Incidence and epidemiology of apple core rot in the Western Cape of South Africa

by

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DECLARATION

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and has not previously in its entirety or in part been submitted at any university for a degree.

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SUMMARY

This study looked at the incidence, etiology and epidemiology of core rot of apples in orchards situated in the Western Cape, South Africa. Core rot is a post-harvest disease, with three symptoms, namely mouldy core (MC), dry core rot (DCR) and wet core rot (WCR). These symptoms are caused by various pathogenic fungi, including *Alternaria* and *Penicillium.* Although MC is not economically important, DCR and WCR are, as they affect the flesh of the fruit. Core rot occurs worldwide in susceptible apple cultivars such as 'Starking' and 'Red Delicious'. These cultivars have a wider, open calyx tube which results in an open core area. In South Africa, core rot of apples are important post-harvest diseases and losses of between 5 and 12% occur in apple cultivars.

An in depth literature search was done on core rot including literature on each core rot symptom, the genuses *Alternaria* and *Penicillium,* molecular identification and techniques, disease incidence and its economic importance, various inoculum sources, pathogenicity of core rot organisms and integrated management of core rot. This study included two research chapters, with seven objectives, namely, to 1, determine the incidence of core rot in apples from commercial orchards both pre- and post-harvest; 2, to identify the causal organisms associated with core rot symptoms; 3, to identify potential sources of inoculum of core rot pathogens and determine whether there is synergism between *Alternaria* and *Tarsonemus* mites associated with core rot; and 4, to determine whether the fungicide Bellis®, used a full bloom application, can be used to manage core rot in South Africa; 5, to identify the species of *Alternaria* and *Penicillium* sampled from core rot symptomatic fruit and inoculum sources (air, apple mummies and mites), using morphological and molecular methods; 6, to compare *Penicillium* species isolated from pre- and post-harvest WCR symptomatic fruit, using molecular species identification methods and 7, to compare and to select the most reliable pathogenicity test for use in future research.

The total decay incidence for Ceres is considerably higher than the previous losses indicated in literature. Pre-harvest core rot, which was confirmed by previous studies, had a higher incidence of each core rot symptom than previously indicated. The two most frequently isolated causal organisms were *Alternaria* and *Penicillium.* Other organisms isolated and then identified from the symptoms were *Fusarium, Cladosporium, Epicoccum, Ulocladium,*

Stemphylium, Phoma, Botryosphaeria, Botrytis, Trichoderma, Verticillium, Paecilomyces and *Gliocladium.*

Three inoculum sources, air, mummies and mites, were regarded as potential sources of infection for core rot. During this study the sources of infection were verified and core rot causing organisms were isolated from these sources. *Alternaria* was isolated from air inoculum samples, but was not found on the other two sources. This dismissed the hypothesis that there was a possible synergism between *Alternaria* and *Tarsonemus* mites. *Penicillium* species were isolated from all three sources, more frequently from the mummies and mites. Bellis® was applied three times during the bloom period. The subsequent results showed a significant difference between the control and Bellis® treated treatments with the treated fruit having a significant higher incidence than the controlled fruit. No control was observed with this result and managing core rot with only Bellis® is not advisable.

Alternaria species were identified using the following genetic loci, ITS, OPA1-3, 2-1 and 10-2 as well as endoPG. Isolates from pre- and post-harvest symptoms and air inoculum were identified using each of the genetic loci. *Alternaria arborescens* was one of the species that was identified. The other isolates obtained were *A. alternata, A. tenuissima, A. gaisen, A. dumosa, A. turkisafria* and *A. perangusta*. Separating combined species was not possible. Another molecular technique, ISSR, was used to identify *Alternaria* species. This technique, after multiple re-runs, did not give consistent results and species could not be identified.

Penicillium species were identified using the genetic loci ITS for isolates collected from pre- and post-harvest symptoms and inoculum sources. Thirteen clades were identified, including the species *P. ramulosum, P.* sp. (aff. *cecidicola*), *P.* sp (aff. *dendriticum*), *P. expansum, P. paneum, P. solitum, P. crustosum , P. brevicompactum, P. novae-zeelandiae, P. glabrum* and *P. rugulosum*. *Penicillium expansum* and *P. ramulosum* had the highest distribution between the isolates. Pre- and post-harvest WCR isolates were identified using the partial beta-tubulin PCR-RFLP method, and comparing different banding-patterns. The species identified using this method were *P. expansum, P. ramulosum, P.* sp. (aff. *cecidicola*), *P.* sp (aff. *dendriticum*), *P. rugulosum, P. chermesinum* and *P. glabrum. Penicillium ramulosum* and *P. expansum* had the highest incidence with *P. ramulosum* occurring more frequently pre-harvest than post-harvest and *P. expansum* occurring more frequently post-harvest.

Five methods, previously published, were compared to select the most reliable pathogenicity test. The methods included surface wounding of an apple with colonised toothpicks, surface wound inoculated with a pipette, inoculation of an open mesoderm core cavity, deep and non-

wounding of apple fruit with colonised toothpicks. The surface wounding with a colonised toothpick gave the most reliable results and can be used in industry as a pathogenicity test for *Alternaria* in apples.

This study contributed to our understanding on the incidence and etiology of core rot in the Western Cape as well as in identifying inoculum sources from where infection can take place in the orchard. The results for the fungicide trial were not as anticipated and more research is required on selecting fungicides for the control of core rot in South African orchards. Although molecular techniques reduce the time in identifying fungal species, it is costly and mistakes can occur due to contamination. Identification of species can be incorrect when using a Genbank as the sequence information may be incorrect. Molecular techniques, though a good tool in identifying species, should be combined with morphological characteristics to ensure more accurate results.

OPSOMMING

Hierdie studie het gekyk na die insidensie, etiologie en epidemiologie van kernvrot in appels vanuit boorde in die Wes-Kaap, Suid-Afrika. Kernvrot is ‘n na-oes iekte, met drie simptome, naamlik beskimmelde kern, droë kernvrot en nat kernvrot. Hierdie simptome word veroorsaak deur verskillende patogeniese swamme, insluitend *Alternaria* en *Penicillium*. Alhoewel beskimmelde kern nie ekonomies belangrik is nie, is droë en nat kernvrot wel belangrik, omdat hulle die vrug se vlees affekteer. Kernvrot kom wêreldwyd voor in vatbare kultivars soos ‘Starking’ en ‘Red Delicious’. Hierdie kultivars het ‘n wye, oop kelkbuis wat ‘n oop kern area veroorsaak. In Suid-Afrika is kernvrot van appels ‘n belangrike na-oes siekte en verliese tussen 5 en 12% kom voor in appel kultivars.

‘n In diepte literatuurstudie is gedoen omtrent kernvrot, insluitend literatuur omtrent elke kernvrot simptoom, die genera *Alternaria* en *Penicillium*, molekulêre identifikasie en tegnieke, siekte insidensie en sy ekonomiese impakte, verskillende inokulum bronne, patogenisiteit van kernvrot organismes en die geïntegreerde bestuur van kernvrot. Hierdie studie sluit in twee navorsings hoofstukke met sewe doelwitte, naamlik om 1, te bepaal wat die insidensie van kernvrot in appels is vanuit kommersiële boorde vir beide voor en na-oes; 2, om veroorsakende organismses wat met kernvrot simptome geassosieër is te identifiseer; 3, om potensiële inokulum bronne van kernvrot patogene te identifiseer en te bepaal of daar ‘n sinergisme tussen *Alternaria* en *Tarsonemus* myte, wat geassosieër is met kernvrot, is; 4, om te bepaal of die fungisied Bellis®, gebruik as ‘n volblom toediening, gebruik kan word om kernvrot in Suid-Afrika te beheer; 5, om die *Alternaria* en *Penicillium* spesies wat uit simptomatiese kernvrot vrugte en inokulum bronne geïsoleer is te identifiseer; 6, om *Penicillium* spesies, wat uit voor en na-oes nat kernvrot simptome geïsoleer is, te vergelyk deur gebruik te maak van molekulêre spesies identifiserings metodes en 7, om die betroubaarste patogenisiteits toets te vergelyk en selekteer vir toekomstige gebruik.

Die totale bederfde insidensie vir Ceres is heelwat hoër as die vorige verliese wat aangedui is in literatuur. Vooroes kernvrot, wat deur vorige studies bevestig is, het ‘n hoër insidensie vir elke kernvrot simptoom gehad as wat voorheen aangedui is. Die twee geïsoleerde veroorsakende organismes wat die meeste voorgekom het was *Alternaria* en *Penicillium*. Ander organismes wat geïsoleer en geïdentifiseer is vanuit die simptome was *Fusarium, Cladosporium, Epicoccum, Ulocladium, Stemphylium, Phoma, Botryosphaeria, Botrytis, Trichoderma, Verticillium, Paecilomyces* en *Gliocladium.*

Drie inokulum bronne, lug, gemummifiseerde vrugte en myte, is geag as potensiële bronne van infeksie vir kernvrot. Gedurende hierdie studie is hierdie bronne bevestig en kernvrot veroorsakende organismes is uit die bronne geïsoleer. *Alternaria* is geïsoleer vanuit die lug inokulum monsters, maar is nie geïsoleer vanuit die ander twee bronne nie. Dus die hipotese dat daar ‘n sinergisme tussen *Alternaria* en *Tarsonemus* myte is, is verwerp. *Penicillium* spesies is geïsoleer vanuit al drie bronne, maar meer gereeld vanuit die gemummifiseerde vrugte en die myte. Bellis® is drie keer gedurende die bot toegedien. Die daaropvolgende resultate het ‘n betekenisvolle verskil tussen die kontrole en Bellis® beheerde behandelings getoon, met die behandelde vrugte wat ‘n betekenisvolle hoër insidensie gehad het as die kontrole vrugte. Geen beheer is waargeneem nie en beheer van kernvrot met net Bellis® word nie aanbeveel nie.

Alternaria spesies is geïdentifiseer deur die volgende genetise lokusse, ITS, OPA1-3, 2-1 en 10-1, asook endoPG. Isolate van voor en na-oes simptome en lug inokulum is geïdentifiseer deur elk van die genetiese lokusse. *Alternaria arborescens* is een van die spesies wat geïdentifiseer is. Ander isolate wat verkry is, was *A. alternata, A. tenuissima, A. gaisen, A. dumosa*, *A. turkisafria* and *A. perangusta*. Om gekombineerde spesies te skei was nie moontlik nie. ‘n Ander molekulêre tegniek, ISSR, was gebruik om *Alternaria* spesies te identifiseer. Hierdie tegniek, na menigte probeerslae, het nie konsekwente resultate gegee nie en spesies kon nie hiermee geïdentifiseer word nie.

Penicillium spesies, versamel vanuit voor en na-oes simptome en inokulum bronne, is geïdentifiseer deur die genetiese lokus ITS. Dertien ‘clades’ is geïdentifiseer, insluitend die spesies *P. ramulosum, P.* sp. (aff. *cecidicola*), *P.* sp (aff. *dendriticum*), *P. expansum, P. paneum, P. solitum, P. crustosum , P. brevicompactum, P. novae-zeelandiae, P. glabrum* en *P. rugulosum*. *Penicillium expansum* en *P. ramulosum* het die hoogste distribusie tussen die isolate. Voor en na-oes nat kernvrot isolate is geïdentifiseer deur die deels beta-tubulin PCR-RFLP metode, en verskillende band patrone te vergelyk. Die spesies geïdentifiseer deur hierdie metode is *P. expansum, P. ramulosum, P.* sp. (aff. *cecidicola*), *P.* sp (aff. *dendriticum*), *P. rugulosum, P. chermesinum* en *P. glabrum. Penicillium ramulosum* en *P. expansum* het die hoogste insidensie gehad met *P. ramulosum* wat meer dikwels vooroes voorkom en *P. expansum* wat meer dikwels na-oes voorkom.

Vyf metodes, wat voorheen gepubliseer is, is vergelyk om die betroubaarste patogenisiteits toets te selekteer. Die metodes sluit in die oppervlak wond van ‘n appel met ‘n gekoloniseerde tandestokkie, oppervlak wond geïnokuleer met ‘n pipette, inokulasie van ‘n

oop mesoderm kern area, diep besering en nie-besering van die appel met gekoloniseerde tandestokkies. Die oppervlak besering met ‘n gekoloniseerde tandestokkie het die betroubaarste resultate gegee en kan in die industrie gebruik word as ‘n patogenisiteits toets vir *Alternaria* in appels.

Hierdie studie het bygedra tot ons kennis van die insidensie en etiologie van kernvrot in die Wes-Kaap sowel as die identifisering van die inokulum bronne, van waar die infeksie in die boord kan plaasvind. Die resultate vir die fungisied proef was nie wat ons verwag het nie en meer navorsing word benodig om fungisiede te selekteer vir die beheer van kernvrot in Suid-Afrikaanse boorde. Alhoewel molekulêre tegnieke die tyd verminder om ‘n swam spesie te identifiseer, is dit wel duur en foute kan voorkom as gevolg van kontaminasie. Identifikasie van spesies kan verkeerd wees indien Genbank gebruik is, omdat die informasie daar nie altyd korrek is nie. Molekulêre tegnieke, alhoewel ‘n goeie manier om spesies te identifiseer, moet gekombineer word met morfologiese karakter eienskappe om akurate resultate te verseker.

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PREFACE

Apple production is an important component of the agricultural industry in South Africa. Post-harvest diseases of apples such as blue mould, bulls eye rot, grey mould and core rot reduces the quality of the fruit and cause economic losses to the industry.

Chapter 1 provides the reader with a broad overview of the disease, core rot, looking at each symptom and the causal organisms responsible for these symptoms. This chapter also gives reference to the etiology of core rot, the sources of inoculum, the economical impact that the disease has on the industry, as well as methods used for controlling the disease through fungicide applications. The identification of fungal organisms to species level using morphological characteristics or molecular methods is also addressed in this chapter.

In **chapter 2**, the incidence, epidemiology and control was evaluated to determine the effect of core rot on the South African industry. Pre- and post-harvest collections determined the incidence of core rot in the Western Cape. Various inoculum sources were evaluated as possible sources for overwintering of organisms known to cause core rot. A new fungicide was evaluated as a possible control measure to use against core rot.

Identifying fungi to species level without a vast knowledge of those specific fungi is difficult. Molecular techniques have simplified the identification process for many fungi, but some answers have not yet been found. In **chapter 3**, *Alternaria* and *Penicillium* isolates collected during chapter 2's trials were identified using various molecular techniques. Pathogenicity tests determined whether the isolates were pathogenic or not.

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Chapter 1: Etiology, epidemiology and control of apple core rot

1.1. Introduction

Apples are an important component of agricultural production in South Africa, with the area dedicated to apple production being second only to dry and table grapes (Anonymous, 2011a). Twenty-one thousand hectares of apples are planted in South Africa, with a total production value of approximately R 3 billion (Anonymous, 2011a). Core rot is one of a number of diseases which impact on the profitability of apple production in South Africa. As symptoms of core rot are largely restricted to the core of the fruit, these symptoms are only revealed once the consumer has bought the fruit, resulting in reduced consumer confidence in the product and fewer repeat purchases of the apple type, leading to economic losses.

There are three symptoms of core rot, namely mouldy core (MC), dry core rot (DCR) and wet core rot (WCR) (Spotts, 1990). These symptoms are caused by various pathogenic fungi and are initially facultative parasites, which grow saprophytically in the seed cavity of the apple and later, under favourable conditions, extend into the mesoderm tissue and cause decay (Combrink and Ginsburg, 1973, Combrink *et al*., 1985, Combrink *et al*., 1987). Published research on core rot can be confusing as the terms mouldy core (MC) and dry core rot (DCR) are often used interchangeably. In this thesis, the term mouldy core is used when fungal hyphae are restricted to the seed cavity and dry core rot is used when decay of the tissue has taken place (Serdani, 1999). Wet core rot (WCR) is used when fungal hyphae are seen in the seed cavity and the surrounding tissue has a soft, wet rot which spreads quickly through the tissue (Combrink and Ginsburg, 1973).

Mouldy core and core rot have been recorded world-wide in countries including Australia, New Zealand, Canada, the United States, the United Kingdom, South Africa and the Netherlands (Spotts, 1990). Many apple cultivars, such as ‘Starking’, ‘Delicious’, ‘Golden Delicious’, ‘Idared’, ‘Fuji’ and ‘Gravenstein’, can be affected with MC and core rot diseases (Spotts, 1990).

In South Africa, core rot of apples are important post-harvest diseases and losses of between 5 to 12% occur in all apple cultivars grown (Combrink and Ginsburg, 1973; De Kock *et al*., 1991; Serdani *et al*., 1998). The only other country besides South Africa that has statistical information with regard to incidence of this disease is Israel, with MC incidence of 7 to 12% for cultivars with open sinuses (Reuveni and Prusky, 2007). Most countries have a

minimum barrier of at least 6% core rot that may be found when processing the food locally and for export (Ellis and Barrat, 1983), while Israel has a 9% average of fruit that may be infected (Reuveni, 2006). Mouldy core is not seen as an economically important disease as it does not impair the flavour or the quality of the fruit itself, only colonizing the core region of the fruit (Carpenter, 1942; Serdani *et al*., 2002). Dry core rot and WCR are economically important as they can affect the tissue of the fruit and reduce the fruit quality.

Cultivars that are susceptible to core rot have a wider, open calyx tube which results in an open core area. Core rot pathogens, *Alternaria* spp., *Penicillium* spp., *Fusarium* spp., *Cladosporium* spp., *Coniothyrium* spp. etc., enter through the calyx tube and penetrate the core area and seed locules (Combrink, 1983). This makes it difficult to control the core rot diseases with fungicides as they cannot reach the core region of the apple. Research on fungicidal control of core rot symptoms has yet to reach a consensus on the timing of sprays and what fungicides to spray (Reuveni and Sheglov, 2002; Reuveni *et al*., 2003; Reuveni, 2006; Reuveni and Prusky, 2007). In South Africa, fungicide application for core rot control was seen as not cost effective as the incidence was too low (Fugler, 1990).

