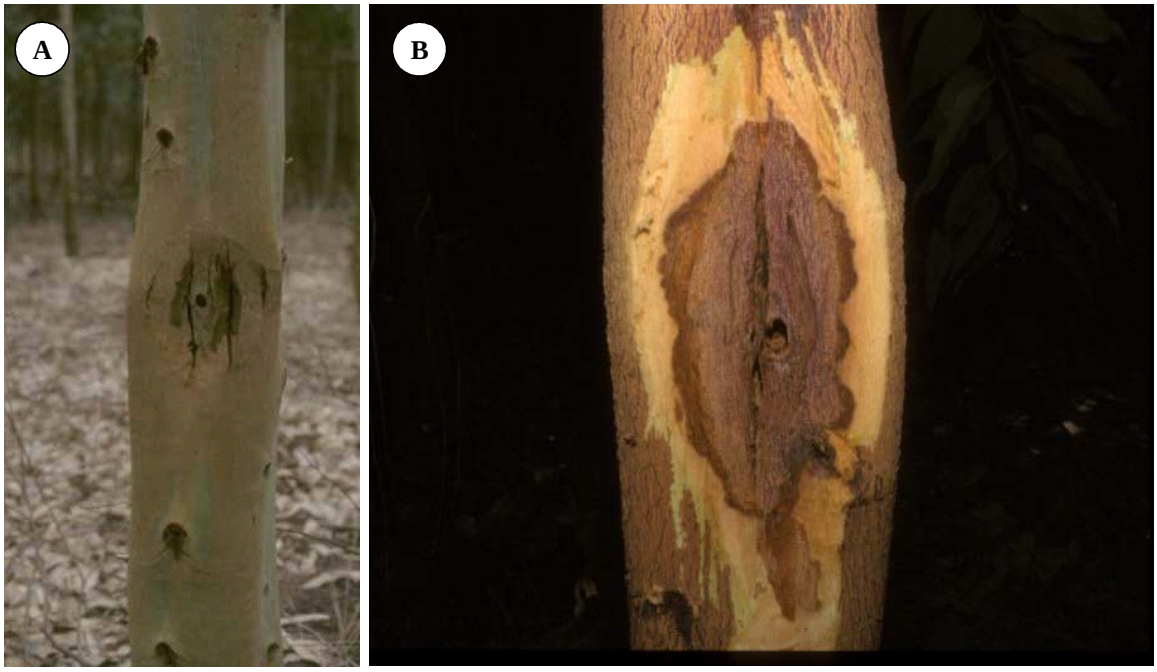

Figure 1: Lesions on a *Eucalyptus* clone after artificial inoculation with *Cryphonectria cubensis*. A: Canker caused by *C. cubensis*. B: Canker exposed under the bark surface.

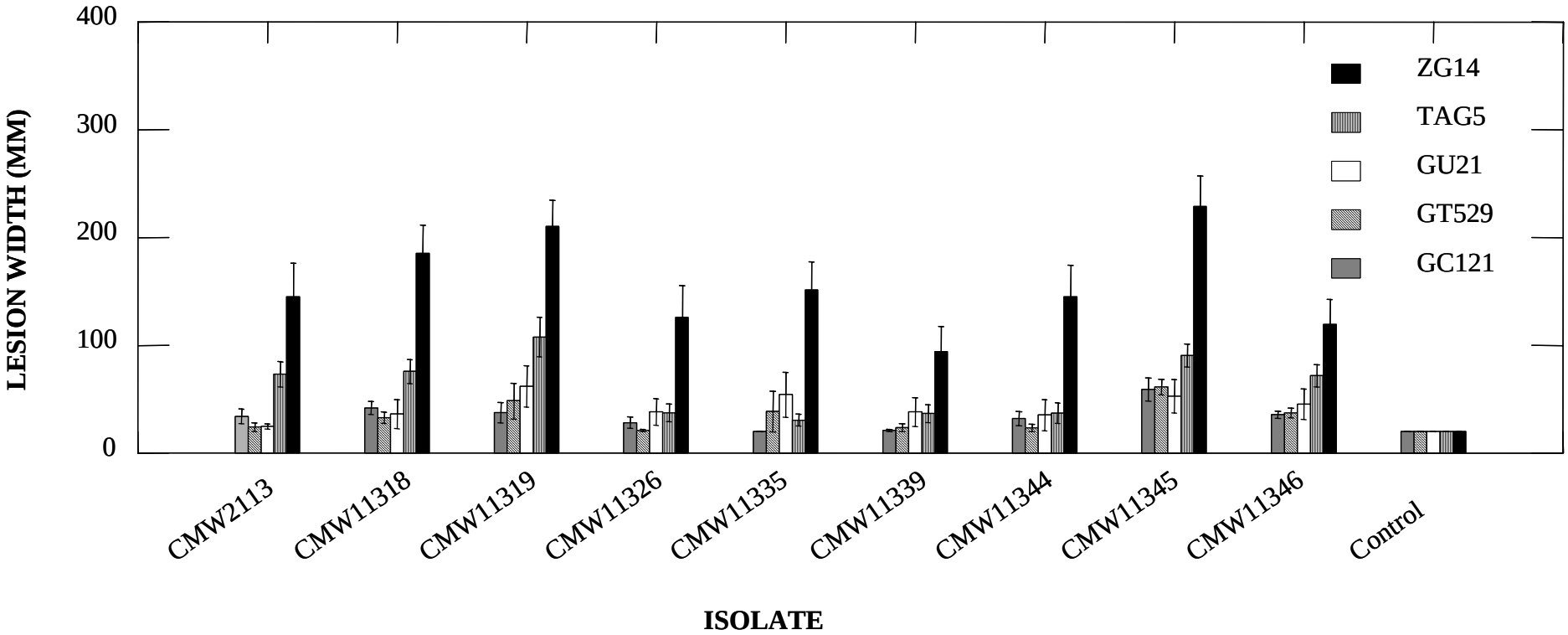

Figure 2: Mean lesion width (mm) ±SEM of the South African *Cryphonectria cubensis* isolates inoculated on the five *Eucalyptus* clones

Chapter 4:

Transfection studies with the *Diaporthe RNA virus* (DaRV) and other *Cryphonectria cubensis* isolates

A portion of this work forms part of a larger study and is published as:

Characterization of Diaporthe perjuncta transfected with Diaporthe RNA virus (DaRV). MOLELEKI, N., VAN HEERDEN, S. W., WINGFIELD, M. J., WINGFIELD B.D. and PREISIG O. (2003). Transfection of *Diaporthe perjuncta* with *Diaporthe RNA virus* (DaRV). *Applied and Environmental Microbiology* 6(7) 3952-3956.

ABSTRACT

Cryphonectria cubensis and *Diaporthe perjuncta* are important pathogens of *Eucalyptus* and grapevine respectively. A positive stranded RNA virus, *Diaporthe* RNA virus (DaRV) from a South African *D. perjuncta* isolate has been fully characterised. RNA transcripts from a cDNA clone of DaRV have also been transfected into an isolate of the peach pathogen *D. ambigua*. Due to the lack of the availability of *D. perjuncta* isolates that are free of viruses, transfection of the natural host could not be attempted at that time. The aim of this study was to expand the transfection studies using the DaRV RNA transcripts. Isolates chosen in this study included, a virus free *D. perjuncta* isolate, a virulent *C. cubensis* isolate and a hypovirulent *C. cubensis* isolate containing the hypovirus CHV1-EP713. Using electroporation, we were able to infect virus-free *D. perjuncta* isolate with DaRV. The transfectant exhibited altered colony morphology in comparison to the original isolate. Furthermore, the vector derived nucleotides which were present in the RNA used for the transfection were not present in the RNA isolated from the transfected isolate. Pathogenicity tests showed that the transfection did not result in a reduction of virulence of the fungus. We were unable to transfect either of the *C. cubensis* isolates included in this study. This suggests that DaRV does not replicate in *C. cubensis* isolates. The successful transfection of *D. perjuncta* for the first time with the virus from this fungus extends the transfection range for this virus.

INTRODUCTION

Mycoviruses are known to occur in a wide range of fungi. Many of these viruses have been well characterised and infect phytopathogens such as *Sphaeropsis sapinea* (Fr.:Fr.) Dyko & Sutton (Preisig *et al.* 1998; Steenkamp *et al.* 1998), *Diaporthe perjuncta* Niessl. (Smit *et al.* 1996; Moleleki *et al.* 2002), *Leucostoma persoonii* (Nits.) Hoehn (Hammar *et al.* 1989), *Ophiostoma novo-ulmi* (Rodgers 1986, 1987), *Cryphonectria cubensis* (Bruner) Hodges (van Heerden, Chapter 2) and *Cryphonectria parasitica* (Murr.) Barr (Day *et al.* 1977; Shapira *et al.* 1991). Many of these viruses cause hypovirulence in the host fungi and are, therefore, of interest as potential biological control agents.