Recently, research highlighted the association between core rot and *Tarsonemus* Canestrini and Fanzago mites (McLeod *et al*., 2008; Van der Walt, 2009). It was suggested that the mites carry the inoculum and deposit it in the core of the apple. The *Tarsonemus* mites make wounds in the fruit cavity wherein the fungus can grow.

Alternaria Nees and *Penicillium* Link species are the two most frequently isolated fungi from core rot symptoms (Combrink and Ginsburg, 1973; Combrink *et al*., 1985, Combrink *et al*., 1987). It is not always possible to identify the species of these organisms morphologically and other identification methods, such as molecular techniques, are needed to identify the causal organisms (Pryor and Michailides, 2002; Prusky *et al*., 2004).

All aspects of core rot development in apples, in particular the different types of core rot symptoms as well as the role of *Tarsonemus* mites in the etiology of core rot was reviewed in this chapter.

1.2. Core rot symptoms

Core rot can be separated into three symptoms, namely mouldy core, dry core rot and wet core rot. Associated with each of these symptoms are specific causal organisms, and each with their own epidemiology and control strategies.

1.2.1. Mouldy core

1.2.1.1. Causative agents

A variety of fungi have been associated with mouldy core of apples, including *Alternaria*, *Botrytis* P. Micheli ex Pers., *Candida* Berkhout, *Cladosporium* Link, *Coniothyrium* Corda, *Fusarium* Link, *Aspergillus* P. Micheli ex Link, *Gloeosporium* Desm. and Mont., *Epicoccum* Link, *Penicillium, Pestalotia* De Not., *Phoma* Sacc., *Sporathrix* Hekt and C.F. Perkins, *Trichoderma* Pers. and *Rhizopus* Ehrenb. (Ellis and Barrat, 1983). Combrink *et al*. (1985) cited *Alternaria alternata* (Fries) Keissler as being the main causal organism of mouldy core in 'Starking' apples in South Africa.

1.2.1.2. Symptoms

Mouldy core, caused by *A. alternata,* is characterized by the growth of mycelium in the core region, as well as in the seed locules of the apple (Reuveni *et al*., 2003). The fungus, which covers the seeds and carpel walls, can differ in colour with white to black mycelium (Ellis and Barrat, 1983). Mouldy core is seen as a precursor of dry core rot (DCR) (Serdani *et al*., 2002).

Mouldy core caused by *Coniothyrium* Corda species has a white to light grey mycelia, which covers the seed locules (Michailides *et al*., 1994). Symptoms are not externally visible and are only seen when the apple has been cut open and the grey mycelium is noticed (Serdani *et al*., 2002). External symptoms are rare but some fruit have a lighter colour and fall prematurely from the tree (Carpenter, 1942; Reuveni *et al*., 2003).

1.2.1.3. Epidemiology and etiology

Flowers and young fruit are sensitive to *Alternaria* infection early in the growing season (Ellis and Barrat, 1983). The spores spread/grow from the external areas of the apple into the open calyx tubes and sporulate in the core region and seed locules during the growth phase of the fruit (Reuveni *et al*., 2003). According to Reuveni *et al*. (2002), the beginning of bloom and full bloom is the most susceptible time in the developing stages for infection by decay causing pathogens such as *A. alternata* to occur. When attempting to control *A. alternata,* the life cycle stages, conidial germination, hyphal growth and decay formation should be targeted as these stages has the highest potential for initiating infection of the fungi in the host and causing disease development in apples (Reuveni *et al*., 2002).

1.2.1.4. Factors that enhance MC

Factors that can enhance MC are wet weather conditions during the spring season that favours mycelial growth and sporulation (Michailides *et al*., 1994). Other factors that influence MC are high relative humidity, mild temperatures and tissue susceptibility (Reuveni *et al*., 2002). Fruit size is also a factor in MC incidence with larger apples having a higher incidence of MC than smaller apples (Serdani, 1999).

1.2.2. Dry Core Rot

1.2.2.1. Causative agents

Dry core rot (DCR), as with MC, has a number of fungi as potential causal organisms. These include the genera *Stemphylium* Wallroth, *Cladosporium*, *Ulocladium* Preuss, *Epicoccum*, *Coniothyrium*, *Alternaria* and *Pleospora herbarum* (Pers. ex Fr.) Rabenh. (Combrink *et al*., 1985; Spotts, 1990; Niem *et al*., 2007). Initially, the main causal agent for DCR in South Africa was *Alternaria alternata* (Combrink *et al*., 1985), however, Serdani (1999) showed that *A. tenuissima* (Nees and T. Nees: Fr.) Wiltshire is the most frequently isolated fungal species from DCR tissue. Other *Alternaria* species frequently isolated from DCR tissue are *A. infectoria* E.G. Simmons and *A. arborescens* E.G. Simmons (Serdani *et al*., 2002).

1.2.2.2. Symptoms

Dry core rot is characterized by a dry rot initiating from the core region, which spreads through the mesoderm (McLeod *et al*., 2008). The tissue texture appears dark and corky with the hue varying between light brown and dark brown. Grey to black mycelium is observed in the core region and in/on the seed locules (Niem *et al*., 2007). Dry core rot develops slowly during fruit development (Combrink, 1983).

External symptoms are rare and this is a great problem for growers because the disease only becomes visible after the fruit has been cut open and mycelium and rot is visible to the consumer (Serdani *et al*., 2002). Some fruit with DCR colour prematurely and can be recognized by a yellow background colour of the skin (Carpenter, 1942). These fruit may ripen and drop prematurely (Serdani, 1999).

1.2.2.3. Epidemiology and etiology

Dry core rot infections take place pre-harvest in the orchard (McLeod *et al*., 2008). The specific time of infection is unclear and can take place before, during or after the bloom

period (Ellis and Barrat, 1983). Researchers are in agreement that the infection route of the fungal disease is through the open calyx tube of the different susceptible cultivars. The flowers are infected and colonized from where the fungus moves into the open calyx and into the core (Spotts, 1990) where it is protected from fungicides.

Fungal spores that penetrated the seed locule through the open calyx tube could cause infection at any stage in the fruit development (Niem *et al*., 2007). According to Niem *et al.* (2007) there was an increased incidence of *A. alternata* colonization between the 14th and 110th day after full bloom.

1.2.2.4. Factors influencing DCR development

Temperature and humidity influence DCR development during the growing season with high relative humidity and mild temperatures favouring disease development and fungal growth (Ellis and Barrat, 1983). Other factors, which predispose fruit to DCR, include low fumaric and malic acid content during the entire growth and development of susceptible cultivars, such as 'Starking' (Combrink, 1983). Resistant cultivars, such as 'Golden Delicious' and 'Granny Smith' have a high fumaric and malic acid content (Combrink, 1983). *Alternaria alternata* grows and sporulates poorly in fruit with a high malic acid content (Combrink, 1983). Fruit size and growth can also contribute to DCR with larger fruit having higher incidences of DCR (Harrison, 1935).

Another factor that can influence DCR is the pH level of the fruit. Niem *et al*. (2007) found that the pH of susceptible cultivars, e.g. 'Red Delicious' were less acidic than resistant cultivars such as 'Golden Delicious', and cultivars with closed calyx sinuses. The endo- and exoglucanase activity levels were also found to be higher because of the higher pH levels in the mesoderm (Niem *et al*., 2007). Different pH levels close to the core influences the start of fungal colonization by activating the specific gene expression (Niem *et al*., 2007). The activity of endo- and exoglucanase may elicit programmed cell death and be a factor in the pathogenicity of the causal organisms, especially *A. alternata* in susceptible cultivars (Ryerson and Heath, 1996; Reuveni *et al*., 2007).

1.2.3. Wet Core Rot

In the 2005/06 season wet core rot (WCR) symptoms were observed in apples, before harvest in South African orchards (McLeod *et al*., 2008; Van der Walt, 2009; Van der Walt *et al*., 2010). The first pre-harvest WCR was seen in South Africa in the Ceres region and the next season 2006/07 in the Grabouw and Ermelo region (McLeod *et al*., 2008; Van der Walt,

2009). This data disagrees with previous statements that WCR was exclusively a post-harvest disease and that infection rarely occurred in the orchard (Serdani, 1999). Although Van der Walt (2009) observed and identified the pre-harvest WCR symptoms, previous research has suggested the probability of infection of WCR causative agents during the pre-harvest period (De Kock *et al*., 1991).

1.2.3.1. Causative agents

Penicillium expansum Link and *P. funiculosum* Thom and to a lesser extent *P. glabrum* (Wehmer) Westling, *P. chermesinum* Biourge and *P. ramulosum* C.M. Visagie and K. Jacobs have been isolated from pre-harvest WCR (Van der Walt, 2009; Van der Walt *et al*., 2010).

Post-harvest WCR is caused by *Penicillium expansum*, which infects the apple during post-harvest handling. The post-harvest treatment for physiological apple scald uses diphenylamine (DPA)-emulsion (Combrink and Ginsburg, 1973), which lowers the surface tension of the spore infested water and they can more readily move into the core region of the apple (Combrink and Ginsburg, 1973). *P. funiculosum*, *P. roquefortii* Thom and *Mucor piriformis* E. Fischer, were later also identified as being causative agents of WCR (Combrink *et al*., 1985; Spotts *et al*., 1988b; McLeod *et al*., 2008). *Fusarium avenaceum* (Fr.) Sacc., encountered in maize and other cereals, also causes WCR in apples and was recently identified pre-harvest in orchards in Slovenia (Sørensen *et al*., 2009). Other fungi associated with WCR include *Botrytis cinerea* Pers. ex. Nocca and Balbis, *Botryosphaeria obtusa* (Schwein.) Shoemaker, *Fusarium* spp. and *Pestalotia laurocerasi* (Westend.) Steyaert (Combrink, 1983).

In the study of Van der Walt (2009) and Van der Walt *et al*. (2010), *Penicillium* species were identified based on macro- and micro-morphology of the isolates as well as using molecular characterisation. For morphological identification of isolates, the isolates were grown on different media to establish growth patterns and colony characteristics such as colour and mycelial structure. The isolates were inoculated using the three point inoculation method as described by Pitt (1979), and Samson and Pitt (1985) onto Czapek Yeast Agar (CYA), Malt Extract Agar (MEA) and 25% Glycerol Nitrate Agar (G25N). The plates were incubated at room temperature for 7 to 21 days. The isolates were then described and characterised using the methods of Pitt (1979), Samson and Pitt (1985) and Okuda *et al*. (2000).

Molecular characterisation of *Penicillium* species can be done using the polymerase chain reaction (PCR) and PCR- restriction fragment length polymorphism (RFLP) of the beta-tubulin gene and internal transcribed spacer (ITS) region. The genomic DNA can be extracted using a modified version of Lee and Taylor (1990), or using CTAB buffer (Doyle and Doyle, 1990). The Bt2a primer (Glass and Donaldson, 1995) and PentubR primer can then be used of for the PCR amplification of the beta-tubulin gene. The ITS-region can be amplified using the primers ITS1 and ITS4 (White *et al*., 1990).

1.2.3.2. Symptoms

Wet core rot can readily be distinguished from the other core rot symptoms. WCR develops from the inside of the core region into the mesoderm of the apple (McLeod *et al*., 2008). The tissue of WCR apples is moist and spongy with a light brown or straw colour lesion (Serdani, 1999). There is a definite barrier between decayed and healthy tissue, and the causal organism in the decayed tissue can be isolated without any difficulty (Serdani, 1999).

Wet core rot, caused by *Fusarium avenaceum,* is visible as white, red or rose-coloured mycelium seen in the core area of the apple with a light-brown wet rot that develops from the core to the mesoderm (Sørensen *et al*., 2009). WCR spreads very fast (Serdani, 1999), and the symptom can sometimes be seen externally after it has spread throughout the tissue. External WCR symptoms resemble Blue mould, which is caused by *Penicillium expansum*.

Besides visual symptoms, *Penicillium expansum* can produce the mycotoxin, patulin that results in the decline of the quality of apple, and can be a human health risk (Batta, 2004). *Fusarium avenaceum* produces high amounts of mycotoxins, such as moniliformin, enniatins, chrysogine, 2-amino-14,16-dimethyloctadecan-3-ol and aurofusarin, in WCR symptomatic fruit causing risk factors in apple products (Gutleb *et al*., 2002, Sørensen *et al*., 2009).

1.2.3.3. Epidemiology

Previous research done in South-Africa indicated the possibility of WCR occurring pre-harvest in the orchards (Combrink *et al*., 1985; De Kock *et al*., 1991; Serdani *et al*., 1998). Van der Walt (2009) confirmed that WCR occur pre-harvest in orchards from the areas Ceres, Elgin and the Langkloof in South Africa. The epidemiology of pre-harvest WCR was not determined and when infection takes place in the orchard is not known (Van der Walt, 2009). Post-harvest WCR has been known for decades as a storage disease of apples (Combrink and Ginsburg, 1973). Research established that WCR was caused by *P. expansum*

from contaminated, dipping water that washed the spores into the core region (Combrink and Ginsburg, 1973; Combrink *et al*., 1985; Combrink *et al*., 1987; Spotts *et al*., 1988b).

A high percentage of apples treated with diphenylamine(DPA)-emulsion for superficial scald were infected with WCR in the pack house (Combrink and Ginsburg, 1973; Combrink *et al*., 1985; Combrink *et al*., 1987; Spotts *et al*., 1988b). The DPA-emulsion lowers the surface tension of the water, which if contaminated allows the spores of the pathogen to be transported easier into the seed cavity and infect the surrounding tissue (Combrink and Ginsburg, 1973). The incidence caused by wet core rot pathogens, *P. expansum* and *M. piriformis* in dip water was between 16.6 and 30% (Spotts *et al*., 1988b).

1.2.3.4. Factors that affect WCR

There are different factors that can influence WCR in apples. Some factors are environmental and cannot be controlled while others are related to the fruit itself. Spotts *et al*. (1988b) concluded that these factors can include the cultivar, the weather during the growing season, and the interaction between dry and wet core rot fungi, fruit shape, postharvest dipping procedure, DPA formulation and fungi present in the dip solutions.

Certain cultivars of apples especially ‘Red Delicious’ and ‘Starking’ have open and wide calyx openings, allowing the fungi to enter the apple with relative easy (Spotts *et al*., 1988b). During dipping procedures in the pack house, water with DPA-emulsion can be contaminated with *Penicillium* conidia, which can wash into the wide opening of the core of the apple and cause WCR (Combrink *et al*., 1985).

1.3. The genus *Alternaria*

1.3.1. Taxonomy

Alternaria is a cosmopolitan genus with many species of economical importance, including saprophytes and phytopathogens that can produce allergens or mycotoxins (Rotem, 1994; Andersen *et al*., 2001; Roberts *et al*., 2012). Some species of *Alternaria* can shift from being saprophytic to pathogenic when associated with a susceptible host (Rotem, 1994). *Alternaria* species can occur on dead plants, food, soil (Neergaard, 1945), agricultural plants (Strandberg, 1992) and as airborne conidia causing allergies (de Hoog and Horre, 2002). Pathogenic *Alternaria* species have been reported in nearly every country in the world (Rotem, 1994).

Alternaria is part of the family Dematiaceae, the order Hyphomycetes, Fungi Imperfecti (Rotem, 1994). The *Alternaria* genus was established in 1817 by C.G. Nees with

A. tenuis as the only species described (Elliot, 1917). In 1819 Fries described the genus *Macrosporium* to differentiate it from other muriform type spores, especially the genus *Cladosporium* (Elliot, 1917). During this time Fries never mentioned *Alternaria* as a genus similar to *Macrosporium* morphologically (Elliot, 1917). This caused confusion in the scientific world as both authors had not described the genus and species to the full extent. Elliot (1917) analyzed *Alternaria* and *Macrosporium*'s characteristics of the conidia, and gave the generic criterion for the form of the *Alternaria* conidia as obclavate, pointed and often having beaks. Wiltshire (1933) resolved the confusion between *Alternaria* and *Macrosporium,* saying they are congeneric but that *Alternaria* is the more appropriate epithet for the genus.

Although the genus name has now been established, *Alternaria* isolates that are small-spored have been in many cases identified incorrectly (Andersen *et al*., 2001). Neergaard (1945) was the first to describe species according to morphological characteristics, but Simmons (1967, 2007) was the pioneer for species identification of the genus *Alternaria. Alternaria alternata* (syn. *A. tenuis*) is the frequently identified species of *Alternaria* (Rotem, 1994), but small-spored *Alternaria* species will be identified as *A. alternata* based solely on conidial size. Simmons identified the bulk of the *Alternaria* species from 1981 to 2003 in the journal Mycotaxon, renaming many *A. alternata* and the species formae specialis isolates as other species. Species occurring regularly as phytopathogens are *A. alternata, A. tenuissima, A. longipes* (Ellis and Everh.) E.W. Mason, *A. arborescens, A. solani* Sorauer, *A. dauci* (Kühn) J.W. Groves and Skolko, *A. infectoria, A. citri* Ellis and Pierce, *A. tangelonis* E.G. Simmons, and *A. mali* Roberts.

Alternaria species cause a few apple diseases, such as Alternaria Blotch, caused by *A. mali*, Alternaria Rot which is caused by *A. alternata* and core rot of apples which is caused by *Alternaria* species (Sawamura, 1990; Spotts, 1990).

1.3.2. Morphology

The general consensus of the morphology of conidia are that they form either in chains or solitary, their form can be typically ovoid or obclavate, they are often beaked, the conidia's colour ranges from pale brown to brown, and the conidia are multicelled with transverse and/or longitudinal septa (Ellis, 1971). Each species differs morphologically either through the form, size and septation of their conidia, the character of the conidial beak, and the dimensions of the conidiophores (Neergaard, 1945).