Cryphonectria cubensis is a serious *Eucalyptus* pathogen in numerous parts of the world including South Africa where it was first reported in 1989 (Wingfield *et al.* 1989). This is a well-studied fungus and could serve as a model organism for various similar tree pathogens. *Diaporthe perjuncta* is a pathogen of *Vitis vinifera* (Melanson *et al.* 2002). A double stranded RNA virus has been found in a slow growing hypovirulent isolate of this fungus (Smit *et al.* 1996). The nucleotide sequence of the dsRNA element has been determined and characterised as the replicating stage of the *Diaporthe RNA virus* (DaRV) genome (Preisig *et al.* 2000; Moleleki *et al.* 2002). Moleleki (2002) used the DaRV in transfection studies and successfully transfected *D. ambigua* a related pathogen of pome and stone fruit, as well as a *Phomopsis* isolate from peach with this virus. Due to a taxonomic confusion at the time, *D. perjuncta*, original host of DaRV (*D. perjuncta*) was never transfected.

The virus (CHV1-EP713) associated with the chestnut blight pathogen *C. parasitica* is one of the dsRNA viruses that confers hypovirulence to its host (Day *et al.* 1977). Other characteristics include altered colony morphology (Anagnostakis 1982; Elliston 1985a, 1985b), reduced or lost sporulation (Anagnostakis 1982; Elliston 1985a), reduced pigmentation (Anagnostakis 1982), reduced oxalate accumulation (Havir & Anagnostakis 1983) and reduced laccase production (Rigling *et al.* 1989). These characteristics are promising for its possible use in biological control. Since the completion of the nucleotide sequence of this hypovirus, it has been used in various transfection and transformation studies (Dawe & Nuss 2001).

Transfection of spheroplasts produced from virus free *C. parasitica* with the full length *in vitro* produced CHV1-EP713 transcripts using electroporation have been successful (Chen *et al.* 1994a). The host range for CHV1-EP713 has been expanded to three species of *Cryphonectria* namely *C. cubensis*, *C. havanenesis* (Bruner) Barr. and *C. radicalis* (Schw.:Fries) Barr., and one species of *Endothia* namely *E. gyrosa* (Schw.: Fries) Fries (Chen *et al.* 1994a). All of these species have been successfully transfected with the *C. parasitca hypovirus* RNA (Chen *et al.* 1994a). A virulent South African *C. cubensis* isolate (CMW2113) has also been transfected with CHV1-EP713, resulting in hypovirulence as well as the production of a bright yellow-orange pigment in culture (van Heerden *et al.* 2001).

The aim of this study was to use the *Diaporthe RNA virus* (DaRV) in transfection studies. We attempted to transfect a *C. cubensis* isolate containing CHV1-EP713, a wild type virus-free *C. cubensis* isolate, and a *D. perjuncta* isolate. This would enable us to determine whether *D. perjuncta* could be transfected by DaRV and to assess the possibility of using such as transfectant isolate as a biological control agent. By including the CHV1-EP713 infected *C. cubensis* isolate, we considered the possibility of co-infecting an isolate with DaRV.

MATERIALS AND METHODS

Isolates and cultural conditions

The isolates selected for this study were the *C. cubensis* isolate (CMW2113-T) transfected with the *C. parasitica hypovirus* CHV1-EP713 (Van Heerden *et al.* 2001), a virus infected *D. perjuncta* isolate (CMW3407) (Smit *et al.* 1996; Moleleki *et al.* 2002) and a virus free *D. perjuncta* isolate (CMW8597). The isolates were all maintained on 2% MEA (Malt extract agar) and are stored in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria.

Preparation of fungal sheroplasts

Fungal spheroplasts were prepared using the method of Royer & Yamashiro (1999) and Moleleki (2002) with minor modifications. The fungal isolates CMW2113-T, CMW2113 and CMW8597 were all grown in 20 ml McCartney bottles containing 5 ml 2% ME (Malt extract) broth for 6 days. The mycelium was removed from the medium and placed in a 6 mm diam. Petri dish. The excess medium was removed with a pipette. Chitinase (0.5% w/v)

(Fluka) and cellulose (1% w/v) (Sigma) were dissolved in 6 ml of 1M Magnesium Sulphate ($MgSO_4.7H_2O$), added to the mycelium and incubated overnight at room temperature. The mixture was then sieved through 120 micron flour gauze (Swiss Milling Company). An equal volume of ice cold 1M sorbitol (Sigma) was added to the resulting spheroplasts. The spheroplast solution was placed in 1.5 ml Eppendorf tubes and centrifuged at 5000 rpm for 5 min at 4ºC. The pellet was then washed with 500 µl ice cold 1M sorbitol before being centrifuged at 5000 rpm for 5 min at 4ºC. The pellet was resuspended in 500 µl STC (1M Sorbitol, 50 mM $CaCl_2$, 50 mM Tris-HCl pH 8) and centrifuged at 5000 rpm for 5 min at 4ºC. The spheroplasts were resuspended in 85 µl sorbitol and stored on ice for a short period before electroporation.

Transfection of the fungal spheroplasts with the in vitro produced DaRV RNA

A full length cDNA copy of the *D. ambigua* RNA virus (DaRV) was cloned in the pGEM®-T Easy Vector (plasmid pDV3) (Preisig *et al.* 2000). The RNA was synthesized from the *Sal* I linearised plasmid. A reaction mixture was prepared containing 150 ng linearised plasmid, 1 mM of each NTP's (ATP, CTP, GTP, UTP), 1 x transcription buffer, 0.5 U T7 RNA polymerase, 1 U RNase inhibitor (Roche Diagnostics). The volume was adjusted to 20 µl. The reaction was allowed to proceed at 30ºC for 2 hours. The transcription products were subsequently analysed on a 1% agarose gel stained with ethidium bromide and viewed under UV light.

Transfections were done by electroporation using a multiporator (Eppendorf) as described by Chen *et al.* (1993) with minor modifications. A mixture was made that contained 85 µl of freshly prepared spheroplasts, 0.12 U RNase inhibitor, 15 µl in vitro produced RNA and was incubated for 5 min on ice. This mixture was then added to a pre chilled 100 µl cuvette (1 mm gap width) (Eppendorf). The spheroplast RNA solution was pulsed 10 times at 1500-2500 Volts with a 5 sec interval between the pulses. For each set of fungi, a negative control transfection was done where the RNA was replaced with water. A volume of 500 µl 1M sorbitol was added to the cuvette and placed on ice for 10 min. Two hundred microlitres of the transfected spheroplasts were placed on the middle of a 90 mm diam. Petri dish. Regeneration medium (48ºC) (0.1% casein hydrolysate, 0.1% yeast extract, 34.2% sucrose, 1.6% agar) was then slowly added to the Petri dish until the transfected spheroplasts were covered with medium. The plates were allowed to solidify and incubated in the dark at room temperature for 1-2 weeks. After sufficient growth, small pieces of agar were arbitrarily cut

from the culture and placed in the centre of a sterile MEA plate. The plates were sealed with parafilm and incubated at 25ºC. After the incubation period, Erlenmeyer flasks containing 200 ml ME broth were inoculated with putatively transfected fungi and incubated at 25ºC for 1 week. The mycelium was then harvested and lyophilised.

Detection of DaRV RNA

Total RNA was extracted from all the isolates used in the transfection process as well as from the naturally DaRV-infected *D. ambigua* isolate (CMW3407) using the High Pure RNA isolation kit (Roche Diagnostics). The detection of DaRV RNA was done by a one step RT-PCR amplification using DaRV specific primers (Preisig *et al.* 2000) and the LightCycler instrument (Roche) with the LightCycler- RNA Amplification Kit SYBR Green I (Roche Diagnostics). A reaction mixture was prepared which contained 0.75 ng total RNA, 0.5 µM primer DaRV5' (5' GGGAAATTTGTGAGATTATCGCC3'), 0.5 µM primer Oli 78 (5' CCTGGGTGACGGTTGTTACAC 3'), 1 x LightCycler-RT-PCR reaction mix SYBR green I, 6 mM $MgCl_2$, 0.4 µl LightCycler-RT-PCR enzyme mix and PCR grade sterile ddH_2O to a total volume of 20 µl. The cycle conditions were a reverse transcription reaction at 50ºC for 10 min, followed by a denaturation step at 95ºC for 10 sec, 35 cycles at 95ºC for 0 sec, 62ºC for 4 sec and 72ºC for 16 sec. The final PCR products were separated on a 1.5% agarose gel stained with ethidium bromide and visualized under UV light.

Sequence of ends of the viral genomes from the transfectants

To determine whether the complete virus was transfected, the distal ends were determined using the RACE approach as described by Frohman (1994). For this procedure a 5'/3' RACE kit was used (Roche Diagnostics). Double-stranded RNA was extracted from the successfully transfected *D. perjuncta* isolate (CMW8597-DaRV) and purified using the BIO 101 RNaid kit (BIO 101; Qbiogene Inc.) (Valverde *et al.* 1990; Preisig *et al.* 1998). The 5' and 3' end were reverse transcribed with primer Oli 73 (5' GTGCCCTGCACAAACAACTC 3') and Oli 75 (5' TCCATCTCACCGGGAGCGGCAG 3') respectively. A poly-A tail was added to the cDNA by the addition of terminal transferase and 2 mM dATP. The tailed cDNA was used in the PCR amplification to determine the 5' and 3' terminal sequence. PCR was performed with an oligo-dT anchor primer and the nested primers Oli 78 (5' CCTGGGTGACGGTTGTTACAC 3') and Oli 81 (5' TTGAACGATGGGTGTAGGTGG 3') for the 5' and 3' ends, respectively. The PCR products were cloned in the pGEM®-T-Easy

vector (Promega). The inserts were sequenced using the ABI PRISM Big Dye Terminator cycle sequencing ready reaction kit (Perkin Elmer). The sequenced products were analysed using an ABI PRISM 3100 automated DNA sequencer (Applied Biosystems).

Pathogenicity tests on apples

The pathogenicity of the *D. perjuncta* isolates CMW3407, CMW8597-DaRV and the non-transfected isolate CMW8597-WT were compared using an apple based test as described by De Lange *et al.* (1998). Prior to inoculation the Golden delicious apples were surface sterilised with 70% ethanol. Discs (15 mm in depth) were removed from the sides of the apples using a 5 mm diam. cork borer. Similar sized mycelial plugs were placed mycelial side down into the wounds and sealed with masking tape to reduce desiccation. Ten apples were each inoculated with the fungal isolates. In a similar manner 10 apples were inoculated with sterile PDA discs which served as negative controls. The apples were incubated at room temperature for ten days whereafter the masking tape was removed and the lesion area measured. The data were analysed using a one way ANOVA (SYSTAT version 7.0.1). The entire trial was repeated once and a Pearson correlation test was performed to compare the two trials

RESULTS

Transfection of the fungal spheroplasts with the in vitro produced DaRV RNA

Diaporthe perjucta isolate (CMW8597) was successfully transfected with the *Diaporthe RNA virus* (DaRV). The transfectant was named CMW8597-DaRV. The successful transfection with DaRV in this study was accomplished using 10 pulses at 2000V during the electroporation. Transfection also occurred at a lower voltage of 1500 V but no transfection occurred at higher voltages. Reverse transcription PCR using the LightCycler with the DaRV specific primer pair, DaRV5' and Oli 78, resulted in an amplicon of ≈350 bp in size in CMW8597-DaRV as well as in the naturally virus infected isolate (CMW3407) (Fig. 1). *Cryphonectria cubensis* isolates, CMW 2113 (virulent) and CMW 2113-T (CHV1-EP713 infected), were not transfected with the *Diaporthe RNA virus* (DaRV) at any of the conditions used in the electroporation (Fig. 1) and no PCR amplification was observed.

Sequence of ends of the viral genomes from the transfectants

Transcription from the *Sal* I linearised plasmid pDV3 resulted in the introduction of 35 vector derived nucleotides at the 3' end of the viral genome. The 5' end of the virus had 61 vector derived nucleotides since T7 RNA polymerase initiates transcription 61 bases before the cDNA insert. Sequence analysis of the 5'/3' RACE PCR products for the 3' and 5' ends of the transfected *Diaporthe RNA virus* isolate (CMW8597-DaRV) showed that the vector-derived nucleotides had not been replicated in the transfected isolate.

Pathogenicity tests on apples

Lesions were observed when the *D. perjuncta* isolates CMW3407, CMW8597-WT and CMW8597-DaRV were inoculated into the apples. No lesions were observed when the inoculation was performed with the sterile PDA discs. Significant differences were observed between the isolates for both the first ($F=45$; $df=3$; $P<0.001$) and the second repeat ($F=33$; $df=3$; $p<0.001$) of this experiment. The Bonferroni pairwise comparison indicated that there were no significant differences between the mean lesion area for CMW8597-DaRV and CMW8597-WT. The Pearson correlation test showed a good correlation of 96% between the two experiments.

Phenotypic changes

When the transfected isolate CMW8597-DaRV was inoculated onto PDA the mycelial growth was slightly changed from the untransfected isolate. The transfected CMW8597-DaRV isolates resulted in slight morphological changes such as the enhanced aerial growth, a fluffy appearance with the production of a slight yellowish pigment when compared to the naturally virus infected and virus free isolate (Fig. 2).

DISCUSSION

In this study we were able to transfect an isolate of *D. perjuncta* (CMW8597) with the *Diaporthe RNA virus* (DaRV). This is the first time that the natural host of DaRV has been transfected with this virus. Although other fungi have been transfected with hypoviruses (Chen *et al.* 1994a; Chen *et al.* 1996, Chen & Nuss 1999; van Heerden *et al.* 2001), this is the first time that *D. perjuncta* has been transfected with a mycovirus and, therefore, extends the range of transfection for spheroplasts of a filamentous fungus

Reverse transcription PCR from the transfected *Diaporthe RNA virus* isolate (CMW8597-DaRV), showed that the dsRNA was derived from DaRV. The determination of the 3' and 5' ends of CMW8597-DaRV showed that there were no vector-derived nucleotides. A similar observation has been made for transformed *C. parasitica* strains, where it was shown that the cDNA derived hypoviral RNA are trimmed of vector derived nucleotides (Chen *et al.* 1994b). It is, therefore, possible that a similar mechanism must be involved in the trimming effect although the virus replication was initiated in this case from a plasmid construct. It is also possible that virus replication only commences exactly at the start of DaRV sequences with the recognition of replication initiation motifs.

The transfected CMW8597-DaRV isolates exhibited slight morphological changes when compared with the virus free isolate. These differences included increased aerial growth, fluffy appearance and the production of a light yellow pigment. Transfection of *C. cubensis* and *C. parasitica* isolates with CHV1-EP713 has resulted in morphological changes in the host (Chen *et al.* 1994a; Chen *et al.* 1996; van Heerden *et al.* 2001). However, the phenotypic changes observed for our study were not as striking as those for the hypovirus *C. parasitica* transfected isolates.

Cryphonectria cubensis isolates, CMW 2113 (virulent) and CMW 2113-T (CHV1-EP713 infected), could not be transfected with the *Diaporthe RNA virus* (DaRV). This may be due to large differences between *C. cubensis* and *D. perjuncta*. Although these fungi reside in the same taxonomic order, they may be insufficiently similar to enable the virus from *D. perjuncta* to replicate in *C. cubensis*. Effective transfection relies on the ability of the fungal recipient to undergo anastomosis after cell wall regeneration to permit the spread of the viral RNA (Nuss *et al.* 2002). However, many fungi can exhibit a filamentous or yeast-like morphology and anastomosis occurs only in the filamentous phase (Nuss *et al.* 2002) The regeneration medium used in this study enhances yeast-like growth (Chen & Nuss, unpublished), which might influence the successful anastomosis and thus the spread of the virus from transfected cells. Further studies will need to be conducted to understand why the transfection was not possible in *C. cubensis*.

No significant differences in the mean lesion area were observed on apples inoculated with virus free and transfected isolates of *D. perjuncta*. Since the naturally virus infected isolates are known to be hypovirulent, this result was surprising. We had expected that the

transfected isolate would exhibit hypovirulence similar to that found in naturally infected isolates (Smit *et al.* 1996). Smit *et al.* (1996) further observed that the hypovirulence factor could spread via hyphal anastomosis to other isolates. For the *C. parasitica* genomes it was shown that the relative contribution of the hypovirus to hypovirulence associated symptoms should be interpreted with caution since the movement of hypoviruses via hyphal anastomisis might lead to the potential transmission of organelles or nuclear genetic information (Chen & Nuss 1999). The other possibility for lack of hypovirulence in transfected isolates might be that the *D. perjuncta* isolate used in this study was relatively non-pathogenic and that its transfection with the virus did not have a significant impact on the virulence.