Alternaria species can be identified using various growth media, such as potato carrot agar (PCA; Simmons, 1992), dichloran rose Bengal yeast extract sucrose agar (DRYES; Frisvad; 1983), malt extract agar (MEA; Pitt, 1979), nitrite sucrose agar (NS; Frisvad, 1981) and V8 juice (V8; Simmons, 1992). Serdani (1999) and Andersen *et al.* (2001) used DRYES media to differentiate between the species *A. alternata, A. infectoria, A. tenuissima, A. longipes* and *A. gaisen* Nagano. The colony colour of each species differs on the media, differentiating the species from each other.

1.3.3. Toxins and secondary metabolites

Alternaria, similar to various other fungal pathogens, produces mycotoxins (Serdani, 1999). Isolates of *Alternaria* species have been identified as 70 different types of secondary metabolites, of which 30 metabolites could be potentially toxic (Robiglio and Lopez, 1995). These include host-specific toxins (HSTs), which are highly pathogenic to a limited number of plants: *A. alternata* f.sp. *lycopersici* (AL-toxin) on tomato, *A. alternata* f.sp. *mali* on apple (AM-toxin), *A. alternata* f.sp. *kikuchiana* on pear (AK-toxin), *A. alternata* f.sp. *fragariae* on strawberry (AF-toxin), *A. alternata* f.sp. *tabaci* on tobacco (AT-toxin), and *A. alternata* f.sp. *citri* on citrus (AC-toxin; Rotem 1994). Other toxins, such as alternariol (AOH), alternariol monomethyl ether (AME), altenuene (ALT), altertoxin 1 (ATX-1) and tenuazonic acid (TA) are non-specific and may occur in more than one host (Logrieco *et al*. 1998).

Specific species of *Alternaria* produces different combinations of secondary metabolites and toxins and this can be used as a tool to identify species (Serdani *et al*., 2002). *Alternaria alternata* produces the metabolites ALT, AOH, AME, ATX-1, TA and tentoxin, whereas *A. tenuissima* produces ALT, AOH, AME, ATX-1 and TA (Bottalico and Logrieco, 1998). *Alternaria infectoria* produces only the metabolites AME and TE (Bottalico and Logrieco, 1998) and *A. arborescens* produced AOH, AME and TE (Andersen *et al*., 2002).

1.4. The genus *Penicillium*

Link described the genus *Penicillium* in 1809 after the Latin word penicillus, which means little brush (Raper and Thom, 1949; Pitt and Hocking, 1997). During this time Link also described three *Penicillium* species: *P. glaucum* Link, *P. candidum* Link, and *P. expansum* (Raper and Thom, 1949). *Penicillium expansum* has been designated as the type species for the genus (Raper and Thom, 1949). The genus is divided into four subgenera *i.e. Aspergilloides* Dierckx, *Penicillium*, *Biverticillium* Dierckx and *Furcatum* Pitt (Pitt and Hocking, 1997).

Penicillium has a world-wide distribution and is one of the most important food and airborne pathogens (Samson *et al*., 2004). It affects a wide range of hosts including various fruits, vegetables, meats, cereals and nuts (Pitt and Hocking, 1997) and is seen as post-harvest fungi, infecting and attacking fruit during storage. The most common disease caused by a variety of *Penicillium* spp. such as *P. aurantiogriseum* Dierckx, *P. brevicompactum* Dierckx, *P. crustosum* Thom, *P. diversum* Raper and Fennell, *P. expansum*, *P. funiculosum*, *P. rugulosum* Thom and *P. verrucosum* Dierckx, is 'Blue Mould' (Rosenberger, 1990).

1.4.1. Morphology

Penicillium species can be identified through colour, colony growth and the roughness or smoothness of the stipes (a hypha supporting a fruiting structure) (Pitt and Hocking, 1997). *Pencillium* species can vary in colours from green, yellow-green, blue-green, gray-green and in some cases the colonies may appear colourless (Raper and Thom, 1949). The Methuen "Handbook of colour" (Kornerup and Wanscher, 1978) is an excellent tool for the identification of the colony colour.

The colony growth and diameter of *Penicillium* species differs between species as well as on different media. The colony growth varies from weak, with an average diameter of 4-14 mm, to strong, with an average diameter of 20-30 mm. The subgenus *Penicillium* can be identified by the penicilli being predominantly terminal terverticillate structures. The phialides are flask shaped but can be needle shaped in some species (Pitt and Hocking, 1997).

Various media can be used to assist with the identification of *Penicillium* species. These media are Czapek-Dox agar (Raper and Thom, 1949), Czapek Yeast Autolysate (CYA) agar (Pitt, 1979), Malt Extract Autolysate (MEA) agar (Raper and Thom, 1949), 2% Malt Extract (ME2) agar (Samson and Pitt, 1985), Oatmeal (OAT) agar (Samson and Pitt, 1985) and Yeast Extract Sucrose (YES) agar (Frisvad, 1981).

1.4.2. Toxins and secondary metabolites

The most important toxin produced by a *Penicillium* species is patulin, which is produced by *P. expansum* (Brain, 1956; Pitt and Hocking, 1997; Batta, 2004). Patulin was first reported as an antibiotic (Anslow *et al*., 1943), and used against the disease "damping-off" (Raper and Thom, 1949). Later, reports of a carcinogenic effect made it an important mycotoxin especially since patulin can be found in processed foods such as juice (Pitt and Hocking, 1997; Scherm *et al*., 2003). Other mycotoxins produced by *Penicillium* species have been identified. These include ochratoxin A and the nephrotoxin citrinin produced by *P.*

verrucosum (Pitt, 1987), xanthomegnin, viomellen and brevianamide A produced by *P. viridicatum* Westling (Pitt, 1987).

Penicillium species produce various secondary metabolites that can be used to identify species (Frisvad and Samson, 2004). Secondary metabolites can play a role in identifying a species through reverse colony colour, and exudate colours (Frisvad and Samson, 2004). Some of these diagnostic metabolites include anacine, aurantiamine, aurantin, penicillic acid, verrucodin and pseurotin (Zhelifonova *et al*., 2009). Other metabolites can play a role in the changes, which occur in fruit during fungal attack (Hadas *et al*., 2007). Gluconic and citric acids are produced by *P. expansum* during infection of the fruit (Prusky *et al*., 2004; Hadas *et al*., 2007). These metabolites acidify the host tissue and increase the colonization of the fungi in the host (Prusky *et al*., 2004; Hadas *et al*., 2007).

1.5. Molecular identification and techniques

Presently, the identification of fungi is more than just identifying the genus and species morphologically. A need has developed for quicker and more accurate identification of fungal species. The use of molecular techniques has been implemented to identify species more accurately and faster especially in circumstances where quarantine organisms are concerned. It is more straightforward to identify some fungal species, such as *Penicillium*, using molecular techniques. Other species have similar genetic strains and more gene regions or techniques are needed to identify the fungi to species level. The disease core rot has two main causal organisms, *Alternaria* and *Penicillium*. Herewith is a description of the techniques used thus far to molecularly identify the fungi to species level.

1.5.1. *Alternaria*

The internal transcribed spacer region (ITS) is one of the most frequently used gene regions for molecular identification of most fungal species. Other gene regions used for identification of fungal species include glyceraldehyde-3-phosphate dehydrogenase (gpd), translocation elongation factor 1 alpha (tef-1α), endopolygalacturonase (endo-PG), mitochondrial small and large subunit (mt SSU and mt LSU), beta-tubulin and anonymous genomic regions (OPA1-3, OPA2-1 and OPA10-2) (Kusaba and Tsuge, 1995; Pryor and Gilbertson, 2000; Pryor and Michailides, 2002; de Hoog and Horre, 2002; Pryor and Bigelow, 2003; Peever *et al*., 2004; Hong *et al*., 2005; Pavón *et al*., 2010).

ITS sequences can differentiate between small- and large-spore *Alternaria* species but has difficulty distinguishing between different small-spore *Alternaria* species. The four

Alternaria species occurring in core rot are *A. alternata, A. tenuissima, A. arborescens* and *A. infectoria* (Kang *et al*., 2002; Serdani *et al*., 2002). When an ITS sequence is analysed *Alternaria infectoria* groups apart from the clade with the other three species (de Hoog and Horre, 2002; Berbee *et al*., 2003; Andersen *et al*., 2009). *Alternaria infectoria* has an additional 26bp insert in the ITS1 sequence (de Hoog and Horre, 2002). Other gene regions can establish a difference between *A. arborescens* and *A. alternata/A. tenuissima* but these latter two species cannot yet be separated into distinct clades (Pryor and Michailides, 2002).

Deoxyribonucleic Acid (DNA) fingerprinting analysis of *Alternaria* species has been used in an attempt to separate species from *A. alternata.* These include random amplified polymorphisms DNA (RAPD), polymerase chain reaction (PCR) - random fragment length polymorphisms (RFLP), and inter simple sequence repeats (ISSR) analyses. RAPDs and PCR-RFLP analysis has been successfully used to separate *A. infectoria* and *A. arborescens* into distinct clades, but *A. alternata* and *A. tenuissima* groups together in a single clade and cannot be distinguished between morphology (Roberts *et al*., 2000; Pryor and Michailides, 2002; Hong *et al*., 2006). Hong *et al*. (2006) looked at ISSR analyses, with the microsatellites $(GACA)_4$ and $(CAA)_5$ to distinguish between the species *A. alternata, A. tenuissima* and *A. arborescens*. *Alternaria arborescens* formed a monophyletic lineage distinct from the *A. alternata* and *A. tenuissima* species groups. The constraint in molecular identification of small-spored *Alternaria* species is still to differentiate between *A. alternata* and various other species such as *A. tenuissima*.

1.5.2. *Penicillium*

Penicillium species have successfully been identified using the internal transcribed spacer (ITS), cytochrome c oxidase 1 (CO1) gene and beta-tubulin gene regions (Lobuglio *et al*., 1993, 1994; Skouboe *et al*., 1999; Seifert *et al*., 2007). Although ITS is a useful region for clarifying subdivisions of the *Penicillium* genus (Lobuglio *et al*., 1993, 1994), it does not always identify specific species closely related to one another (Skouboe *et al.,* 1999). Cytochrome c oxidase 1 (CO1) gene area (400 – 600bp) has been used to barcode the *Penicillium* genus, but was found to have a lower sequence divergence than beta-tubulin, and, as with ITS, was not able to identify closely species from another. Beta-tubulin is the most divergent gene used to identify *Penicillium* species (Van der Walt *et al*., 2010) and can distinguish closely related species from another (Seifert *et al*., 2007).

Fingerprinting techniques, such as RAPDs and PCR-RFLPs have been used to identify fungi to species level. Pianzolla *et al*. (2004) found that the RAPD technique is

useful to separate subspecific genera, but it is not sufficiently reliable to identify species. Van der Walt *et al*. (2010) used the beta-tubulin PCR-RFLP technique with restriction enzymes *Hae*III and *Rsa*I to distinguish between *Penicillium* species isolated from core rot symptoms. Their results were confirmed through sequence analysis (Van der Walt, 2009; Van der Walt *et al*., 2010).

1.6. Disease Incidence and Economic Importance

Agrios (2005) defined the incidence of disease as the number or proportion of plant units such as leaves or fruit that are diseased in relation to the total number of units examined. The incidence of disease indicates the possible need for control measures to manage the fungal population in the orchard.

The incidence of applecore rot varies from season to season, area to area, and from farm to farm. In Israel, the incidence of core rot from 1997 until 2002 ranged between 7 and 12% (Reuveni *et al*., 2002). In 2003, a very high incidence of up to 40% core rot (mainly mouldy core) was recorded in Israel (Reuveni *et al*., 2003; Reuveni, 2006; Reuveni *et al*., 2007). Sporadic, high disease incidences in orchards are not uncommon. Raina *et al*. (1971) recorded a disease incidence of 40% in an orchard in India, where the average disease incidence was 10%. In the USA, incidences of between 38 and 66% have been recorded (Ellis and Barrat, 1983).

In South African orchards the incidence of core rot varied between 3.3 and 12% (Combrink and Ginsburg, 1973; De Kock *et al*., 1991; McLeod *et al*., 2008). Recent specific data have indicated the range in incidence of pre-harvest wet core rot (WCR) to be between 0 and 1.7%, and that of dry core rot (DCR) in ‘Oregon Spur’ and ‘Top Red’ cultivars to be between 0.4 and 6% (Van der Walt, 2009). Mouldy core (MC) incidence varied between 0 and 16% (Van der Walt, 2009). Recently, Sørensen *et al.* (2009) evaluated the incidence of wet core rot of apples in Slovenia and found an average incidence of 5% symptomatic apples. Internationally, the incidence of core rot cannot exceed 6 – 9% if the fruit is to be exported or sold as fresh fruit on the local market (Ellis and Barrat, 1983; Reuveni *et al*., 2002).

The economic importance of the various core rot symptoms differ (Combrink *et al*., 1985; Spotts, 1990; McLeod *et al*., 2008) with DCR and WCR, impacting both on quantity and quality of apples sold, as well as on the consumer confidence in the product (Ellis and Barrat, 1983). Mouldy core is not as economically important because it is restricted to the core cavity of the apple (McLeod *et al*., 2008).

In 2011, South Africa was ranked 12th overall of the major apple producing countries (Anonymous, 2011a). South Africa's main export markets are United Kingdom, Asia and the Far East (Anonymous, 2011a). The most popular cultivars exported are 'Golden Delicious', 'Granny Smith', 'Royal Gala', 'Cripps' Pink', 'Fuji', 'Top Red' and 'Starking' (Anonymous, 2011a). Open calyx cultivars such as 'Fuji', 'Top Red' and 'Starking' are susceptible to core rot infection (Spotts, 1990). In 2011 these three cultivars covered an area of 4594 hectares (20.96%) of South African apple farming area (Anonymous, 2011a).

The 2010/2011 total production of apples for South Africa was 766 622 tons, of which 318 993 tons were exported (Anonymous, 2011a). Of the exported apples 35 673 tons (11.18%) were open calyx cultivars (Anonymous, 2011a). In Europe and the United States the total production of apples are 7 million and 4 million tons, respectively (Anonymous, 2011a), of which estimations for open calyx end cultivars 'Fuji', 'Ida Red' and 'Red Delicious' were approximately 1.5 million tons each (Anonymous, 2011a).

If we use the average of 6% infection with core rot, calculated based on what is known, then South Africa, Europe and United States will lose approximately 5946 and 250 000 tons respectively of apples due to this disease. The significance of this for the South African market is of great importance. South African producers received R6210/ton for exported apples in 2011(Anonymous, 2011a) and a loss of 5946 tons at a price of R6210/ton would have resulted in a loss of R37 million in revenue.

1.7. Inoculum sources

Inoculum of a pathogenic organism can initiate infection when according to Agrios (2005) "...any part of the pathogen comes in contact or is brought into contact with a susceptible host organism such as a plant." Inoculum does not necessarily need to infect immediately, as some organisms can be latent in or on host tissue, such as blossoms, fruit, and other parts of the plant. Inoculum can overwinter as conidia or as survival structures, until favourable environmental conditions occur for the inoculum to infect the plants. Sources of inoculum can be alternative hosts near an orchard, such as shrubs and other plants or weeds, as well as debris from the orchard such as leaf litter, grasses, prunings, dropped fruit, mummified fruit on the ground as well as in trees. Inoculum sources are important factors in the epidemiology of plant diseases. A pathogen needs protection and sustenance during unfavourable conditions until the conditions become favourable for infection. Alternative hosts such as weeds and surrounding shrubs and trees can become inoculum sources, as well as the debris of the trees itself. In spring the inoculum is transported to the host plant through

the wind, insect movements and through rain dispersal (Agrios 2005). Potential inoculum sources for core rot are the buds on the trees, pruning debris left in the orchard, mummified fruit and airborne conidia (Serdani *et al*., 1998; Van der Walt, 2009). Post-harvest inoculum can be found on infested packing bins and contaminated flumes (Combrink *et al*., 1985; Spotts *et al*., 1988a).

1.7.1. Alternative hosts, weeds and crop debris

Alternative hosts are defined by the dictionary of Plant Pathology as those plant species which are hosts of one pathogen (Holliday, 1998). Crops and trees near an orchard can be a host to different pathogens and can become potential sources of inoculum. In other fungal plant diseases alternative hosts were shown to contribute as an epidemiological factor. Alternative hosts can be any type of tree, shrub, crop or even weeds. Weeds are defined by Baker (1974) as species that have no detected human value, but interfere with human activities and can cause economic, and ecological losses (Mack and Lonsdale, 2001).

To survive the winter season many pathogens survive on crop debris, growing as saprophytes or using specialised resting structures, such as chlamydospores or sclerotia (Sussman, 1968; West *et al*., 2008). *Botrytis cinerea* was collected from several different weed species, such as white sweet clover (*Melilotus alba* Medikus.), annual bluegrass (*Poa annua* L.), common chickweed (*Stellaria media* (L.) Vill.), common mallow (*Malva neglecta* Wallr.), quackgrass (*Elymus repens* (L.) Gould), and dandelion (*Taraxacum officinale* G. H. Weber ex Wiggers) (Spotts and Serdani, 2006). *Alternaria* species were reported on the wild plants *Helianthus annuus* (Jeffery *et al*., 1984), in wild Apiaceae (Netzer and Kenneth, 1969) and in *Fumaria muralis* (Soteros, 1979).*Alternaria* pathogens overseason in or on debris of wild plants and in seeds (Rotem, 1994). They also overwinter in host tissue, for example the bark, nodes and internodes of perennial trees (Tanaka *et al*., 1989).

Penicillium species occurs on storage bins and can overwinter on the wood (Lennox *et al*., 2003; Spotts *et al*., 1988a). *Penicillium* colonises rotten fruit left at the bottom of the bin (Combrink *et al*., 1987), and any debris left in the orchard, such as leaf litter (Lennox *et al*., 2003).