The slight effect on the morphology as well as the inability of the transfected strain to induce hypovirulence, reduces the potential usefulness of DaRV as a biological control agent. However, results of this study have shown the potential of expanding the transfection system to other viruses. Further, the transfection of other fungal viruses for which full genome sequences are available might lead to the identification of a suitable biological control agents for other pathogens.

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Table 1: Mean lesion area on golden delicious apples ten days after inoculation with the natural infected (CMW3407) isolate, DaRV transfected (CMW8597-DaRV) isolate, and the virus free isolate (CMW8597) of *Diaporthe perjuncta*.

Isolate	Mean lesion area[a]		Significant differences[b]
	Repeat 1	Repeat 2	
Agar	113±0	113±0	a
CMW3407	355±33	369±11	b
CMW8597-DaRV	629±41	1024±80	c
CMW8597-WT	611±49	891±126	c

[a]Mean lesion area (mm^2) were determine by calculating the lesion area with the formula area=πr^2 with the radius being the (horizontal diameter + vertical diameter) / 4.

[b]Isolates with different letters differed significantly from each other (p=0.001) as indicated for the Bonferroni pairwise comparison.

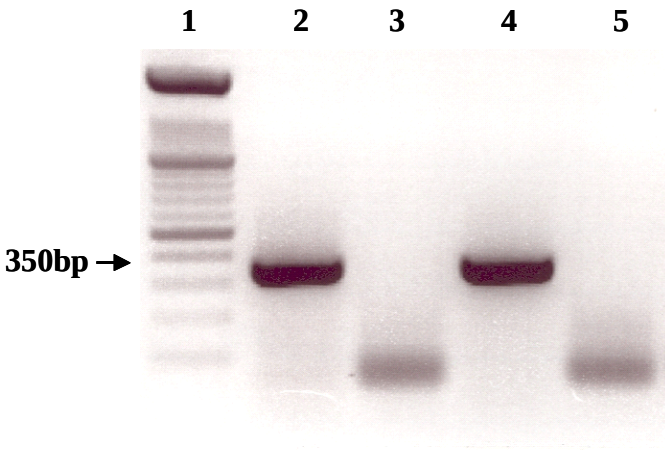

Figure 1: Confirmation of successful transfection with the *Diaporthe RNA virus* (DaRV) using reverse transcription (RT) PCR. The products were separated on a 1.5% agarose gel, stained with ethidium bromide and visualised under UV light. Lane 1: 100 bp molecular weight marker, Lane 2: *D. perjuncta* isolate, CMW8597-DaRV, Lane 3: CMW8597 water transfected, Lane 4: *D. perjuncta* natural virus infected isolate, CMW3407- positive control Lane 5: *C. cubensis* isolate, CMW2113-T after transfection,

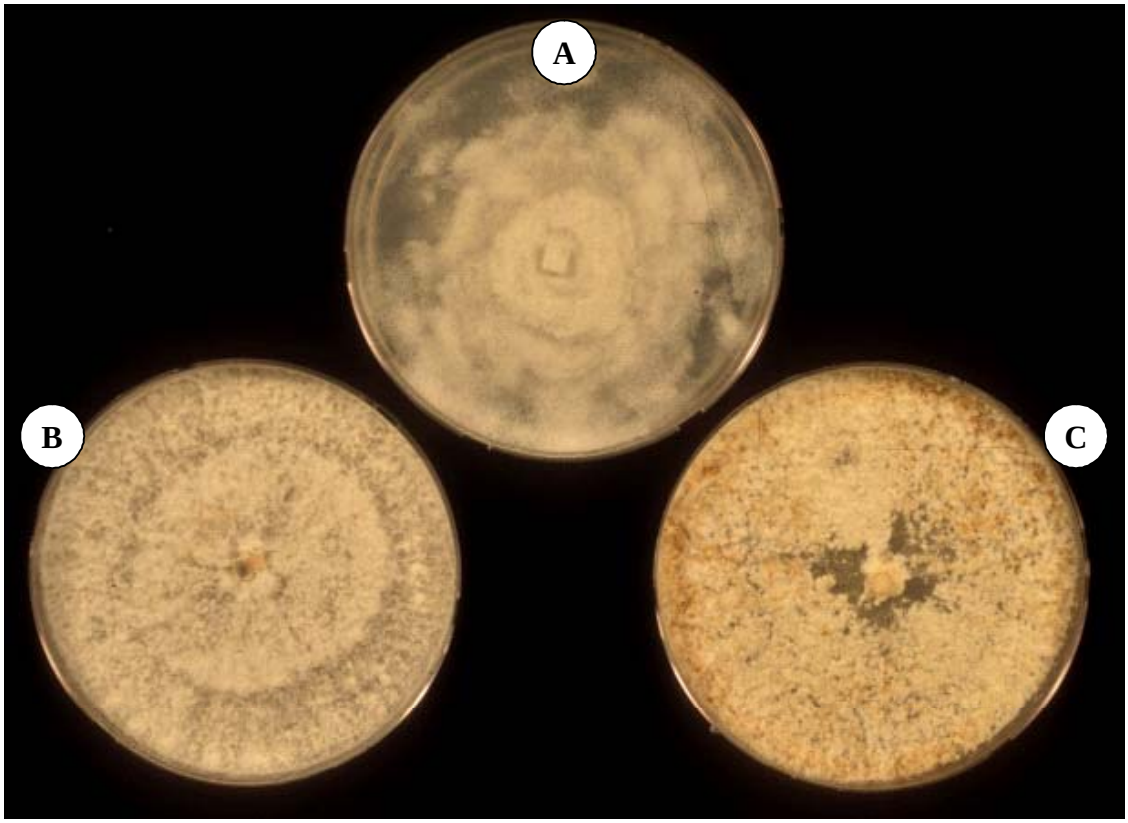

Figure 2: Phenotypic changes associated with *Diaporthe perjuncta* isolates after transfection with DaRV. The transfected isolate shows the production of aerial growth, fluffy appearance with the production of a slight yellowish pigment. A: *D. perjuncta* isolate (CMW3407) naturally virus infected. B: *D. perjuncta* isolate (CMW8597) untransfected. C: *D. perjuncta* isolate (CMW8597-DaRV) DaRV transfected.

Chapter 5:

Biological control of Cryphonectria canker of *Eucalyptus* using an isolate transfected with the *C. parasitica* hypovirus

ABSTRACT

Cryphonectria cubensis is one of the most serious *Eucalyptus* pathogens in South Africa. Previously, a virulent South African *C. cubensis* isolate was transfected with the full-length coding strand of the *C. parasitica hypovirus* (CHV1-EP713). The transfectant isolate was shown in a limited laboratory study to impart hypovirulence. Here, we report on the use of the transfectant isolate in the first field trial evaluating its potential as a biological control agent. The field trial was established using one-year-old *Eucalyptus grandis* clones ZG14 and TAG5, known to be susceptible to Cryphonectria canker. Inoculations with the transfected *C. cubensis* isolate were characterised by significantly smaller lesions than those associated with the virulent, virus-free isolate. Co-inoculation on single trees with both the virulent and virus-containing isolate resulted in the significant reduction in the size of the lesions. Treatment of already established Cryphonectria cankers with the transfectant isolate at four points around the periphery of cankers did not lead to a significant reduction in canker size, but did alter the morphology of the cankers. The virus was also shown to be transmitted via hyphal anastomosis to the virulent isolates causing the cankers.

INTRODUCTION

Cryphonectria cubensis (Bruner) Hodges is an ascomycetous fungus that causes a severe canker disease on *Eucalyptus*. Since the discovery of *C. cubensis* in South Africa (Wingfield *et al.* 1989) the need to reduce the impact of this disease has grown. Currently, the most effective means to manage the impact of Cryphonectria canker is through breeding and selection of disease-tolerant planting stock (Alfenas *et al.* 1983; Wingfield 1990; van Heerden & Wingfield 2002). Another exciting, but possibly longer term approach could be through biological control using double stranded (ds) RNA viruses linked to hypovirulence (van Heerden *et al.* 2001).

The possibility of using dsRNA viruses as biological control agents to reduce the impact of plant diseases has been considered for various pathogens. The best studied system is that of the causal agent of chestnut blight, *Cryphonectria parasitica* (Murr) Barr. This emerged after Biraghi (1950) observed healing chestnut blight cankers in Italy, and the subsequent identification of hypovirulent *C. parasitca* strains (Grente 1965). The causal agents of this hypovirulence were later shown to be double stranded RNA elements (Day *et al.* 1977). These viruses were later assigned to the genus *Hypovirus* in the virus family *Hypoviridae* (Hillman *et al.* 2000). The hypoviruses are transmitted either vertically to the conidia (asexual spores) or via cytoplasmic exchange, after hyphal anastomosis (Nuss 1996).

Various field experiments have been conducted in an effort to establish whether the impact of chestnut blight can be reduced using hypoviruses. One of the first of these trials was in France between 1966 and 1974, where Grente & Berthelay-Sauret (1978) developed an inoculation technique for blight affected chestnut trees. In this procedure, growers were supplied with a mixture of hypovirulent *C. parasitca* isolates, which they then inoculated at the edges of existing cankers (Grente & Berthelay-Sauret 1978; Heiniger & Rigling 1994). These cankers started to heal and mortality decreased. Biological control has also been successfully applied in Italy where similar application techniques have been used (Heiniger & Rigling 1994). In North America, cankers on trees have been treated successfully but spread of naturally occurring hypoviruses has not occurred (Anagnostakis 1982). In contrast, transgenic hypovirulent strains have shown effective transmission of the virus in field trials (Nuss 2000).

Recently, the full length coding strand of the *C. parasitica hypovirus* (CHV1-EP713) was transfected into a virulent South African *C. cubensis* isolate and the transfectant was shown to be stable (van Heerden *et al.* 2001). This resulted in pronounced morphological changes in the recipient fungus, such as the production of a bright yellow-orange pigment, reduced sporulation and growth reduction. Preliminary greenhouse inoculations showed that the transfected isolate was significantly less virulent than the wild type virus free isolate.

The aim of this study was to use the CHV1-EP713 transfected *C. cubensis* isolate in a first field experiment and to test whether it shows bio-control characteristics on established trees in the field. The trial was also designed to test whether the transfected isolate could slow the expansion of already established cankers.

MATERIALS AND METHODS

Isolates used

A virulent South African *C. cubensis* isolate (CMW2113) was selected for this study. This isolate has been used in a suite of previous studies on Cryphonectria canker in South Africa and is known to represent the higher order of pathogenicity in the local *C. cubensis* population (van Heerden & Wingfield 2001). In addition, another South African *C. cubensis* isolate CMW11336 was selected to consider the potential of the biocontrol isolate to reduce the expansion of cankers caused by a different isolate of *C. cubensis*. The isolate (CMW2113-T), previously transfected with the *C. parasitica hypovirus* CHV1-EP713 (van Heerden *et al.* 2001), was used in this study as the potential biological control agent. All three isolates are maintained in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa.

Evaluation of field bio-control characteristics

One-year-old stems of the *Eucalyptus grandis* clones ZG14 and TAG 5 were used for this inoculation study. Trees were inoculated by removing a cambial disc with a 20 mm diam. cork borer, and approximately 150 cm from the ground. Similar sized agar discs from the edges of actively growing cultures on 2% malt extract agar (MEA) were placed in the wounds with the mycelium side towards the wound. The wounds were sealed with masking tape to reduce desiccation. Twenty trees of each clone were inoculated with each of the isolates CMW2113 and CMW2113-T. In addition to inoculation of trees with single isolates,

the two isolates CMW2113 (virulent) and CMW2113-T (transfected) were co-inoculated into trees. Here, isolates were inoculated on the same trees, alongside each other, with inoculation points 5 mm apart from each other. Ten trees of each clone were inoculated with a sterile MEA plug that acted as a control.

Six weeks after inoculation, the masking tape was removed from the inoculation sites and lesion lengths, lesion widths and tree circumferences were measured. Lesion areas were computed from lesion lengths and widths. These values were analysed using a one way ANOVA (SYSTAT version 7.0.1) to test for significant differences in lesion size associated with the various inoculations. Tree circumference was used a co-variat in the analysis. A post-hoc pairwise Bonferroni comparison was also performed to determine isolate differences based on their lesion areas.

Inhibition of canker expansion

To determine whether the expansion of actively growing cankers can be halted, newly established cankers were treated with the virus transfected isolate. The cankers were established on trees of the *E. grandis* clone ZG 14. Thirty trees were each inoculated with the South African *C. cubensis* isolates CMW2113 and CMW11336. These trees were left for six weeks for the cankers to become well established.

In order to treat the cankers, wounds (20 mm in diam.) were made in healthy tissue at the periphery of the cankers. The wounds were made at four sites at the edges of the actively growing six-week-old canker (Fig. 1). Two of these were placed at the canker periphery in horizontal positions across the centers and at opposite sides of the canker. The other two inoculation points were also placed at the canker periphery but vertically across the centre and at opposite ends of the canker. Wounds were made in the healthy tissue at the periphery of the cankers. Similar sized discs of the actively growing transfectant isolate CMW2113-T on MEA agar were placed into the wounds (Fig. 1). The wounds were sealed with masking tape to prevent desiccation. Fifteen trees of each isolate were inoculated in this manner. The remaining 15 cankered trees were left untreated and acted as controls. After six weeks, cankers were inspected and canker lengths, widths and tree circumferences were measured.

The data were analysed using a one way ANOVA (SYSTAT version 7.0.1). The data were found to be inordinately skewed, not meeting the assumptions of parametric testing and were

thus log transformed to normalise the distribution. A post-hoc pairwise Bonferroni comparison was performed on the data.

Re-isolations were made from each of three cankers caused by the two *C. cubensis* isolates (CMW2113 and CMW11336), which were subsequently treated with the virus infected isolate. The re-isolations were made from different positions at the canker periphery (Fig. 4). Some of these positions were selected to be as far away as possible from the point at which the virus infected isolate had been inoculated. Others were specifically from points adjacent to the areas where the virus infected isolate had been placed. Four-five re-isolations were made per canker (Fig. 4) and these were to determine whether the virus could be retrieved from parts of the cankers distant from the sites where the virus transfected isolates had been placed. This would make it possible to determine whether the virus had been transferred from the virus containing isolate to the virus free virulent isolates

To confirm that the retrieved isolates from the treated cankers caused by isolate CMW11336 represented the original fungus and not overgrowth from the virus-infected isolate (CMW2113-T), vegetative compatibility tests (VCG's) were done. These were with all the retrieved isolates from the cankers caused by CMW11336 and subsequently treated with the transfectant isolate. The procedure for testing VCG's was the same as that described by van Heerden and Wingfield (2001), using a medium containing Bromcresol green.

In order to screen the isolates retrieved from the cankers for the presence of the virus they were grown in 2% Malt extract broth. Total RNA was extracted from freeze dried mycelium using the High Pure RNA Isolation Kit (Roche Diagnostics). For detection of the virus, a one step RT-PCR reaction was performed using a LightCycler (Roche Diagnostics) with the LightCycler- RNA Amplification Kit SYBR Green I (Roche Diagnostics). The primer pair RSDS10 and BR43 was selected to determine the presence of CHV1-EP713. Primer RSDS 10 (5'-GCCTATGGGTGGTCTACATAGG-3') corresponds to the 5´-terminal sequence of CHV1-EP713 coding strand and primer BR43 (5'-GGATCCACTGTAGTAGGATCAA-3') is complementary to nucleotide positions 566-545 of the CHV1-EP713 coding strand (van Heerden *et al.* 2001). A reaction mixture was prepared containing 0.75 ng total RNA, 0.5 µM primer RSDS10, 0.5 µM primer BR43, 1 x LightCycler-RT-PCR reaction mix SYBR green I, 6 mM $MgCl_2$, 0.4 µl LightCycler-RT-PCR enzyme mix and PCR grade sterile ddH_2O to a total volume of 20 µl. The cycle conditions were a reverse transcription reaction at 50ºC

for 10 min, followed by a single cycle at 95ºC for 10 sec, 35 cycles at 95ºC for 0 sec, 62ºC for 4 sec and 72ºC for 16 sec. The data were analysed based on the melting curves of the PCR products. A melting temperature of 87ºC would indicate that a virus specific product had been obtained. The PCR products were also separated on a 1.5% agarose gel stained with ethidium bromide and visualised under UV light.

RESULTS

Evaluation of bio-control characteristics

Distinct lesions were observed six weeks after inoculation with the virulent *C. cubensis* isolates. No lesion development was observed for the control inoculations. Lesion size differed significantly for the two *C. cubensis* isolates, and the control isolate used in this inoculation on both the ZG14 *E. grandis* clone ($F=22.9$; $df=3$; $p<0.001$) and the *E. grandis* clone, TAG5 ($F=113.3$; $df=3$; $p<0.001$). The post-hoc Bonferroni pairwise comparisons showed that isolate CMW2113 gave rise to cankers significantly larger to those associated with the CMW2113-T and the control inoculations on both clones (Fig. 2). For example on clone ZG14, CMW2113 gave rise to lesions with a mean length of 123±11 mm and for clone TAG 5 these were a mean of 135±4 mm (Table 1). Lesions caused by the hypovirus-transfected isolate CMW2113-T did not differ significantly from inoculations using a sterile MEA plug on either of the tree clones (Fig. 2; Table 1).