Other crops may be used as an alternative host such as *Botrytis cinerea* on blackberries in pear orchards (Spotts and Serdani, 2006) or *A. alternata* in poplar trees where they overwinter as mycelia or conidia in fallen leaves and buds (Xu *et al*., 1984).

1.7.2. Airborne inoculum

Airborne conidia can be a major inoculum source to many different crops and hosts. Many devastating epidemics, for example late blight of potato, false smut of rice and chestnut blight have been spread from field to field, neighbour to neighbour and across countries through airborne conidia (West *et al*., 2008). It is important to understand the source of inoculum as part of the epidemiology of the disease.

Conidia are small and light, thus making it easy for them to be dispersed in air currents, through splashing of water droplets and by insects, such as bees and ants carrying the conidia on their bodies (Coertze and Holz, 1999). Virulent pathogen conidia are dispersed through air dispersal onto susceptible hosts and if environmental conditions are favourable disease can develop (West *et al*., 2008). New pathotypes, mating types or mutants of pathogens can be introduced to susceptible areas through wind dispersal (West *et al*., 2008). Airborne conidia can cause disease from the orchard through harvest to the packaging line. Airborne fungi are opportunistic and move into any wound or crack on the fruit and cause infection.

Alternaria is an important causal organism of many diseases, for example, *A. brassicae* that causes dark leaf and pod spot of oilseed rape (West *et al*., 2008), *A. solani* that causes early blight of potato and tomato (Rotem, 1959), and *A. alternata* that causes a wide range of diseases including core rot (Carpenter, 1942). The main mechanism for distributing *Alternaria* species from one host to another is through air dispersal, but in some cases splash dispersal can occur (Rotem, 1994).

Alternaria conidia are good air travellers as their large bodies make it easier for them to float on the air currents and their dark pigmentation protects them from radiation (Gregory, 1973). Factors necessary for *Alternaria* conidia to be released is wind at a velocity above 7-11 km/h (Strandberg, 1977). Aylor (1990) established that for *Alternaria* conidia a brief gust or moderate winds was all that was necessary for dispersal. A low humidity in the air is also necessary for dispersal (Rotem, 1994). It has been established that under normal conditions *Alternaria* conidia can be dispersed around noon or later (Rotem, 1994) when it was the warmest and driest part of the day and the greatest wind speed (Gregory, 1973).

Airborne *Penicillium* species have been found in orchards and pack houses in the United States (Spotts and Cervantes, 2001). They established that three conidia/litre of *Penicillium* conidia in the air was enough to cause significant levels of decay in storage (Spotts and Cervantes, 2001). *Penicillium* species are aggressive colonisers of both soil and

plant litter. They can be dispersed as dry or wet conidia in the orchard, landing on harvested fruit and storage bins (Spotts and Cervantes, 2001; Lennox *et al*., 2003). The bins are transferred to pack houses, where conidia are dispersed through air currents and handling to surfaces and dumping water, increasing the inoculum levels (Spotts and Cervantes, 2001; Lennox *et al*., 2003). Levels between 0 and 1020 conidia/litre have been found in packinghouses in Oregon and Washington (Spotts and Cervantes, 2001).

Other causal organisms dispersed through air currents in the orchard are *Botrytis cinerea,* which causes grey mould, and *Cladosporium* species, which causes leaf mould (West *et al*., 2008). Both these fungi are causal organisms of core rot of apple (Combrink *et al*., 1985).

Different sampling procedures can be used to establish the density of the airborne population, for example through gravitational deposition of spores or by volumetric spore trapping devices (Kennedy *et al*., 2000). Lennox *et al*. (2003) used a portable air sampler to establish the density of airborne fungi in d'Anjou pear orchards.

1.7.3. Mummies

Fruit that drop early due to natural as well as mechanical thinning can become a major source of inoculum in the orchard when not controlled effectively (Hong *et al*., 1997; Cox and Scherm, 2001; Holb and Scherm, 2007). Most of the early dropped fruit become shrivelled and mummified hence the term 'mummies'. The mummies serve as a source of food and protection for fungi that need to overwinter before infecting an orchard in the next season (Holb and Scherm, 2007). There are two requirements for mummified fruit to function as a source of inoculum namely that the fungi must survive the winter and that the environmental conditions must be conducive for the fungus to sporulate during the bloom period (Hong *et al*., 2000).

Mummies can be the primary inoculum source for many different diseases on different types of crops and are especially important in initiating brown rot disease of stone fruit caused by *Monilinia fructicola* (G. Winter) Honey (Hong *et al*., 2000; Landgraf and Zehr, 1982). *Monilinia* Honey species can overwinter as mycelium (Villarino *et al*., 2010) on mummified fruit, and under favourable environmental conditions the mycelia produce either conidia or ascospores (Luo *et al*., 2001; Luo *et al*., 2005).

Botrytis cinerea also infects mummies of wild blackberries as an alternative host and overwinters in the mummies to become an inoculum source in the spring when the conidia are released and spread to the pear host plants to cause infection (Spotts and Serdani, 2006).

Blueberries, almonds and pistachios are infected by *Monilinia vaccinii-corymbosi* (Reade) Honey, *Colletotrichum acutatum* J.H. Simmonds and *Botryosphaeria dothidea* (Moug. ex Fr.) Ces and de Not. respectively, due to the infection of overwintered conidia on mummified fruit (Adaskaveg *et al*.,1998; Michailides, 1991; Miles and Schilder, 2009).

Research on mummies in apple orchards have been investigated as a possible inoculum source for overwintered conidia. *Physalospora obtusa* and *Botryosphaeria dothidea* (Sutton, 1981) have been isolated from mummified apples from the orchard floor as well as from the trees. Mummies from the twigs and branches in apple orchards in Europe have been colonised by the fungus, *Diplodia seriata* De Not. (Trapman *et al*., 2008). *Penicillium* species are aggressive colonisers (Lennox *et al*., 2003) and have been found on the mummy surface and on the inner tissue of the mummified fruit (Hong *et al*., 1996; Hong *et al*., 2000). *Penicillium* species were recovered from mummified stone fruit with a recovery incidence of above 20% (Hong *et al*., 2000). During orchard surveys *Alternaria, Botrytis* and *Cladosporium* have been identified on stone fruit mummies (Hong *et al*., 2000). In South Africa both *Penicillium* and *Alternaria* have been isolated from mummified apples (Combrink *et al*., 1994).

Although mummies have been established as an inoculum source in the stone fruit orchards for initiating brown rot, it has not yet been determined whether they are an inoculum source for the fungi causing core rot in apples. *Alternaria* and *Penicillium* have been isolated from mummified apple fruit (Combrink *et al*., 1994), but relevant research on the survival of these pathogens on mummies has not been established. After the overwintering period the fungi that have colonised in the mummies, germinate, form fruiting bodies such as apothecia or produces conidiaphores (Sutton, 1981; Miles and Schilder, 2009). The conidia are dispersed through wind or rain dispersal into the orchard.

We can speculate and say that *Alternaria* and *Penicillium* either overwinter as mycelia or conidia, as is the case with *Monilinia fructicola* (Villarino *et al*., 2010) or that these fungi revert to their saprophytic phase waiting for favourable environmental conditions before infecting the host plant (West *et al*., 2008). Van der Walt (2009) observed that *Tarsonemus* mites were isolated from the mummies. Although the significance of the mites in the mummies has not yet been determined, it is possible that they play a role in the infection process of core rot in apples.

1.7.4. Mites as vectors of plant diseases

Mites can play an important role in the dispersal of fungal conidia as well as causing various diseases. In the orchard ground cover plant-feeding and fungi-feeding (phytophagous) mites and their predators occur (Skubala *et al*., 2006). Mites frequently found in South-African, European and American orchards are the European red mite, *Panonychus ulmi* Koch and the two-spotted spider mite, *Tetranychus urticae* Koch (Alston, 1994; Coli *et al*., 1994; Pringle, 2001; Reding *et al*., 2001; Pringle and Heunis, 2006). *Panonychus ulmi* has also been observed in orchards which are severely affected by Alternaria blotch (Filajdić *et al*., 1995). Management of these mites is through the application of acaricides and miticides and biological control through the use of predatory mites.

Phytophagous mites in the genus *Tarsonemus* have been found to vector *Ophiostoma minus* (Hedgcock) H. and P. Sydow on *Protea* L. spp. (Roets *et al*., 2007; Roets *et al*., 2008; Roets *et al*., 2009) and *O. minus* on pine trees (Lombardero *et al*., 2000; Lombardero *et al*., 2003; Hofstetter *et al*., 2006; Hofstetter *et al*., 2007). In recent studies *Tarsonemus* mites were also found in apples infected with core rot (Michailides *et al*., 1994; McLeod *et al*., 2008; Van der Walt, 2009). Van der Walt (2009) hypothesised that the *Tarsonemus* mites vector the core rot causing fungi into the core region of the susceptible apples. *Alternaria* and *Penicillium* are the two most common fungi associated with core rot (Combrink *et al*., 1985). *Alternaria* and *Penicillium* has also been isolated from Oribatid mites such as *Paradamacus claviceps* Hermann, and it is speculated that these mites disperse the fungal species onto plants (Renker *et al*., 2005).

Siteroptes avenae Muller, a mycophagous mite, has been associated with glume infections of wheat caused by *Fusarium poae* (Peck) Wollenweber (Kemp *et al*., 1996). *Siteroptes avenae* was examined under the light microscopy while it fed on *F. poae*. Two sac-like structures were observed which was later revealed as two elongated sporothecae that contained conidia of this fungus. When the mites move over the mycelia the conidia are gathered into the sporothecae from where it is later discharged (Kemp *et al*., 1996). The blue-staining fungus, *Ophiostoma minus*, is vectored in the sporothecae of the mites, *Tarsonemus krantzi* Smiley and Moser, *T. ips* Lindquist, and *T. fusarii* Cooreman (Klepzig *et al*., 2001; Lombardero *et al*., 2000; Lombardero *et al*., 2003). The southern pine beetle, *Dendroctonus frontalis* Zimmermann vectors the *Tarsonemus* mites on their exoskeleton into the phloem of surrounding trees from where the mites distribute the fungi (Lombardero *et al*., 2000, 2003; Klepzig *et al*., 2001; Hofstetter *et al*., 2006; Hofstetter *et al*., 2007). Both *D. frontalis* and

Pityokteines Fuchs have a relationship with the *Tarsonemus* mites that can be characterised as a phoresy (Lombardero *et al.*, 2000; Pernek *et al.*, 2008). According to Gullan and Cranston (2005) phoresy (adj phoretic) can be defined as the phemomen of one individual being carried on the body of a larger individual of another species. The *Tarsonemus* species carry different ophiostomatiod fungi of the genera *Ceratocystis* Ellis and Halst, *Ceratocystiopsis* H.P. Upadhyay and W.B. Kendr., *Ophiostoma* Goid., and *Grosmannia* Syd. and P. Syd in sporothecae that transport and deposit the fungi onto susceptible host tissue (Pernek *et al.*, 2008).

In South Africa, the spider mites *Tetranychus urticae* and *Panonychus ulmi* are found in the apple producing areas, of Elgin, Ceres and the Langkloof (Pringle, 2001). These mites cause bronzing of the leaves and in severe cases, leaf drop. This is occurs due to the mites feeding on the leaves and removing the sap and chloroplasts from the leaf (Pringle and Heunis, 2006). Mites have a history of becoming resistant to acaricides and due to the lack of control in managing spider mite with miticides combined with the high costs of doing so, biological control has been investigated as an alternative control option (Pringle and Heunis, 2006). Predatory mites have been researched as possible biological control agents. *Galendromus occidentalis* Nesbit, which is insecticide resistant, was brought over from the United States and released in the apple orchards as a biological control agent in the Western Cape (Pringle, 2001). Mite control with *G. occidentalis* was disappointing as predation was insufficient, and two cyhexatin applications were necessary to reduce the number of spider mites (Pringle, 2001). Later, *Phytoseiulus persimilis* Athias-Henriot was discovered in apple orchards, usually when high spider mite populations were observed. They were able to reduce the numbers of prey but only after a high mite population had developed. Another biological control agent, *Neoseiulus californicus* McGregor, was recorded in apple orchards and it successfully controlled both *T. urticae* and *P. ulmi*. *Neoseiulus californicus* reduced the number of miticide application required, particularly in the Elgin area (Pringle, 2001). When testing for the effectiveness of *N. californicus* as a biological control agent in apple orchards in South Africa, *Euseius adoensis* Van der Merwe and Reyke was frequently observed in the orchards. *E. adoensis* survived better than *N. californicus* when mite populations were low because they reproduced quickly with only a diet of pollen (Pringle and Heunis, 2006). In apple orchards in the UK the mites, *Panonychus ulmi* and *Aculus schlechtendali* Nalepa were controlled by the predatory mite, *Typhlodromus pyri* Scheuten (Solomon, 1993).

1.7.4.1. *Tarsonemus* mites

Tarsonemus mites have been collected from many plant species, fungi, litter, and soil as well as from stored food and products (Zhang, 2003). The mite belongs to the family Tarsonemidae [subfamily Tarsoneminae] and more than 500 species are found in the genus (Gerson *et al*., 2003; Lindquist, 1986). *Tarsonemus* mites are fungivorous by nature and can act as vectors of the fungi they come into contact with (Lindquist, 1986; Lombardero *et al*., 2003; Hofstetter *et al*., 2006). They effectively become part of the disease cycle of the fungi (Van der Walt, 2009). In South Africa, *Tarsonemus* mites have become the primary vector transferring the non-pathogenic *Ophiostoma* species to *Protea* infructescences (Roets *et al*., 2007, 2008). The *Tarsonemus* species, *T. ips, T. krantzi* and *T. endophloeus* were found on two species of pine beetles, *Dendroctonus frontalis* (the southern pine beetle) and *D. brevicomis* (the western pine beetle) (Moser *et al*., 1995). These *Tarsonemus* mites carry the heterothallic fungus *Ceratocystiopsis ranaculosus* Perry and Bridges in sporothecae and the mites are vectored onto the pine trees on the back of the beetle (Moser *et al*., 1995).

In 1994, Michailides *et al.* hypothesized that *Tarsonemus* mites might be a vector transporting fungi to the core of the apple. They suggested the mites feed on *Coniothyrium* spores and then enter the core region through the open calyx and as they enter the sinus area, they transfer the minute spores into the core area (Michailides *et al*., 1994). The mites also cause small wounds that can give entry to the pathogens (Michailides *et al*., 1994). Laboratory tests showed that apples inoculated with both the fungal pathogen, *Coniothyrium*, and the mite had an almost 50% increase in DCR incidence (Michailides *et al*., 1994).

In South Africa the *Tarsonemus* mite was present in a relative high percentage (20 to 80%) of core and calyx regions of red apple cultivars and they were associated with core rot pathogens, *Alternaria* and *Penicillium* (Van der Walt, 2009). Low incidences of the mites were found in the buds and blossoms of apples but were found at a high incidence in mummies collected from the orchard floor (McLeod *et al*., 2008).

Research on the ecology of *Tarsonemus* mites in the apple orchards and the association with core rot diseases and fungi found that *Tarsonemus* mites colonize during several important apple developmental stages, buds, blossoms, small diameter fruit, mature fruits and mummies, in the growing season (Van der Walt, 2009). At the beginning of the season high incidences of mites are found in the previous seasons mummies, suggesting that the mites are potentially overwintering in or on the mummies. From here they emerge in spring and move to the blossoms and buds where they colonize the fruit calyx end and then

move later into the core (Van der Walt, 2009). It is likely that the *Tarsonemus* mites start vectoring the fungi, *Penicillium* and *Alternaria*, at the beginning of the season as soon as they emerge from the mummies (Van der Walt, 2009).

The *Tarsonemus* mites reported by Michailides *et al*. (1994) were identified as *Tarsonemus confuses*. It was established that the South African *Tarsonemus* mite is not this species (Van der Walt, 2009). Three species were identified in the South African *Tarsonemus* collection from apple, namely *Tarsonemus waitei* Banks; a new putative species with close similarity to *Tarsonemus mixtus* Kaliszewski; and a second putative new species with closest similarity to *Tarsonemus bilobatus* Suski (Van der Walt *et al*., 2011). *Tarsonemus* mites may play a role in the epidemiology of apple core rot diseases because of the significant association of mites within the core region of 'Red Delicious' fruits with core rot, as well as their association with core rot pathogenic fungi (Van der Walt, 2009). Control of *Tarsonemus* mites might prevent core rot development in orchards (Michailides *et al*., 1994). Neither Van der Walt (2009) nor Michailides *et al*. (1994) referred to methods of *Tarsonemus* control in the orchards, but sanitation practices involving mummy removal might control the population of mites by removing their breeding habitat.

Tarsonemus species have been used as biological control agents against grape phylloxera (Forneck *et al*., 1998). The *Tarsonemus* attack and feed on the phylloxera as well as their eggs and morphs. There is insufficient knowledge of *Tarsonemus* species making it difficult to use them as biological control agents (Forneck *et al*., 1998).

1.8. Pathogenicity of core rot organisms

The isolation of fungi from symptomatic plant material does not necessarily mean that they are the primary causal organism. To verify this, pathogenicity tests needs to be performed and Koch's postulates followed to identify the primary causal organism. Some fungi are endophytic and may or may not become pathogens. An endophyte is defined as "an organism which completes its life cycle in a plant which shows no external sign of the infection" (Holliday, 1998). Fungi can also vary in their ability to cause disease, which is why the most appropriate pathogenicity test needs to be designed.

Pathogenicity and virulence are terms that have been difficult to define, specifically because scientists cannot agree on a specific definition. In Agrios' Plant Pathology (2005) the term pathogenicity is defined as "...the ability of the parasite to interfere with one or more of the essential functions of the plant and thereby to cause disease." Andrivon (1993, 1995) wrote a letter to the editor of the journal Phytopathology to simplify the definitions of

pathogenicity and virulence, and a debate with Hunt (1994) ensued with both parties finally agreeing upon the definitions of Whetzel (1929) for pathogenicity, "...is the ability of an organism [pathogen] to produce disease" and virulence "...is the measure of pathogenicity..."