Where the transfected isolate CMW2113-T was inoculated alongside the virulent isolate (CMW2113), lesion size associated with the virulent isolate was significantly reduced. This combined inoculation resulted in significant smaller lesions than those associated with CMW2113 on both clones (Fig. 2). The resulting mean lesion lengths for clone ZG14 and clone TAG5 were 69±8 mm and 72±2.5 mm respectively (Table 1).

Inhibition of canker development

There were no significant difference in the size of cankers for the hypovirus treated and the virulent CMW2113 cankers ($F=0.005$; $df=1$; $p=0.903$) (Fig. 3). The mean lesion length for the cankers caused by the untreated CMW2113 canker was 222±15 mm and the mean lesion width was 107±8 mm. Cankers caused by this fungus, which were subsequently treated with the transfected isolate, had a mean lesion length of 238±19 mm and a mean lesion width of 104±10 mm. There were also no significant difference in the size of cankers caused by the

hypovirus-treated and the untreated CMW11336 cankers (F=0.008; df=1; p=0.859) (Fig. 3). The mean length for the untreated canker caused by CMW11336 was 185 ±11 mm and the mean width was 59± 8mm. The hypovirus treated canker had a mean lesion length of 133±19 mm and a mean lesion width of 72±4 mm.

The canker morphology of the treated cankers were irregular in shape. *Cryphonectria cubensis* was reisolated from selected sections of the cankers that had been treated with the virus containing isolate, CMW2113-T. The RT-PCR with the total RNA extracted to determine the presence of the virus resulted in a 600 bp amplicon (Fig. 4 & 5). These results show that the virus was transferred from the hypovirulent isolate CMW2113-T to both isolates CMW2113 and CMW11336. The VCG tests showed that the fungi reisolated from the different parts of the cankers caused by isolate CMW11336, belong to the same VCG as CMW 11336 and not the transfected isolate (CMW2113-T).

DISCUSSION

In this study we have provided the first illustration of the impact of the *C. parasitica hypovirus* (CHV1-EP713) on Cryphonectria cankers caused by *C. cubensis* under field conditions. Inoculation of *Eucalyptus* trees with the hypovirus-transfected *C. cubensis* isolate (CMW2113-T) resulted in lesions significantly smaller than those caused by the virulent isolate CMW2113. These results are similar to those of van Heerden *et al.* (2001) in which the transfected isolate (CMW2113-T) reduced virulence under greenhouse conditions. The combination inoculation, in which the virulent and virus transfected isolates were co-inoculated on the same tree at the same time, has shown that the virus infected isolate CMW2113-T reduces the development of the virulent isolate. These results, therefore, show that CMW2113-T is able to reduce canker expansion if it develops together with the virulent isolate.

Where the transfectant isolate was inoculated at the periphery of expanding cankers, canker development was not significantly reduced. A similar method was applied in the biological control program instituted by the French Ministry of Agriculture in an effort to reduce the impact of chestnut blight. In that protocol, blighted chestnut trees were treated annually with mixtures of hypovirulent compatible *C. parasitica* strains by placing them in holes around cankers caused by *C. parasitica* (Grente & Berthelay-Sauret 1979; van Alfen 1982;

MacDonald & Fulbright 1991). This method was shown to be effective. However, *C. cubensis* is a very aggressive pathogen on *Eucalyptus* and will generally kill susceptible trees such as those inoculated in this study in less than six months after inoculation (van Heerden, unpublished). It was, therefore, necessary to assess results in the early stages of development. The method used in this study to reduce Cryphonectria cankers was not effective and other methods using the transfectant isolate as a potential bio-control agent for the reduction of the cankers will be required.

Our study has further shown that the virus CHV1-EP713 is transmitted via hyphal anastomosis to the virulent virus-free isolates. This was observed for both the isolates inoculated in this study and confirmed using VCG tests to identify the isolate genotypes, and RT PCR to determine the presence of the virus, thus indicating the effective movement and establishment of the virus in two different isolates. The virus was also observed in all the sections of the cankers that were the furthest away from the point of inoculation of the virus-infected isolate. Van Heerden *et al.* (2001) have shown in a laboratory experiment that CHV1-EP713 is transmitted via hyphal anastomosis to many individuals of a population of isolates of the fungus from South Africa. Since it is known that the efficacy of any biological control strategy relies on the effective dissemination of the virus, our observation was encouraging.

Inoculation of the CHV1-EP713 transfected isolate in all the experiments resulted in the formation of very small lesions. This suggests that the transfectant strain is ecologically unfit and would probably not survive under natural conditions. A similar observation was made for other CHV1-EP713 transgenic isolates released in a field experiment (Anagnostakis *et al.* 1998). Although that virus was shown to be transmitted to the ascospores of *C. parasitica*, it failed to persist at the release site for more than two years (Anagnostakis *et al.* 1998). MacDonald and Fulbright (1991) have also noted that hypovirulent strains used in North America were highly curative, but they had a poor capacity to colonise and produce spores. This resulted in limited persistence and poor prospects for biological control under natural conditions. One means of overcoming this impediment will be to use an alternative virus for biological control. CHV1-Euro 7 has suitable characteristics such as enhanced colonisation and spore production (Chen & Nuss 1999; Dawe & Nuss 2001). Using a strain such as this could balance the ecological fitness of the biological control agent.

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Table 1: Mean lesion size for the virulent and virus transfected South African *Cryphonectria cubensis* isolates inoculated on *Eucalyptus grandis* clones ZG14 and TAG5.

Isolate [a]	Clone	Mean lesion length (L) (mm) ±SEM	Mean lesion width (W) (mm) ±SEM	LxW [b]
CONTROL	ZG14	20 ± 0.0	20 ± 0.0	400±0.0 a
CMW2113-T	ZG14	20 ± 0.0	20 ± 0.0	400±0.0 ab
COMB	ZG14	69±8	35±2	2635±422 ac
CMW2113	ZG14	123±11	47±4	6186±793 d
CONTROL	TAG5	20 ± 0.0	20 ± 0.0	400±0.0 a
CMW2113-T	TAG5	20 ± 0.0	20 ± 0.0	400±0.0 ab
COMB	TAG5	72±2.5	32±0.8	2312±111 c
CMW2113	TAG5	135±4	40±2	5351±340 d

[a] Isolates: CMW2113-T is the hypovirus (CHV1-EP713) transfected South African *C. cubensis* isolate. CMW2113 virulent South African *C. cubensis* isolate. COMB is the combination inoculation where CMW2113 and CMW2113-T were inoculated into trees together, alongside each other with inoculation points 5 mm apart.

[b] Isolates with the same letter do not differ significantly from each other.

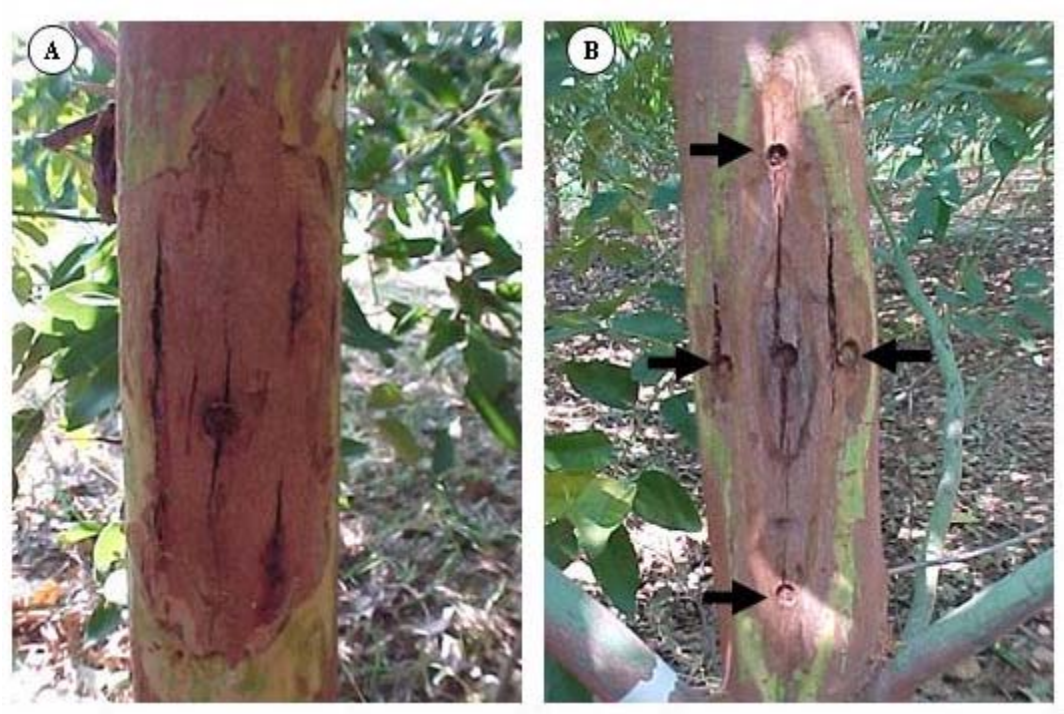

Figure 1: Lesions on the *Eucalyptus grandis* clone (ZG14) 12 weeks after inoculation with *C. cubensis* isolate CMW2113. A: Untreated canker. B: Canker that was inoculated on four sides with virus containing isolate, CMW2113-T, with the inoculation sites indicated by the arrows.