Unfortunately the terms pathogenicity and virulence are not just used in the scientific field of plant pathology, but they are also used in the scientific fields of medicine, microbiology, evolutionary ecology, insect pathology and more (Thomas and Elkinton, 2004). Each of these fields has different definitions for these two terms. The term virulence according to insect pathologists can be defined as the LD_{50}, which is the dose that will kill 50% of the insects in contact with the pathogen (Thomas and Elkinton, 2004). In medicine there are different definitions for virulence. It can be defined as the degree of pathogenicity or as the relative capacity to overcome available defences (Casadevall and Pirofski, 1999) or as the severity of the disease symptoms among the infected hosts (Thomas and Elkinton, 2004).

Understanding the definitions for the terms pathogenicity and virulence, increases the knowledge of the relationship to disease causing organisms (pathogens). This enables scientists to understand why endophytes can cause disease on another host under favourable conditions thereby becoming pathogenic. Pathogens can have endophytic qualities, under unfavourable environmental conditions and wait till these conditions change to favourable. The question as to why certain organisms cause disease and others do not have been extensively researched.

Toxins, such as the AB- and AM-toxin from *Alternaria brassicicola* (Schw.) Wiltshire and *A. alternata* have been shown to have a host specific reaction when in contact with a plant and can cause disease (Johnson *et al.*, 2000, 2001; Cramer and Lawrence, 2003, 2004). The host specific toxins (HSTs) have been shown to be part of the pathogenesis of the organism (Johnson *et al*., 2000). Other factors that have been investigated and determined to be part of the pathogenicity of an organism are the environmental pH of a fungi during pathogenicity (Prusky *et al*., 2004; Hadas *et al*., 2007), melanin has been reported to be involved in the pathogenicity of *Magnaporthe* R.A. Krause and R.K. Webster and *Colletotrichum* Corda spp., but not in *Alternaria alternata* (Kawamura *et al*., 1999), cell wall degrading proteins and inhibitory proteins (Vadlapudi *et al*., 2011). The disruption of specific homologs (genes similar to one another) can lead to the inability of the pathogen to infect its host (Lawrence *et al*., 2008).

To verify whether a pathogen can cause disease on a specific host, pathogenicity tests need to be done. These tests also verify if the use of certain pathogenicity factors mentioned

previously can be suppressed, by using wild-type isolates and mutant isolates (Johnson *et al*., 2000). Pathogenicity tests are designed to suite the specific host-pathogen interaction being investigated. For foliar diseases, leaves can be inoculated *in vivo*, with the leaves still on the plant or *in vitro*, where the youngest leaves have been removed and inoculated with conidial suspensions (Benaouf and Parisi, 1998), or plants can be sprayed with conidial suspensions until runoff (Gilchrist and Grogan, 1976; Parisi and Lespinasse, 1996; Gonzalez *et al*., 2000) after which the trees are incubated at high relative humidity.

For pathogenicity tests performed on fruit, most tests involve wounding of the fruit. Using pressure syringes conidial suspensions are inoculated into the fruit through the calyx end to cause similar lesions to wound pathogens (Combrink and Ginsburg, 1973; Spotts *et al*., 1988a; Franck *et al*., 2005). A variety of pathogenicity tests for apple fruit diseases have been documented. For example, wounding of the mesoderm using a sterile pipette tip followed by inoculating conidial suspension of the pathogen into the wound (Niem *et al*., 2007; Reuveni *et al*., 2007) or the use of a conidia covered needle to make a wound in the mesoderm (Sommer *et al*., 1974), and colonized toothpicks inserted into the mesoderm (Serdani *et al*., 2002) and the calyx end (Van der Walt *et al*., 2010) of the apple have been used as pathogenicity tests for core rot organisms, as was cutting the apple longitudinally and inoculating the core of the apple with conidial suspension (Niem *et al*., 2007).

1.9. Integrated management of core rot

Integrated pest management (IPM) has become important in the control of most plant diseases. The consumer wants less fungicidal residue and more environmentally friendly products. Integrated pest management uses both biological as well as cultural based tactics to control plant diseases. Sanitation has become a big part of the IPM program by reducing inoculum incidence through removing alternative hosts, debris and decayed fruit (MacHardy, 2000). New fungicides that specifically target apple core rot and sanitation can form part of the components needed for an IPM strategy against this disease.

1.9.1. Fungicides

Research has been done in South Africa with various fungicides to determine their efficacy in the control for core rot of apples. At present there are no registered fungicides in this country for the control of this disease. The past decade has seen new research done with fungicides against apple diseases especially from Israel and the United States. New fungicides have come onto the market to reduce post-harvest diseases by controlling them

pre-harvest. Specific core rot diseases are controlled with specific fungicides and new fungicides are important in the integrated management of the disease.

1.9.1.1. Fungicides used for control core rot symptoms

Mouldy Core The control of mouldy core (MC) and *Alternaria* spp., using fungicidal foliar sprays e.g. benomyl, captan, dodine, iprodione, mancozeb, or combinations of these fungicides, have been unsuccessful (Table 1) (Reuveni *et al*., 2002). Developing bud samples taken during trials were already colonized with *Alternaria* spp. and later as the fruit developed the stamens, pistils, calyx tubes and core cavity was colonized by fungi identified as *Acremonium* Link sp. and *Alternaria alternata* (Serdani, 1999). This complicates control using fungicides, because once inside the core region, the pathogen is protected from most fungicides (Serdani, 1999). The timing of the sprays is essential to protect the susceptible calyx. Taylor (1955) and Raina *et al*. (1971) suggested that this period is 3-6 wk before harvest whereas Ellis and Barrat (1983) were of the opinion that fungicides should be applied during or shortly after full bloom. Serdani (1999) suggested that fungicidal sprays should be applied before full bloom as infection takes places earlier than expected.

Recent studies were undertaken to find a fungicide to control MC development. These fungicides included ß-aminobutyric acids, potassium phosphates, azoxystrobin, difenoconazole, polyoxin B, trifloxystrobin, mixtures of DMI fungicides and captan, bromuconazole and Signum (Table 1) (Reuveni and Sheglov, 2002; Reuveni *et al*., 2003; Reuveni, 2006; Reuveni and Prusky, 2007). Most of the fungicides attempt to control one or more stages in the life cycle of *A. alternata*.

To ensure that the fungicide application is effective, the timing of the application is crucial. Reuveni and Sheglov (2002) conducted trials with polyoxin B, trifloxystrobin, difenoconazole and azoxystrobin. The trials were done *in vitro* to observe the germination and mycelial growth of *A. alternata*. The fungicides inhibited the conidial germination and reduced the mycelial growth of *A. alternata*. These trials were unfortunately not repeated in the field and field control could not be verified. In 2003, Reuveni *et al.* undertook trials with potassium phosphate and beta-aminobutyric acids. The fungicides were sprayed three times during the bloom period, at 50% bloom, at full bloom and at 80 to 90% petal fall. During the bloom period, potassium phosphate and beta-aminobutyric acids reduced fruit infection by 40 to 78%. Trials with Signum, a premix fungicide containing pyraclostrobin+nicobifen, and bromuconazole, a sterol biosynthesis inhibitor (Reuveni 2006), and with mixtures of DMI fungicides and captan (Reuveni and Prusky, 2007) were also undertaken. The fungicides were

applied during the beginning of bloom at the pink cluster stage, at 80% bloom and at the beginning of petal fall. The Signum and bromuconazole reduced infected fruit 30 to 80%, and mixtures of DMI fungicides with captan reduced infected fruits 40 to 60% when applied during the bloom period. Potassium phosphite inhibited mycelial growth and conidial germination, whereas ß-aminobutyric acids induced defence mechanisms against causative organisms (Reuveni *et al*., 2003). Polyoxin B and trifloxystrobin inhibited the germination of conidia, Polyoxin B and difenoconazole inhibited mycelial growth and azoxystrobin and trifloxystrobin inhibited decay formation (Reuveni and Sheglov, 2002). Signum controlled decay formation and inhibited conidial and mycelial germination (Reuveni, 2006). Bromuconazole controlled decay formation (Reuveni, 2006). DMI mixtures with captan effectively controlled MC (Reuveni and Prusky, 2007). Some of these fungicides have been shown to reduce MC incidence by 40 to 80% in orchards (Reuveni *et al*., 2003; Reuveni, 2006; Reuveni and Prusky, 2007). Although these fungicides showed potential in reducing MC, there are currently no fungicides registered specifically for MC internationally or in South Africa.

Dry core rot Most of the fungicides used to control dry core rot (DCR) of apple, are sprayed as foliar sprays during the bloom period because no specific infection period, has been linked to infection by fungi associated with DCR and thus control of the disease is difficult. It is also difficult to control the disease with contact fungicides once the fungus is inside the core region (Serdani, 1999).

There is currently no effective chemical control for DCR available in South Africa. Several fungicides, including captab, tebuconazole, dichlofluanide, captan, benomyl, dodine, iprodione, procloraz and mancozeb, have been used during the bloom period but showed no real consistency in controlling DCR pathogens (De Kock *et al*., 1991; Ellis and Barrat, 1983). There is no registered chemical fungicide for core rot pathogens in South Africa, but it is believed that controlling MC pathogens will prevent DCR from occurring. Physical control measures have had an impact on controlling DCR, including using resistant cultivars, orchard management practices and ideal storage conditions (Chaunzhen *et al*., 1993; Mouat, 1953). Good airflow and light penetration reduces fungal growth and should be considered when pruning orchards (Mouat, 1953; Van der Walt, 2009). These control measures reduces the possibility of infection, either through removal of inoculum sources or reducing favourable infection environments.

Biological control can be an effective new method to control apple core rot development. *Gliocladium roseum* Bain. and *Stachybotrys elegans* (Pidopl.) W. Gams, applied as biocontrol, act as parasites of *A. alternata* spores causing them to disintegrate and appear to "collapse" (Turhan, 1993). *Pseudomonas syringae* van Hall has shown good promising results in inhibiting *A. alternata* during development of MC in the field and under cold storage conditions and shows potential in controlling DCR (Teixidó *et al*., 1998).

Wet core rot The etiology and epidemiology of pre-harvest wet core rot (WCR) of apples has not been determined thus making disease control very difficult. Good orchard practices can be used to manage WCR, by removing decayed fruit, fallen fruit, mummies, leaves and branches from underneath the trees can reduce the inoculum sources and thus lessen disease incidence. De Kock *et al*. (1991) found that only procloraz reduced the pre-harvest WCR incidence. Most of the control for WCR is for post-harvest disease such as, good sanitation practices in the pack houses that can reduce spore counts in the drench water, flumes and dip water (McLeod *et al*., 2008; Spotts, 1990). This can be achieved by removing infected fruit from the bins before they are dipped and chlorinating the water regularly to reduce the inoculum in the water (Combrink *et al*., 1987; McLeod *et al*., 2008, Spotts, 1990). Cultivars such as 'Starking' and 'Red Delicious' with open calyx tubes that was exposed to a higher concentration of DPA-emulsion had a higher incidence of WCR due to DPA lowering the surface tension of the water and allowing the conidia to be washed into the calyx (Combrink *et al*., 1987; Spotts *et al*., 1988b). When the water was sanitized with thiabendazole and chlorination a decrease in WCR pathogens was observed (Combrink *et al*., 1987). The use of a DPA wettable powder instead of a DPA emulsion lessened WCR incidence (Combrink and Ginsburg, 1973). Alternatives to drenching such as using in-line applicators instead of dipping the fruit can be applied as control measures (Combrink *et al*., 1994; Spotts, 1990).

Products registered for use in South African pack houses to control postharvest decay are benomyl (Benomyl®), captab (Captab®), chlorine dioxide, *Cryptococcus albidus* (Saito) Skinner (YieldPlus®), dimethyl didecyl ammonium chloride (Sporekill®), iprodione (Rovral Flo®) and thiabendazole (Tecto®) (Nel *et al*., 2003). Although these fungicides are registered for postharvest use in pack houses they are not specifically registered as core rot fungicides.

1.9.1.2. Experimental fungicides

Bellis® is a new fungicide registered in 2005 for the pre-harvest protection against powdery mildew and apple scab (Anonymous, 2011b). Bellis®, is also registered in the United States as Pristine® (Anonymous, 2011b), and is a protectent and systemic fungicide with a broad-spectrum activity, which enables the fungicide to control pre-harvest as well as major storage rots caused by *Gloeosporium*, *Botrytis*, *Alternaria* and *Penicillium* (Anonymous, 2011b; Creemers and van Laer, 2006; Xiao and Boal, 2009; Xiao and Boal, 2010, Yin *et al*., 2011). Bellis®/Pristine® has been used against pre-and post-infection of *Botrytis cinerea* on table grapes (Serey *et al.*, 2007), controlling strawberry rot (Sallato *et al*., 2007; Wedge *et al*., 2007) and controlling *Monilinia fructicola* that causes brown rot of stone fruit (Schnabel *et al*., 2004).

Bellis® consists of two active ingredients, pyraclostrobin (12.8% w/w) and boscalid (28.2% w/w), and is marketed as a water dispersible granule applied for a maximum of four sprays per annum. The suggested time for the application of Bellis® is at the end of petal drop and can be applied as late as seven days before harvest (Creemers and van Laer, 2006).

Pyraclostrobin is a Quinone outside inhibitor (QoI) fungicide, from the FRAC group 11. The mode of action for this fungicide is the inhibition of complex III through the inhibition of the electron transport chain in the cytochrome bc1 (ubiquinol oxidase) at Qo site (Anonymous, 2007; Avenot *et al*., 2008). Pyraclostrobin is a broad spectrum fungicide and can be used against Ascomycetes (*Uncinula necator* (Schw.) Burr.), Basidiomycetes (*Puccinia* Pers. spp.), Deuteromycetes (*Alternaria solani*) and Oomycetes (*Pythium aphani-dermatum* (Edson) Fitzp.) (Bartlett *et al*., 2002).

Boscalid is a pyridine carboximide from the FRAC group 7. Although both pyraclostrobin and boscalid affect the respiration of the fungus, boscalid's mode of action inhibits the quinine reduction activity of the complex II (Anonymous, 2007; Avenot and Michailides, 2007). The inhibition of complex II inhibits the germination of spores, the elongation of the germ tube, the mycelial growth of the fungus and the sporulation of the spores (Avenot and Michailides, 2007). Thus boscalid inhibits all developmental stages of fungi, giving it control over pathogens which have already developed resistance to other chemical classes of fungicides (Avenot and Michailides, 2007; Myresiotis *et al*., 2008). Boscalid is a broad spectrum fungicide and can be used against *Botrytis cinerea, Sclerotinia* Fuckel spp., *Alternaria* spp. and *Phoma* spp. (Anonymous, 2011b).

Pristine® is sprayed on various host crops including on pistachio trees for the control of Alternaria blight (Avenot and Michailides, 2007; Avenot *et al*., 2008) and on apples against *B. cinerea* and *Penicillium expansum* (Xiao and Boal, 2009; Kim and Xiao, 2010; Xiao and Boal, 2010; Kim and Xiao, 2011).

The resistance and sensitivity of fungi to fungicides is important in the control of plant diseases. In fungicide resistance management establishing a baseline sensitivity is very important as it can illustrate the shift of the pathogen from sensitive to resistant (Myresiotis *et al*., 2008). The baseline sensitivity of *Botrytis cinerea* for pyraclostrobin and boscalid were determined based on the inhibition of spore germination for both fungicides and mycelial growth inhibition for boscalid (Myresiotis *et al*., 2008).

Isolates of *A. alternata, B. cinerea* and *P. expansum*, resistant to pyraclostrobin have been found in pistachio and apple orchards, with a lesser degree of resistance to boscalid (Avenot and Michailides, 2007; Xiao and Boal, 2009; Kim and Xiao, 2010; Xiao and Boal, 2010; Kim and Xiao, 2011). *Botrytis cinerea* with resistance against pyraclostrobin and boscalid have been reported on grape and kiwi (Kim and Xiao, 2011). This is a concern as only one spray of Pristine® near harvest had been applied and resistance had occurred after only four consecutive years of application (Kim and Xiao, 2010; Yin *et al*., 2011).

A possible explanation for the rapid development of resistance is that both active ingredients inhibit single-sites which make them prone to resistance development in pathogens (Avenot and Michailides, 2007). Dual resistance occurred frequently among *B. cinerea* isolates obtained from commercial orchards sprayed with Pristine®, whereas all isolates obtained from these orchards before the use of Pristine® were sensitive to the fungicides pyraclostrobin and boscalid (Kim and Xiao, 2010; Kim and Xiao, 2011). This suggests that the use of Pristine® induced the development of resistance against these two fungicides through selection pressure (Kim and Xiao, 2010). Cross resistance between pyraclostrobin and boscalid does not occur confirming different sites of action of these fungicides (Avenot *et al*., 2008).

1.9.2. Sanitation

There are different strategies for using sanitation as a control practice against plant pathogens. Removing inoculum sources reduces the density and incidence of fungi. Sanitation practices used involve clearing the orchard floor of leaf litter, cuttings, decayed fruit and mummified fruit. Leaf litter can be shredded to powder and incorporated into a compost mixture to eradicate fungal spores (Holb, 2006 and 2008). Other sanitation practices

include adding urea to leaf litter, using plastic to cover to orchard floor and removing infected stems and twigs through winter pruning (Holb, 2006 and 2008). These sanitation practices have worked very well in reducing apple scab, *Venturia inaequalis* (Cooke) Wint. incidence in orchards (Holb, 2006 and 2008).

Sanitation practices also apply to the packing lines at the pack houses. Sanitizing the dip suspension is an important part of keeping inoculum density low. Removing any decayed fruit from the packing line, sanitizing the bins after use and disinfecting the flume water keep the inoculum density low (Spotts *et al*., 1988a). There are no specific sanitation practices for core rot in apples, but possible practices for dry core rot and mouldy core is removing the alternative hosts, weeds and debris in the orchards as well as the mummified fruit and this might lower the inoculum incidence. Wet core rot incidence occurring in the pack house can be lowered by removing heavily infected fruit before the fruit is dipped in the flume water. This will prevent an inoculum build-up in the water.

1.10. Conclusion

Core rot of apples is caused by several fungi of which the main causal pathogens of dry core rot are *A. alternata* and *A. tenuissima* and for wet core rot, *P. expansum* and *P. funicolosum.* Wet core rot has been established as both a pre- and post-harvest disease. Pre-harvest WCR has very low incidence of infected fruit in the orchard. While it might not be worthwhile to try and control it, we do not have enough information at this time to quantify if there is a relationship between pre- and post-harvest WCR incidence. Dry core rot and WCR are economically more important than MC because the tissue of the fruit is affected. Either DCR or WCR can be more prevalent in a season depending on the amount of infected fruit reaching the consumer. The epidemiology and etiology of core rot disease complex is still very unclear, and much research is needed on the subject. Open calyx cultivars are still some of the most popular cultivars planted in South Africa and in the rest of the world although there has been a decrease in ‘Starking’ and ‘Top Red’ cultivar areas planted per hectare in South Africa (Anonymous, 2011a). Other open calyx cultivars such as ‘Idared’ and ‘Fuji’ have increased in area per hectare planted in South Africa and throughout the world. Over a million cartons of ‘Top Red’ and ‘Fuji’ and approximately 300 000 cartons of ‘Starking’ are exported to overseas markets from South Africa (Anonymous, 2011a). Currently, no research has been recorded for breeding specifically for closed calyx cultivars although most new varieties have a closed calyx (Anonymous, 2011a). New varieties are not as susceptible to core rot yet most South African consumers still prefer open calyx cultivars, such as

‘Starking’, ‘Fuji’ and ‘Top Red’ (Anonymous, 2011a). New techniques to identify core rot symptoms are important to the industry especially if the technique is non-destructive. Shenderey *et al*. (2010) research reprted on a non-destructive method using near-infrared spectroscopy (NIRS) to identify ‘Red Delicious’ apples infected with mouldy core and core rot symptoms. Although this is a new technique it shows promising results as it can be used to distinguish between diseased and healthy fruit (Shenderey *et al*., 2010). This makes it possible to remove decayed fruit before storage and export.

The study will focus on verifying the identity of the different fungi causing core rot symptoms as well as identifying the most important inoculum sources of these core rot organisms. Research will be undertaken on pre-harvest WCR, to establish whether this symptom has any affect on post-harvest WCR incidence. In conclusion, fungicide trials with Bellis® fungicide will establish it’s ability to reduce the development of core rot in apples.

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Table 1: FRAC table of fungicides mentioned in literature to control core rot of apples.

Trade Name	Active ingredient	Chemical class	Concentration	FRAC group	FRAC #	Mode of action
Bellis® / Pristine®	pyraclostrobin	Strobilurin	12.8% w/w	C3	11	Penetrant
	boscalid	pyridine carboxamide	28.2% w/w	C2	7	Contact
Captan	captan	phthalimides	500 g/L	M4		Contact
	dodine	guanidines		M7		Contact
Rovral	iprodione	dicarboximide	255 g/L	E3	2	Contact
Dithane	mancozeb	dithiocarbanate	750 g/kg	M3		Contact
	B-aminobutyric acid	plant defense activator				Contact
	potassium phosphates	Inorganic compound				Systemic
Score	difenoconazole	azole	250 g/L	G1	3	Systemic
	polyoxin B	polyoxins		H4	19	Contact
Flint	trifloxystrobin	Strobilurin	500 g/kg	C3	11	Penetrant
Granit	bromuconazole	triazole	200 g/L	G1	3	Penetrant
Signum	pyraclostrobin	Strobilurin		C3	11	Penetrant
Signum	nicobifen	pyridine carboxamide		C2	7	Contact
Captan	captab	phthalimides	500 g/L	M4		Contact
Folicur	tebuconazole	triazole	250 g/L	G1	3	Systemic
Euparen Sulphur Dust	dichlofluanid	sulphamides	980 g/kg	M		Contact
Omega	procloraz	Imidazole	450 g/L	G1	3	Penetrant
Chlorine dioxide	chlorine dioxide	Inorganic compound	20 g/L			Contact
Sporekill	dimethyldidecylammonium chloride	Quarternary ammonium compound	120 g/L			Contact
Benomyl	benomyl	benzimidazole	500 g/kg	B1	1	Systemic

Chapter 2: Incidence, epidemiology and control of apple core rot in the Western Cape, South Africa

2.1 Abstract

Core rot is a major contributor to postharvest apple losses in South Africa, contributing to losses of 5 to 12% annually. Disease symptoms can manifest in three forms namely: dry core rot, wet core rot and mouldy core. A variety of causal organisms are responsible for core rot, with *Alternaria* and *Penicillium* being the most frequently isolated from symptomatic fruit. In previous studies, *Tarsonemus* mites were found in the core region of susceptible red cultivars. The aims of the study were firstly: to identify the incidence of core rot decay in susceptible apple orchards through isolations from symptomatic fruit and identification of fungi isolated; secondly: to determine the main inoculum sources of apple core rot pathogens in South African apple orchards through evaluation from air sampling and laboratory evalution of the mummies and mites; and lastly: to determine whether adequate control of core rot causal organisms can be achieved through orchard spray applications of the fungicide, Bellis® and laboratory evaluation through isolations from symptomatic fruit and identification of fungi isolated.

Results from this study indicated a total decay incidence of core rot of between 1.8 to 22.1% for Ceres and 9.5 to 9.7% for the Vyeboom region. The evaluation of the incidence of core rot was higher pre-harvest than in previous studies. *Alternaria* and *Penicillium* were the two most frequently isolated fungi in relation to other fungi found. A high incidence of *Penicillium* species were observed from the mummy washing plates. Other fungi identified were *Trichoderma* and *Verticillium* species. *Penicillium* and *Trichoderma* species were observed from the mite washings, whereas *Alternaria, Penicillium, Fusarium, Cladosporium* and *Botrytis* species were identified in the air samples. These fungi have been previously isolated from apples showing core rot symptoms. A significant difference (P = 0.010) was observed between the treatments of the fungicide Bellis® with the treated fruit having a higher incidence of core rot symptoms than the control fruit.

The incidence of pre-harvest core rot decay differed from that found in previous studies, being higher than previously described, with *Alternaria* and *Penicillium* spp. representing the most frequently isolated fungi. *Alternaria* was not found on mummies or on *Tarsonemus* mites but was found in the air. This study has the potential to pave the way for

future research into orchard sanitation and integrated disease management programs for the control of core rot.

2.2. Introduction

Core rot of apples is a descriptive name given to indicate decay within the apple core. Symptoms include mouldy core (MC), dry core rot (DCR) and wet core rot (WCR) (Carpenter, 1942; Combrink and Ginsburg, 1973) (Fig. 1). Cultivars with an open calyx-end, such as 'Red Delicious' (chance seedling), 'Starking' (sport of 'Red Delicious'), 'Gravenstein' (chance seedling) and 'Idared' ('Jonathan' x 'Wagener') are more susceptible to core rot causing organisms than cultivars with closed calyx-ends (Miller, 1959; Spotts, 1990). The causal organism most frequently isolated from MC and DCR is *Alternaria* Nees species, whereas *Penicillium* Link species are frequently isolated from apples with WCR symptoms (Combrink and Ginsburg, 1973).

Globally, to date, the incidence of core rot has been reported to be between 4 and 15% (Raina *et al*., 1971; Combrink and Ginsburg, 1973; Ellis and Barrat, 1983; De Kock *et al*., 1991; Reuveni *et al.*, 2002). The United States has recorded incidences of between 0 to 16% for mouldy core (Michailides *et al*., 1994), with some orchards recording very high incidences of core rot between 38 and 66% (Ellis and Barrat, 1983). India has recorded an average of 10% incidence (Raina *et al*., 1971), with Israel having recorded 4 to 15% incidence (Reuveni *et al.*, 2002). South Africa's recorded incidence for core rot is between 4 and 12% (Combrink and Ginsburg, 1973; De Kock *et al*., 1991).

Inoculum sources are important factors in the epidemiology of plant diseases. These sources protect and give nutrition to pathogens during unfavourable environmental conditions, and when susceptible host tissue is not available. These sources can be alternative hosts or weeds, or debris from the orchard such as leaf litter, grasses, prunings, dropped fruit, mummified fruit on the ground as well as in trees (Rotem, 1994; Spotts and Serdani, 2006).

Many devastating epidemics, for example, potato late blight and chestnut blight have been caused through airborne inoculum (West *et al*., 2008). Conidia can be small and light and can travel on wind currents and be dispersed onto susceptible hosts (Coertze and Holz, 1999). Core rot causing organisms, *Alternaria* and *Penicillium* (Combrink *et al*., 1985) can also cause various other diseases in different hosts. Both *Alternaria* and *Penicillium* have been noted to spread through air dispersal (Rotem, 1994; Spotts and Cervantes, 2001; Lennox *et al*., 2003). The dark pigmentation of *Alternaria* conidia protects the fungus against radiation (Gregory, 1973). *Penicillium* species have been found in the air in orchards and in

pack houses (Spotts and Cervantes, 2001; Lennox *et al*., 2003). Very few conidia of *Penicillium* are necessary for significant decay levels (Spotts and Cervantes, 2001). Other core rot causing organisms that are airborne are *Botrytis* Micheli ex Pers. and *Cladosporium* Link (Lennox *et al*., 2003; Serdani and Spotts, 2006; West *et al*., 2008).

Natural as well as mechanical thinning results in fruit dropping onto the orchard floor, where it shrivels and mummifies as the season progresses (Hong *et al*., 1997; Cox and Scherm, 2001; Holb and Scherm, 2007). The mummies serve as protection and a source of nutrition for fungi that need to overwinter or where environmental conditions are unfavourable for infection (Holb and Scherm, 2007). Mummies are the primary inoculum source for various diseases such as *Monilinia* Honey species on stone fruit (Landgraf and Zehr, 1982; Hong *et al*., 2000), *Botrytis cinerea* Pers. ex Nocca and Balbis overwintering on blackberries, as primary cause of Gray mould in pears (Spotts and Serdani, 2006) and *Botryosphaeria dothidea* (Moug. ex Fr.) Ces and de Not. in apple orchards (Sutton, 1981). *Penicillium* and *Alternaria* have been recovered during orchard surveys on mummies (Hong *et al*., 2000). Combrink *et al*. (1994) isolated *Penicillium* and *Alternaria* from mummies found in South African orchards.

Van der Walt (2009) observed mites in and on apple mummies in South African orchards, and hypothesised that these mites vector the core rot causing fungi into the apple core region. The mites were identified as *Tarsonemus* Canestrini & Fanzago species from the family Tarsonemidae (Lindquist, 1986). Michailides *et al*. (1994) were the first to report *Tarsonemus* mites collected from apples showing the core rot symptom, mouldy core. The *Tarsonemus* species collected during their trials were identified as *T. confusus* Ewing (Michailides *et al*., 1994). The South African *Tarsonemus* mites collected were identified as *T. waitei* Banks and two new putative species with a close similarity to *T. mixtus* Kaliszewski and *T. bilobatus* Suski (Van der Walt *et al*., 2011) were reported. *Tarsonemus* species are known as fungi-eating (fungivorous) mites and can vector fungal conidia in specialised ‘sacs’ called sporothecae (Lindquist, 1986; Lombardero *et al*., 2003; Pernek *et al*., 2008). The mite carries the conidia into the open calyx of susceptible fruit, such as ‘Red Delicious’ where it disperses the conidia into the core (Michailides *et al*., 1994). The mites also create wounds, which enhances the infection of the fungus (Michailides *et al*., 1994).

Acceptable control of core rot has been achieved through foliar sprays of different fungicides e.g. benomyl, captan, dodine, iprodione, and mancozeb (Ellis and Barrat, 1983; Combrink *et al*., 1985). Reuveni and associates have experimented with various fungicides to

control *Alternaria* species from apple core rot including trifloxystrobin, azoxystrobin, difenoconozole, potassium phosphate and a mixture of DMI fungicides together with captan (Reuveni and Sheglov, 2002; Reuveni *et al*., 2003; Reuveni and Prusky, 2007).

Currently, there are no fungicides specifically registered for the control or management of core rot of apples in South Africa. The fungicide, Bellis®, registered as Pristine® in the United States since 2005, is a pre-mixture fungicide consisting of pyraclostrobin and boscalid. It is registered as a broad spectrum fungicide for the control of storage rots, caused by fungi such as *Gloeosporium* Desm. & Mont., *Botrytis*, *Alternaria* and *Penicillium* as well as against powdery mildew (*Uncinula necator* (Schw.) Burr.) (Anonymous, 2011b). The fungicide's host range includes pome fruit, stone fruit, grapes and berries (Schnabel *et al*., 2004; Sallato *et al*., 2007; Serey *et al*., 2007; Anonymous, 2011b). Although registered in the United States, Bellis® is not registered for storage rots in South Africa (van Zyl, 2011). In South Africa, Bellis® is registered on tomatoes and potatoes against early blight (van Zyl, 2011).

Apples produced in South Africa are either, exported to Europe and Asia, sold locally, or used for juicing. In 2011, South Africa produced 766 622 tons of apples, with 231 285 tons going to the local market and 318 993 tons being exported (Anonymous, 2011a). Core rot is an important disease to South African producers as apples exported from South Africa to our overseas markets carry a zero percent tolerance of the disease in red apple cultivars (personal communication with M. Reineke). Thus knowledge of the degree of disease incidence, present in commercial orchards, and from where the causal organisms infects the fruit is necessary to be able to improve management of the disease.

In this chapter, the incidence of core rot in commercial apple orchards and the causal organisms from the symptomatic tissue is determined. The objectives of the experiments described in this chapter were to 1, determine the incidence of core rot in apples from commercial orchards both pre- and post-harvest; 2, to identify the causal organisms associated with core rot symptoms; 3, to identify potential sources of inoculum of core rot pathogens and determine whether there is synergism between *Alternaria* and *Tarsonemus* mites associated with core rot; and 4, to determine whether the fungicide Bellis®, used as a full bloom application, can be used to manage core rot in South Africa.

2.3. Materials and methods

2.3.1. Decay incidence of core rot in Starking's

2.3.1.1. Pre-harvest collection of fruit

Three commercial farms, (CA, CB and CC) with histories of high incidence of core rot, were selected in the Witzenberg Valley, Ceres, South Africa. Pre-harvest collection was made in March, two weeks before commercial harvest, in 2009 and 2010, from each farm. In 2009, healthy-looking fallen fruit were collected from the orchard floor. Ten trees were used as replicates per orchard with twenty fruit collected from underneath each tree. In 2010, the replicates were increased from ten to 40 trees for each orchard. Two commercial farms (VA and VB) in Vyeboom, South Africa were included in the trial.

2.3.1.2. Post-harvest collection of fruit

In 2009, fruit from the three farms in Ceres were commercially harvested early in April. The harvested fruit from two of the farms was stored at a local commercial pack house under regular atmosphere for four to five months. Fruit from the third farm was stored under controlled atmosphere for seven months. In 2010, the fruit was harvested in the middle of April and stored under regular atmosphere for six months. The fruit was treated with diphenylamine (DPA, Chempak, Paarl, RSA), YieldPlus® (*Cryptococcus albidus*, Anchor Yeast, Cape Town, RSA) and iprodione (Rovral®, Bayer, Isando, RSA). After storage, bins were taken from the rooms and ten replicates of 100 fruit were selected each from the Ceres (2009 and 2010) and Vyeboom farms (2010).

2.3.1.3. Isolations and identification of causal organisms

Both pre- and post-harvest fruit were examined in the Post Harvest Laboratory, Department of Plant Pathology, Stellenbosch University, South Africa. Fruits were surface sterilized with 70% ethanol for 1 min and allowed to air dry. The surface sterilized fruit were cut in half longitudinally and evaluated for core rot symptoms, including MC, DCR and WCR. Isolations were made from symptomatic core tissue and from the edge of symptomatic mesoderm tissue, by excising small portions of the tissue and transferring it onto potato dextrose agar (PDA, Becton Dickinson and Company, Sparks, MD, USA). The plates were incubated at room temperature (22°C) for seven days before being examined for fungal growth.

2.3.2. Morphological identification of causal organisms

Fungi isolated from affected tissue and grown on PDA were identified morphologically. Morphological identification was done using keys and line drawings of known core rot causing organisms (Barnett and Hunter, 1998) and identified to genus level.

2.3.3. Inoculum Sources

2.3.3.1. Air inoculum collection

Using a portable air sampler (Burkard Manufacturing Co Ltd., Hertfordshire, UK, Patent No. 8819423.8), conidia density in the air was determined in 2009 and 2010. The Burkard portable air sampler is a volumetric air quality monitor, capturing spores onto PDA plates. The manufacturer rates the impaction efficiency of this sampler at 100% for 5µm particles at the 20 litres/min sampling rate. Samples were taken from the designated three orchards (CA, CB and CC) in Ceres, with inoculum being collected onto PDA plates.

Air samples were collected at ten sites per orchard for two time intervals (1 and 3 min), with each site replicated three times. The plates were incubated for seven days at room temperature, and fungal colonies sub-cultured onto PDA before being identified morphologically.

2.3.3.2. Collection of mummies and mites

Mummies were collected from the same designated orchards where air samples were taken in 2009 and 2010. Following a modified protocol of Hong *et al.* (2000), sampling was done at ten sites per orchard, with one tree representing each site, and ten mummies being collected for each site. Mummies were transported to the laboratory in brown paper bags sealed in plastic bags.