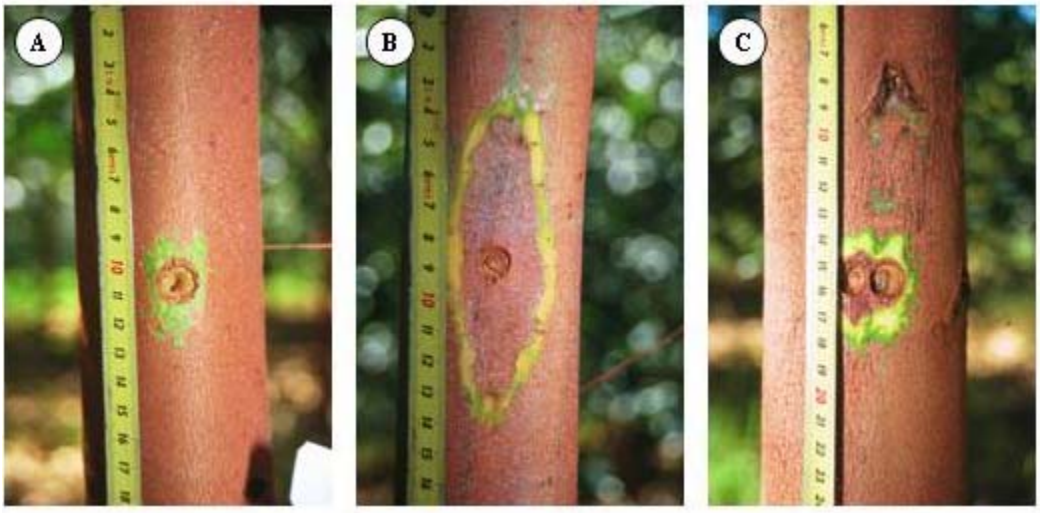

Figure 2: Lesions on the *Eucalyptus grandis* clone ZG14, after artificial inoculations with virulent and a virus transfected *Cryphonectria cubensis* isolates. A: Lesion caused by the hypovirus infected isolate CMW2113-T. B: Lesion caused by the wild type virulent virus free isolate CMW2113. C: Lesion caused by the combination inoculation with both CMW2113-T on the left and CMW2113 on the right.

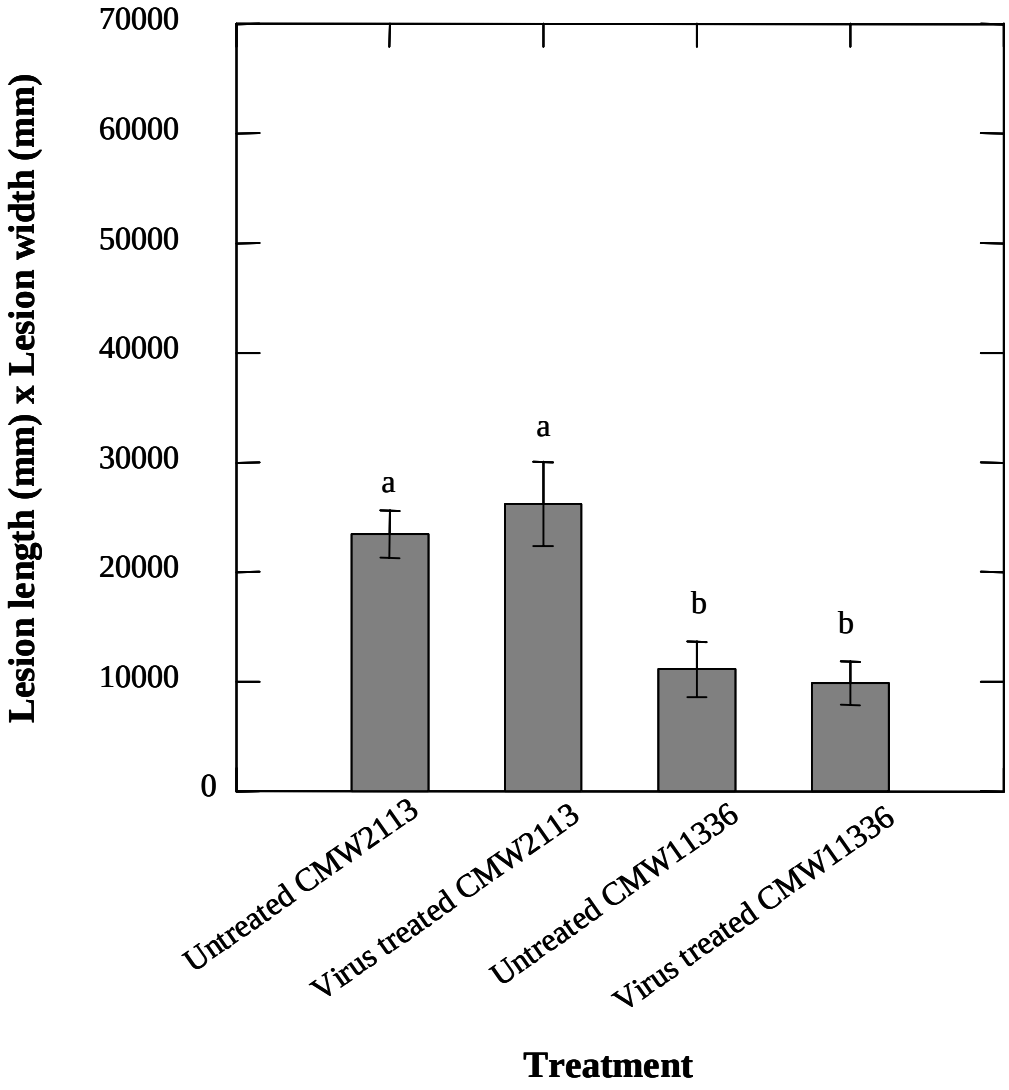

Figure 3: Mean lesion length (mm) x mean lesion width (mm) (± SEM) of virus treated and the untreated *Cryphonectria cubensis* cankers in an investigation to determine whether the hypovirus (CHV1-EP713) transfected *C. cubensis* isolate (CMW2113-T) can reduce actively growing cankers from expanding. The cankers were caused by two virulent *C. cubensis* isolates CMW2113 and CMW11336. Columns with the same letter do not differ significantly from each other ($P<0.001$) indicating that the virus infected isolate did not cause a reduction in canker expansion.

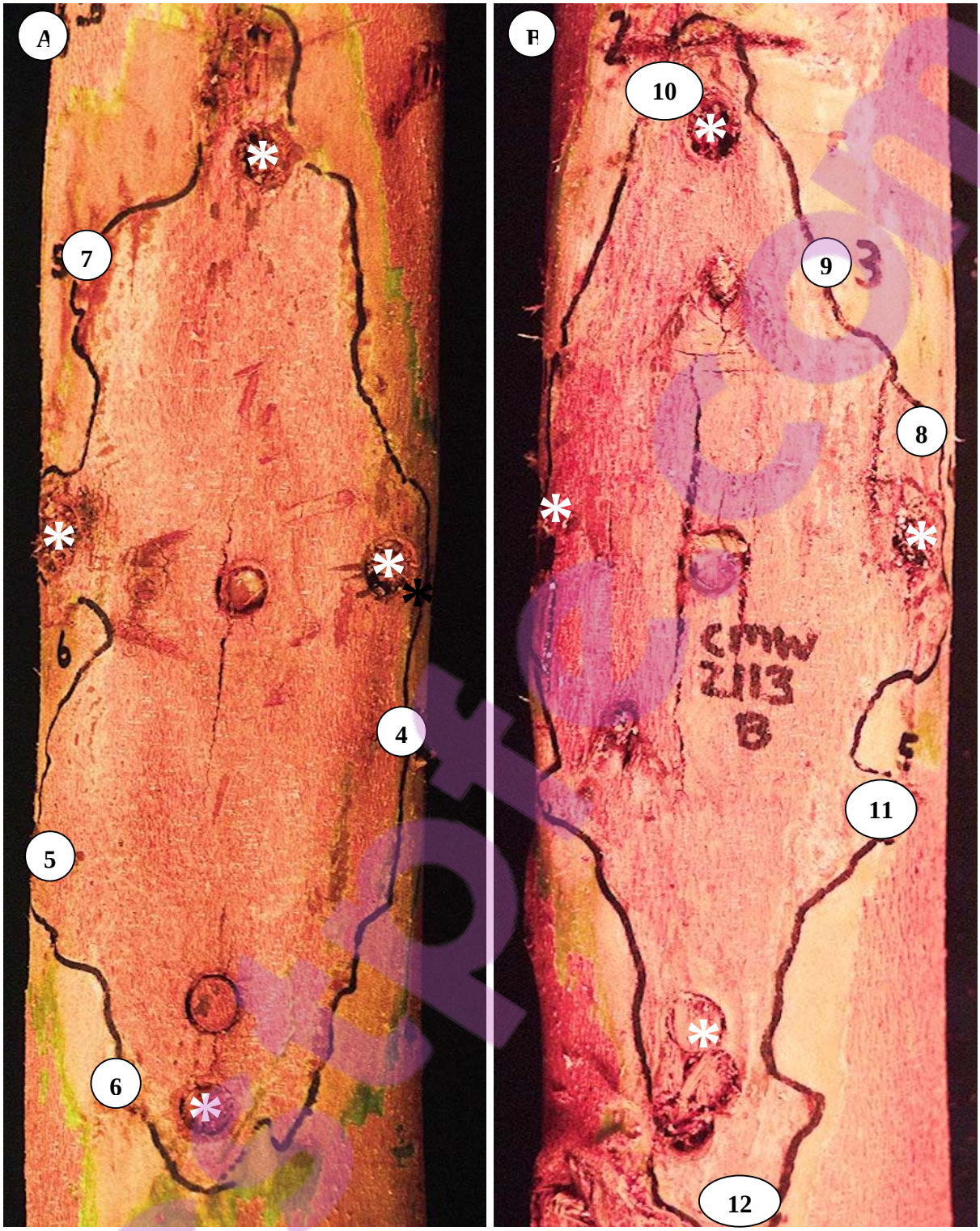

Figure 4: Cankers caused by two virulent South African *Cryphonectria cubensis* isolates. A. Canker caused by isolate CMW11336. B: Canker caused by isolate CMW2113. Both these cankers were treated with the hypovirus (CHV1-EP713) transfected isolate on four corners on the canker periphery. These positions are indicate by a ✻ . Reisolations of the fungus with the purpose to determine the presence of the virus at different positions on the canker were also done and are indicated by the numbers 4-12.

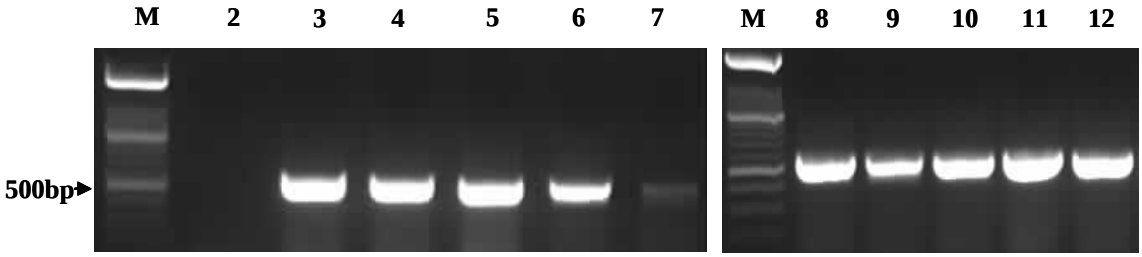

Figure 5: Confirmation of the presence of the hypovirus (CHV1-EP713) using reverse transcription PCR. The PCR products were separated on a 1.5% agarose gel stained with ethidium bromide and visualised under UV light. Lanes M: 100 base pair (bp) molecular weight marker. Lanes 2: Virus free isolate which was used as a negative control. Lane 3: Hypovirus transfected isolate CMW2113-T as positive control. Lanes 4-6 and 8-12: RT-PCR amplicons derived from fungi re-isolated from the canker and corresponds to the positions on the cankers indicated in Figure 4. Presence of the virus is indicated by a 600 bp amplicon.

SUMMARY

Cryphonectria cubensis is an ascomycetous fungus that causes a serious canker disease on *Eucalyptus* trees in many parts of the world. The importance of the disease has led to numerous studies involving the taxonomy, genetic diversity and the control of Cryphonectria canker. However, there remain many questions pertaining to the disease that have not been considered. The objectives of the studies presented in this thesis were, therefore, to investigate the possibility of biological control of Cryphonectria canker, to evaluate the currently used disease screening strategy in South Africa and to establish a transfection system with dsRNA elements in *Diaporthe*, which is closely related to *Cryphonectria*.

The introductory chapter of this thesis provides a review of the literature pertaining to *Cryphonectria cubensis*. In addition literature on hypovirulence in fungi is also extensively reviewed, with a special emphasis on the genus *Cryphonectria*.

The aim of study in the second chapter of the thesis was to screen the South African *C. cubensis* population for the presence of dsRNA viruses. Two viruses were identified and the full sequence of these elements showed a strong homology to the mitochondrial viruses (mitoviruses) within the family *Narnaviridae*. We, therefore, named the viruses *Cryphonectria cubensis* mitovirus 1 (CcMV1) and *Cryphonectria cubensis* mitovirus 2 (CcMV2). The two viral genomes are 2601 nucleotides and 2639 nucleotides in size respectively and encode for a protein that probably functions as an RNA-dependant RNA polymerase (RdRp). Pathogenicity studies indicated that the viruses do not result in a significant reduction in pathogenicity of *C. cubensis.*

In the third chapter, results of a study to consider whether different *Eucalyptus* clones responded similarly to various South African *C. cubensis* isolates, are presented. The aim was, therefore, to evaluate the current *C. cubensis* resistant screening method used on *Eucalyptus* spp. in South Africa. The statistical analysis of the inoculation data showed a significant isolate x clone interaction. This data also suggest the possibility of vertical resistance, which is different to previous assumptions.

Transfection studies (Chapter 4) involving a positive stranded RNA virus, *Diaporthe RNA virus* (DaRV) from a South African *D. perjuncta* isolate are presented here. In this study, a

virus free *D. perjuncta* isolate, a virulent *C. cubensis* isolate and a hypovirulent *C. cubensis* isolate containing the hypovirus CHV1-EP713 were chosen to be transfected with DaRV. By using electroporation, it was possible to infect a virus free *D. perjuncta* isolate with the *Diaporthe RNA virus*, thus extending the transfection range of this virus. The resulting transfection led to altered colony morphology but did not lead to a reduction in pathogenicity. We were also not successful in attempts to transfect isolates of *C. cubensis* with DaRV, indicating that the virus does not replicate in this host.

In a previous study a virulent South African *C. cubensis* isolate was transfected with the *Cryphonectria parasitica hypovirus* CHV1-EP713. This resulted in the fungus becoming hypovirulent. Chapter five of this thesis presents the results of a study to evaluate the potential use of this virus in the biological control of Cryphonectria canker in South Africa. A field trial was established and existing cankers were treated with the transfected isolate. The treatment of the cankers did not lead to a significant reduction in canker size, but did alter the morphology of the cankers. The virus was also shown to be transmitted via hyphal anastomosis to the virulent canker causing isolates. In addition the co-inoculation on single trees with both the virulent and virus-containing isolate, resulted in a significant reduction in the size of the lesions. This study also showed that the transfected *C. cubensis* isolate are characterised by significantly smaller lesions than those associated with the virulent, virus-free isolate.

Cryphonectria cubensis and the associated canker disease of *Eucalyptus* threaten the forestry industry in South Africa. The overall aims of the studies presented in this thesis were to gain a more complete understanding of this fungus and to evaluate potential control strategies. Each of these chapters should contribute towards a better understanding of the viruses associated with *C. cubensis* and other important aspects of Cryphonectria canker, which will hopefully lead to enhanced control strategies of the disease in South Africa.