Mites were retrieved from collected mummies with a thin needle and placed in 1ml sterile distilled water (SDW). Glass beads were placed in the SDW solution containing mites and vortexed for 1 min. One hundred microliter of the mite's washing water was plated out onto each of three PDA plates. Plates were incubated at room temperature for 7 days after which fungi colonies were morphologically identified.

2.3.3.3. Assessment of mummy and mite samples

In the laboratory, each mummy was placed in 10 ml of SDW with a drop of Tween 80 / litre of water. The mummy was shaken for 10 minutes at 160 rpm on a rotary shaker, and then sonicated for 10 min (Spotts and Serdani, 2006). Fungi in each mummy's conidial

washing solution were identified and counted using a haemocytometer. One hundred microliters of each of the mummy's washing solution (1:100) was spread onto each of three PDA plates. Plates were incubated at room temperature for 7 days after which fungi colonies present were morphologically identified.

2.3.4. Assessment of control using the fungicide, Bellis®

The efficacy of the fungicide, Bellis® (WG, 12.8% w/w pyraclostrobin and 25.2% w/w boscalid, BASF, Cheadle, UK) was evaluated in a single season's trial in 2008. Three designated Starking cultivar apple orchards (A, B and C) located in the Witzenberg Valley, Ceres were treated with the fungicide (0.8 kg/ha; 1010 litre/ha) at three spray application intervals (10% and 80% bloom and at petal fall) from end of October to mid November 2008. Untreated Starking orchard blocks adjacent to each of the treated orchards served as controls.

2.3.4.1. Fruit collection pre-harvest

In March 2009, two weeks before commercial harvest, dropped fruit beneath ten treated and ten control trees were collected following a randomized block design for each of the three orchards. The fruit that had fallen were split into two groups, fallen fruit that were visibly decayed and fallen fruit that were visually healthy.

Of the visually healthy fruit, twenty fruit were sampled, selecting the twenty biggest fruit underneath the tree. The biggest apples were sampled because of the reported correlation between the size of the apple and it's susceptibility to core rot (Harrison, 1935). This was done for all the replicates. Both the experimental treatment and the control treatment fruit were sampled in the same manner.

2.3.4.2. Fruit collection post-harvest

Fruit were harvested in orchard A, B and C in the beginning of April 2009. The fruit from orchard B and C were stored at regular atmosphere (RA, -0.5°C) whilst fruit from orchard A were stored under controlled atmosphere (CA, -0.5°C, 2.5% CO_2 and 1.5% O_2). The fruit from orchard B and C were kept in RA for four months, with orchard A's fruit retrieved after seven months in CA. Post-harvest treatment of the fruit was 1-MCP applied through fogging the storage rooms. Bins from each farm were taken out after storage and ten replicates of 100 fruit were sampled.

2.3.4.3. Pre-and Post-harvest fruit assessment for core rot

The fruit collected from pre- and post-harvest sampling were cut in half using a knife sterilized after each fruit was cut. The fruit were assessed either as healthy, with mouldy core, wet core rot or dry core rot symptoms.

Isolations were made from fruit with mouldy core, wet core rot or dry core rot symptoms and plated onto PDA amended with streptomycin. The isolates were grown for a week at room temperature where after it was identified using morphological characteristics.

2.3.5. Statistical analysis

Disease incidence and inoculum sources for decay incidence, as well as disease incidence and causal organisms from Bellis® fungicide treatments, were subjected to appropriate analysis of variance (ANOVA). Fisher's LSD was calculated to identify significant differences in disease incidence parameters and inoculum sources for decay incidence experiments, as well as for disease incidence parameters and causal organisms for Bellis fungicide treatments. A confidence interval of 95% was used. To establish whether the data was normally distributed, the chi-square of the data was analysed. Due to a high kurtosis value the data was not normally distributed and adjustments to the data had to be made. The data was transformed using logit transformation formula l=log((g+0.5)/(n-g+0.5)) (Snedecor and Cochran, 1967). All statistical analysis was done using statistical analysis software Addinsoft XLSTAT Version 2011.2.06 (www.xlstat.com).

2.4. Results

2.4.1. Morphological identification of isolates

The isolates collected from pre- and post-harvest collections, inoculum sources and the pre- and post-harvest collections from the Bellis® treated and untreated fruit were identified to genus level through microscopy. These isolates included *Alternaria, Penicillium, Fusarium, Cladosporium, Epicoccum, Ulocladium, Stemphylium, Phoma, Botryosphaeria, Botrytis, Trichoderma, Verticillium, Paecilomyces* and *Gliocladium.*

2.4.2. Decay incidence and causal organisms

2.4.2.1. Decay Incidence – Ceres orchards

The total decay incidence of core rot was determined by analysis of variance (ANOVA) which indicated a significant three factor interaction between year, farm and pre-

or post-harvest collection on the total decay incidence of core rot (P <0.0001) (Table 1). Significant interactions were also observed between farms and pre- or post-harvest collections (P<0.0001) and between the different year and pre- or post-harvest collections (P<0.0001). The total incidence of core rot pre-harvest was between 1.8% and 7.1% and post-harvest was between 11.9% and 22.1% (data not shown).

The total decay was separated into each type of decay and the four factor interaction between year, farm, pre- and post-harvest collection and type of decay was significantly (P = 0.040) indicated with ANOVA (Table 2). Significant interactions were also observed between the different farm, pre- and post-harvest collections and types of decay (P <0.0001), between the different years, farms and pre- and post-harvest collections (P = 0.003), between the different pre- and post-harvest collections and types of decay (P<0.0001), between the different farms and pre- and post-harvest collections (P<0.0001), between the different years and types of decay (P<0.0001) and between the different years and farms (P = 0.002).

Interactions between the year, farm, pre- and post-harvest collection and type of decay revealed significant differences (Table 3). The incidence of the total decay was between 1.8 and 7.1% pre-harvest (mean of 4.6%) and between 11.9 and 22.1% post-harvest (mean of 14.7%) (Table 3). Mean relative recovery for MC was between 3.0 and 16.4% (mean of 8.4%), DCR was between 2.1 and 15.5% (mean of 6.6%) and WCR was between 0.4 and 12.5% (mean of 4.4%).

2.4.2.2. Causal Organisms – Ceres Orchards

Analysis of variance indicated a significant four factor interaction between farm, pre- and post-harvest, type of decay and causal organisms on the incidence of core rot for 2009 (P=0.036) (Table 4). Significant interactions were also observed between the different pre- and post-harvest collections, types of decay and causal organisms (P<0.0001), between the different farms, pre- and post-harvest collections and causal organisms (P=0.007), between the different farms, pre- and post-harvest collections and types of decay (P=0.005), between the different types of decay and causal organisms (P<0.0001), between the different pre- and post-harvest collections and causal organisms (P<0.0001), between the different pre- and post-harvest collections and types of decay (P<0.0001) and between the different farms and causal organisms (P<0.0001).

Analysis of variance for the influence of farm, pre- and post-harvest, type of decay and causal organisms on the incidence of core rot for 2010 indicated a significant three factor interaction between pre- and post-harvest collections, types of decay and causal organisms

(P<0.0001) (Table 4). Significant interactions were also observed between the different farms, pre- and post-harvest collections and causal organisms (P<0.0001), between the different farms, pre- and post-harvest collections and types of decay (P=0.002), between the different types of decay and causal organisms (P<0.0001), between the different pre- and post-harvest collections and causal organisms (P=0.000), between the different pre- and post-harvest collections and types of decay (P<0.0001) and between the different farms and pre- and post-harvest collections (P<0.0001).

Significant interaction between the farms, pre- and post-harvest collections, types of decay and causal organisms (P=0.036) was observed for 2009, but no significant difference was observed for 2010 (Table 4). The symptom with the highest mean incidence of causal organisms was MC (13.636% and 9.797%), followed by DCR (6.192% and 9.410%) and WCR (4.339% and 4.438%) for the seasons 2009 and 2010 (Table 5).

The causal organisms that occurred more frequently in the orchards were *Alternaria*, *Penicillium* and *Fusarium* as well as other causal organisms which were grouped together. In 2009 the causal organisms recovered from symptomatic tissue were as follows: *Alternaria* had the highest mean recovery (16.8%), followed by *Penicillium* (10.8%), Other (2.8%) and *Fusarium* (1.8%) (Table 5). In 2010 similar results were recovered with the highest mean recovery from *Alternaria* (18.8%) followed by the combined other organisms (6.5%), *Penicillium* (5.2%) and *Fusarium* (1.1%) (Table 5).

2.4.2.3. Decay Incidence – Vyeboom Orchards

Analysis of variance indicated no significant interactions between farm and pre- and post-harvest collection on the total decay incidence of core rot (P=0.696) (Table 6). A significant difference was observed between the pre- and post-harvest collections. The analysis of variance for the influence of the farm, pre- and post-harvest collection and type of decay on the incidence of core rot indicated significant interactions between the farms, pre- and post-harvest collections and the types of decay (P=0.045) (Table 7). Significant interactions were also observed between the different pre- and post-harvest collections and types of decay (P<0.0001) and between the different farms and types of decay (P<0.0001).

Significant interactions between the farms, pre- and post-harvest collections and types of decay were obtained (Table 7). The mean relative recovery for MC was between 8.5 and 9.3% (mean of 8.9%), DCR was between 4.7 and 8.0% (mean of 6.2%) and WCR was between 0.6 and 4.8% (mean of 1.7%) (Table 8).

2.4.2.4. Causal Organisms – Vyeboom Orchards

Analysis of variance indicated a significant four factor interaction between farm, pre- and post-harvest collection, type of decay and causal organisms on the incidence of causal organisms (P=0.010) (Table 9). Significant interactions were also observed between the different pre- and post-harvest collections, types of decay and causal organisms (P=0.001), between the types of decay and causal organisms (P<0.0001), between the different pre- and post-harvest collections and causal organisms (P<0.0001), between the different pre- and post-harvest collections and types of decay (P<0.0001), between the different farms and causal organisms (P=0.000) and between the different farms and types of decay (P<=0.003) (Table 9).

The symptom with the highest mean incidence of causal organisms was MC (11.2%) followed by DCR (10.4%) and WCR (3.1%) (Table 10). *Alternaria* was the organism with the highest relative mean recovery (17.3%), followed by the combined other causal organisms (10.0%), *Penicillium* (4.8%) and *Fusarium* (0.8%) (Table 10).

2.4.3. Inoculum Sources

Analysis of variance indicated a significant four factor interaction between year, farm, inoculum source and causal organism on the incidence of causal organisms (P=0.002) (Table 11). Significant interactions were also observed between the different years, inoculum sources and causal organisms (P<0.0001), between the different years, farms and causal organisms (P=0.013), between the different years, farms and inoculum sources (P<0.0001), between the different inoculum sources and causal organisms (P<0.0001), between the different farms and causal organisms (P=0.013), between the different farms and inoculum sources (P<0.0001), between the different years and causal organisms (P=0.002), between the different years and inoculum sources (P<0.0001), and between the different years and farms (P<0.0001).

The source with the highest mean incidence of causal organisms was air (25.0%), followed by mummies (23.5%) and then mites (10.5%) (Table 12). *Penicillium* had the highest mean relative recovery of causal organisms from the inoculum (43.2%), followed by the other causal organisms (19.7%), *Alternaria* (13.4%) and *Fusarium* (1.1%) (Table 12). *Alternaria* occurred only in samples from the inoculum source, air, whereas *Penicillium* occurred on all the inoculum sources (Table 12).

2.4.4. Decay and Causal organism incidence of fungicidal trial with fruit treated and not treated with Bellis®

2.4.4.1. Decay Incidence

Analysis of variance indicated a significant four factor interaction between farm, pre- and post-harvest collection, treatment and type of decay on the incidence of core rot (P=0.007) (Table 13). Significant interactions were also observed between the different farms, pre- and post-harvest collections and types of decay (P=0.000), between the different pre- and post-harvest collections and types of decay (P<0.0001), between the different farms and types of decay (P=0.010) and between the different farms and pre- and post-harvest collections (P<0.0001). There was also a significant difference observed between the treatments (P=0.010) (Table 13). The symptom with the highest mean decay incidence of fruit treated and untreated with the fungicide Bellis® was MC (9.5%), followed by DCR (5.3%) and WCR (3.9%) (Table 14).

2.4.4.2. Causal Organisms

Analysis of variance indicated a significant two factor interaction between the different types of decay and causal organisms (P<0.0001) (Table 15). Significant interactions were also observed between the different farms and causal organisms (P=0.005), between the different farms and types of decay (P=0.040) (Table 15).

Analysis of variance indicated a significant four factor between farm, treatment, type of decay and causal organisms on the incidence of core rot for post-harvest collections (P=0.033) (Table 15). Significant differences were also observed between the different farms, types of decay and causal organisms (P=0.046), between the different farms, treatments and types of decay (P=0.050), between the different types of decay and causal organisms (P<0.0001), between the different farms and causal organisms (P=0.012), between the different farms and types of decay (P=0.000) and between the different farms and treatments (P=0.012) (Table 15).

Significant interaction between the farms, treatments, types of decay and causal organism for post-harvest collections were observed, with no significant interactions observed for pre-harvest collection (Table 15). The symptom with the highest mean incidence of causal organisms was MC (9.4% and 16.6%), followed by DCR (9.2% and 5.1%) and WCR (5.2% and 3.2%) for pre- and post-harvest collection (Table 15). *Alternaria* was the organism with the highest relative mean recovery from both pre- and post-harvest collections

(12.6% and 22.7%), followed by *Penicillium* (12.5% and 7.5%), the collection of other causal organisms (3.3% and 2.8%) and *Fusarium* (3.3% and 0.3%) (Table 16).

2.5. Discussion

Core rot is a disease that occurs world-wide on susceptible apple cultivars. Although not seen as an economically important disease (Serdani *et al*., 2002), as it has an incidence of less than 5%, it has the potential to become an epidemic. Orchards with high incidence of core rot run the risk of fruit not being allowed to be exported or used on the local markets and instead will have to be processed, which in turn will result in economic losses.

For the season 2011 the estimated value for fruit destined for export from South Africa was R6210/ton, locally marketed fruit R4326/ton and processed fruit R736/ton (Anonymous, 2011a). The average amount of fruit harvested per orchard of 'Starking' is 42 tons/ha. Core rot is an undetected disease until processed or eaten by the consumer. This makes it impossible to know the true extent of the disease in an orchard.

Estimated calculations are made by sub-samples as was done in this trial's data collection. If an orchard has more than 10% core rot the fruit is classed as substandard and will be sent to be processed. The difference between exporting fruit and sending the fruit to be processed is a tenfold loss of income to the producers.

In South Africa, core rot of apples are important post-harvest diseases and losses of between 5 and 12% occur (Combrink and Ginsburg, 1973; De Kock *et al*., 1991; Serdani *et al*., 1998). As previously mentioned international incidence of core rot varied between 4 and 15 percent (Raina *et al*., 1971; Combrink and Ginsburg, 1973; Ellis and Barrat, 1983; De Kock *et al*., 1991; Reuveni *et al*., 2002), with some orchards having incidences above 40 percent. A study done in South Africa for the seasons 2005/06 and 2006/07 showed pre-harvest incidence of 0 to 1.7% for WCR, 0.4 to 6% for DCR and 0 to 16% for MC from fruit sampled from the trees (Van der Walt, 2009). Compared to this study, where the pre-harvest incidence for WCR was 1.0 to 12.5%, DCR was 5.0 to 15.5% and MC was 3.0 to 16.4% for the Ceres farms and for the Vyeboom farms WCR was 0.9 to 4.8%, DCR was 4.7 to 8.0% and MC was 8.5 to 9.3%, the pre-harvest incidence of the previous study was considerably lower. The current study's pre-harvested fruit were sampled from the ground instead of picked from the trees (Van der Walt, 2009). The difference between the incidences for each core rot symptom for the two trials could be due to the area from where the fruit was sampled. Carpenter (1942) observed that fruit infected with core rot fungi dropped

prematurely, thus fruit from underneath the tree could possibly have a higher incidence than fruit still attached to the tree.

Significant interactions occurred for the year, farm, pre- and post-harvest collection and type of decay for the core rot incidence in Ceres orchards (P= 0.040). The significant three-factor interaction (farms x pre- and post-harvest collection x types of decay) in the analysis of disease incidence for both Ceres and Vyeboom is believed to be caused by the interactions between the different decay symptoms (MC, DCR and WCR) and the pre- and post-harvest collections. Although the farms occur in different areas (Ceres and Vyeboom) the interaction is the same for the types of decay and pre- and post-harvest collection.

Abiotic influences such as temperature, humidity and rainfall can increase incidence of core rot on the farms, especially if it established favourable conditions to cause disease. The influence between pre- and post-harvest collections and types of decay can be due to infections happening pre-harvest as well as post-harvest. It has been shown that apples treated for scald with diphenylamine (DPA), such as the fruit used in this study, had an increase chance of developing wet core rot post-harvest (Combrink and Ginsburg, 1973; Spotts *et al*., 1988a and b). Currently many pack houses use 1-methylcyclopropene (3.3% w/w 1-MCP, Smartfresh^{SM}, Rohm and Haas, Paris, France) as an inhibitor of ethylene and have moved away from using DPA (Kim and Xiao, 2008). Unfortunately, 1-MCP does not control disease and studies have shown an increase in incidence and development of decay (Janisiewicz *et al*., 2003; Leverentz *et al*., 2003; Kim and Xiao, 2008).

Core rot is caused by various pathogenic fungi, infecting the core region and developing as the season progresses (Combrink and Ginsburg, 1973; Ellis and Barrat, 1983). *Alternaria* spp. and *Penicillium* spp. are the most frequently isolated organisms from MC and DCR and from WCR (Combrink *et al*., 1985). The current trial looked at the causal organisms isolated from the symptomatic fruit. *Alternaria, Penicillium* and *Fusarium* were frequently isolated as well as other causal organisms, which included *Cladosporium, Botrytis* and *Verticillium.* The most frequently isolated causal organism for both pre- and post-harvest was *Alternaria,* followed by *Penicillium.* The disease complex varied from area to area and year to year. The results of this study concur with what has been published by Combrink *et al*. (1985), Ellis and Barrat (1983) and Spotts *et al*. (1988a) on the causal organisms isolated from core rot symptoms.

Inoculum from a pathogenic organism can infect when the pathogen comes in contact with a susceptible host (Agrios, 2005). Pathogens do not always come in contact immediately

with a susceptible host and need to find an alternative source from where they can infect when conditions are favourable. Potential inoculum sources for core rot are the buds on trees, pruning debris left in the orchard, mummified fruit and airborne conidia (Serdani *et al*., 1998; Van der Walt, 2009). Post-harvest inoculum can be found on infested packing bins and contaminated flumes (Combrink *et al*., 1985; Spotts *et al*., 1988a). The inoculum sources chosen for this trial are intermediary hosts for pathogens that can cause core rot.

Known pathogens that infect through the air are *Alternaria, Penicillium, Cladosporium* and *Botrytis* (Gregory, 1973; Spotts and Cervantes, 2001; Lennox *et al*., 2003; West *et al*., 2008). During the current trial various core rot causing organisms were collected using the portable air sampler. *Alternaria* had the highest incidence of the pathogenic organisms collected.

Fruit that drop prematurely (natural or mechanical thinning) can become a major source of inoculum if not removed from the orchard (Hong *et al*., 1997). These fruit become mummified as the season progresses and can serve as a source of food and protection for fungi in unfavourable conditions. Hong *et al*. (2000) did a survey of stone fruit mummies to establish what organisms occurred on and in these mummies. They found predominantly *Penicillium,* but also observed *Alternaria*, *Cladosporium* and *Botrytis* (Hong *et al*., 2000). The same causal organisms were isolated from apple mummies sampled from orchards with high core rot incidences.

Michailides *et al*. (1994) observed mites, later identified as *Tarsonemus confusus,* in the core region of apples. They hypothesised that the mites vectored the core rot causing fungi into the core region through the open calyx end. In South Africa the *Tarsonemus* mites were found in the core region of ‘Red Delicious’ apples and was associated with *Alternaria* and *Penicillium* (Van der Walt, 2009). Van der Walt *et al*. (2011) identified that these *Tarsonemus* mites are not the species *T. confusus* but *T. waitei* and two new putative species.

During the current study *Alternaria* was not observed in the washings of the mummies or from the mites collected. Haemocytometer counts show low colony forming units (CFU)/ml of *Alternaria* in the mummy and mite washings (data not shown). This could be due to the inhibiting effect of *Penicillium,* which occurred in high incidences in mummies and mites, and in low incidences in the air. *Fusarium* occurred in low incidences in the air and mites, whereas the other core rot causing organisms occurred with varying incidences in all the inoculum sources.

Previous research (Van der Walt, 2009) suggested that synergism between the *Tarsonemus* mites and *Alternaria* species occur. The current study's results show no synergism between the mites and *Alternaria*, but there is a possible connection between *Penicillium* and the mites. It is possible that the size of the conidia play a role in the vectoring of the fungi. It is also possible that the mites do vector *Alternaria* conidia, from the opening of the calyx end into the core, but not from the mummies. These results indicate the increased necessity to use integrated pest management (IPM), including practicing sanitation in removing mummies from the trees and the orchard floor.

Yearly increases in costs on managing plant diseases with chemical control, increases the demand for integrated pest management. Integrated pest management looks at every aspect of the disease cycle to spray more effectively and reduce the amount of fungicide spray applications.

Bellis® (12.8% w/w pyraclostrobin and 28.2% w/w boscalid) has been registered in the USA as a broad spectrum fungicide that controls storage rots and in South Africa it is registered on tomatoes and potatoes for early blight. Bellis® has also been used against pre- and post-harvest infection of *Botrytis cinerea* on table grapes (Serey *et al*., 2007), controlling strawberry rot (Sallato *et al*., 2007; Wedge *et al*., 2007) and controlling *Monilinia fructicola* (G. Winter) Honey that causes brown rot of stone fruit (Schnabel *et al*., 2004). Pyraclostrobin is a quinine outside inhibitor (QoI) fungicide with a mode of action as an inhibitor of mitochondrial respiration, blocking the electron transfer at the cytochrome complex (Reuveni, 2006; Anonymous, 2007). Boscalid is a pyridine carboxamide with a mode of action for the inhibition of the complex II targeting the succinate-dehydrogenase (Reuveni, 2006; Anonymous, 2007).

In the 2002 and 2003 season, Reuveni (2006) conducted an experiment using the pre-mixed fungicide, Signum® (BASF, Cheadle, UK), a mixture of pyraclostrobin (6.7% w/w) and boscalid (26.7% w/w). Reuveni's trials resulted in a decrease of mouldy core symptoms with a control of 45 to 60% on Starking apples. The results showed that mouldy core was controlled in inoculated fruit as well as in naturally infected fruit (Reuveni, 2006).

Reuveni (2006) only looked at the control of MC and found 30 to 40% of the apples were naturally infected with MC. Signum® at various concentrations reduced the number of fruit infected with MC between 45 and 80%. The current trial with Bellis® had a natural infection rate for MC of between 3.0 and 16.4% before harvest. The fruit treated with Bellis® had a MC incidence, which ranged between 4.6 and 18.0%. The fungicide treated fruit had a

significantly higher incidence of decay than the untreated control fruit. When comparing the current results with the results found by Reuveni (2006), control of core rot could not be managed during this trial with the fungicide Bellis®.

Reasons for this phenomenon may be climate orientated (although Israel and South Africa have similar climate areas) as well as human error where the fungicide was incorrectly applied. Recent research done with Pristine® on pistachio against *Alternaria* spp. and on apple against *Botrytis cinerea* shows a resistance build up against pyraclostrobin as well as to a lesser degree against boscalid (Avenot and Michailides, 2007; Kim and Xiao, 2010; Kim and Xiao, 2011). The dual resistance occurring within these isolates reduces the effect of the fungicide against the pathogens in the orchard (Kim and Xiao, 2010). A possibility for the resistance occurring within four consecutive seasons with only one to three sprays per season is the simultaneous spraying of other fungicides within the same FRAC group (Anonymous, 2007; Kim and Xiao, 2010).

The orchards used in this trial was sprayed with Flint® 50 WG (a.i. trifloxystrobin), Strobi® and Ardent® 50 SG (a.i. kresoxim-methyl), which are all part of the FRAC group 11 together with pyraclostrobin. Resistance studies were not conducted, but the possibility of resistance occurring in the orchards can be due to the same FRAC group fungicides sprayed during the same season.

The relevance of this study to the industry is of importance due to the high inoculum levels encountered. The knowledge of where the inoculum originates increases the possibility to manage the disease either through improved spray programmes or integrated management practices. This study has resulted in the confirmation of three inoculum sources that contain the causal organisms of core rot especially *Alternaria* and *Penicillium*, which are the main organisms isolated from the core rot symptoms.

The inadequate control of core rot with the fungicide, Bellis®, raises questions of possible resistance to the active ingredients of the fungicide. Future research can focus on whether the spray programme used to control the disease is adequate, including looking at the various fungicides used and if the spray programme is rotated to reduce the possibility of resistance. To reduce the high inoculum, management practices needs to be looked at, including the increasing removal of overwintering sources from the orchards.

In conclusion, the incidence of core rot for the seasons 2009 and 2010 was higher than anticipated. Pre-harvest WCR was confirmed during these trials, having a higher incidence than post-harvest WCR. Higher incidences of all the core rot symptoms was found pre-

harvest, indicating that the incidence of core rot post-harvest could be a lot higher if infected fruit do not fall prematurely. The possible inoculum sources, air, mummies and mites, were verified to spread and overwinter core rot causing organisms. Air is a source for various fungi, most commonly *Alternaria* and mummies and mites are sources for *Penicillium*. A possible synergism between *Alternaria* and the *Tarsonemus* mite as a vector was not found, but the mite did show possible synergism with *Penicillium*.

Knowing what the incidence of core rot is and from where infection may be caused, lays the foundation to controlling the disease. Bellis® was used as a possible fungicide control however a higher incidence of core rot symptoms was found in the treated fruit than in the untreated control fruit. Adequate control was not achieved with Bellis® as a possible fungicide against core rot.

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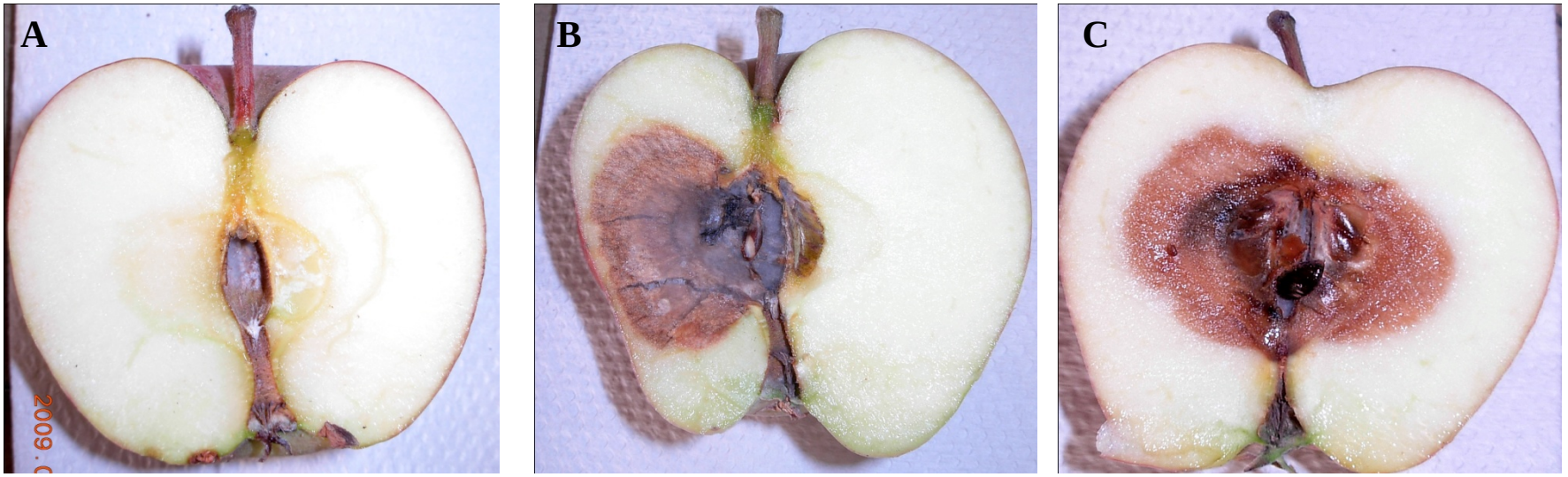

Figure 1: Core rot symptoms found in Starking apples a) mouldy core, b) dry core rot and c) wet core rot.

Table 1: Analysis of variance on the total decay incidence of core rot symptoms found on farms in the Witzenberg Valley, Ceres for the seasons 2009 and 2010.

Source	DF	Mean squares	F	Pr > F
Year (Y)	1	52.172	6.880	0.009
Farm (F)	2	207.433	27.356	< 0.0001
Pre/Post (P/P)	1	4333.416	571.477	< 0.0001
Y*F	2	10.943	1.443	0.239
Y*P/P	1	145.241	19.154	< 0.0001
F*P/P	2	81.208	10.709	< 0.0001
Y*F*P/P	2	151.149	19.933	< 0.0001
Error	198	7.583		
Corrected Total	209			

Table 2: Analysis of variance on the mean incidence[a] of core rot symptoms found on farms in the Witzenberg Valley, Ceres for the seasons 2009 and 2010.

Source	DF	Mean squares	F	Pr > F
Year (Y)	1	24.972	42.125	< 0.0001
Farm (F)	2	12.164	20.519	< 0.0001
Pre/Post (P/P)	1	51.465	86.815	< 0.0001
Types of Decay	2	18.463	31.145	< 0.0001
Y*F	2	3.802	6.413	0.002
Y*P/P	1	2.273	3.834	0.051
Y*TD	2	12.403	20.922	< 0.0001
F*P/P	2	7.163	12.084	< 0.0001
F*TD	4	0.678	1.144	0.335
P/P*TD	2	22.807	38.473	< 0.0001
Y*F*P/P	2	3.570	6.021	0.003
Y*F*TD	4	0.310	0.522	0.719
Y*P/P*TD	2	0.399	0.673	0.511
F*P/P*TD	4	4.133	6.971	< 0.0001
Y*F*P/P*TD	4	1.493	2.518	0.040
Error	594	0.593		
Corrected Total	629			

[a] The ANOVA was run on the logit transformed data to normally distribute the data.

Table 3: The mean[a] percentage incidence of core rot symptoms found during pre- and post-harvest collections from farms in the Witzenberg Valley, Ceres for the seasons 2009 and 2010.

	2009						2010					
	CA		CB		CC		CA		CB		CC	
	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post
MC	3.000 d-g	8.189 b-c	16.382 a	7.900 b-d	6.000 b-e	10.600 a-b	4.270 d-f	4.616 d-f	8.281 c-e	11.300 a-b	9.917 a-b	10.400 a-b
DCR	5.000 c-f	2.234 h-j	9.625 b-c	2.200 h-j	6.000 b-e	2.100 h-i	8.219 b-c	4.416 e-h	11.683 b	7.800 b-d	15.500 a	4.800 d-g
WCR	1.000 f-i	1.613 i-j	6.204 b-e	2.100 g-i	12.500 a-b	0.400 k	4.575 d-f	3.524 f-h	7.090 c-e	3.000 g-i	9.750 b	0.900 j-k

[a] Means indicated by different letters which differed at the 5% level according to Fischer's least significant difference (LSD) test. The t-values were gained from the LSD test run on the logit transformed data of the causal organisms.

Table 4: Analysis of variance on the mean incidence[a] of causal organisms obtained from core rot symptoms found on farms in the Witzenberg Valley, Ceres for the seasons 2009 and 2010.

	2009				2010			
Source	DF	Mean squares	F	Pr > F	DF	Mean squares	F	Pr > F
Farm (F)	2	10.388	18.106	< 0.0001	2	44.149	60.627	< 0.0001
Pre/Post (P/P)	1	60.192	104.915	< 0.0001	1	253.007	347.441	< 0.0001
Types of Decay (TD)	2	20.005	34.868	< 0.0001	2	15.088	20.720	< 0.0001
Causal Organism[b]	3	38.238	66.649	< 0.0001	3	69.941	96.046	< 0.0001
F*P/P	2	17.539	30.570	< 0.0001	2	10.028	13.771	< 0.0001
F*TD	4	0.074	0.130	0.972	4	0.658	0.904	0.461
F*CO	6	1.152	2.007	0.063	6	0.572	0.785	0.581
P/P*TD	2	8.924	15.554	< 0.0001	2	12.555	17.241	< 0.0001
Pre/Post*CO	3	8.835	15.399	< 0.0001	3	4.589	6.302	0.000
TD*CO	6	15.256	26.592	< 0.0001	6	19.927	27.365	< 0.0001
F*P/P*TD	4	2.147	3.742	0.005	4	3.101	4.259	0.002
F*P/P*CO	6	1.708	2.977	0.007	6	4.375	6.008	< 0.0001
F*TD*CO	12	0.798	1.391	0.165	12	0.627	0.861	0.587
P/P*TD*CO	6	3.210	5.596	< 0.0001	6	8.732	11.992	< 0.0001
F*P/P*TD*CO	12	1.067	1.860	0.036	12	0.890	1.222	0.261
Error	648	0.574			1728	0.728		
Corrected Total	719				1799			

[a] The ANOVA was run on the logit transformed data to normally distribute the data.

[b]The causal organisms consisted of *Alternaria, Penicillium, Fusarium* and Other. The group other was the combined data for the following fungi: *Cladosporium, Botrytis, Ulocladium, Stemphylium, Gliocladium, Paecilomyces, Verticillium, Epicoccum, Phoma, Botryosphaeria* and *Trichoderma*.

Table 5: The mean[a] percentage incidence of causal organisms from core rot symptoms found during pre- and post-harvest collections from farms in the Witzenberg Valley, Ceres for the seasons 2009 and 2010.

2009

	CA								CB								CC							
	Alternaria		*Penicillium*		*Fusarium*		Other		*Alternaria*		*Penicillium*		*Fusarium*		Other		*Alternaria*		*Penicillium*		*Fusarium*		Other	
	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post
MC	16.111 c-f	58.296 a	12.222 d-f	8.834 j-r	6.667 d-h	0.000 w	0.000 f-k	3.982 p-w	32.963 b-d	45.690 a-b	11.012 h-o	9.444 j-r	3.482 n-v	2.937 p-w	2.857 o-w	2.262 p-w	11.040 g-n	54.221 a	15.706 f-k	21.048 d-h	5.833 j-r	1.538 r-w	0.000 n-v	1.111 t-w
DCR	4.444 d-h	9.960 i-p	3.889 c-h	1.880 t-w	6.111 c-g	0.000 w	20.556 b-e	3.281 p-w	16.976 g-l	11.270 h-n	13.832 g-m	1.667 s-w	2.222 o-w	0.714 s-w	1.429 p-w	3.222 p-w	19.397 e-i	14.420 g-m	5.929 i-q	3.419 p-w	0.000 n-v	0.000 v-w	4.000 l-t	0.000 v-w
WCR	0.000 f-k	3.587 p-w	5.000 d-h	9.179 j-r	2.500 e-i	0.000 w	2.500 e-i	1.000 v-w	0.000 r-w	1.714 q-w	15.227 f-j	17.429 g-m	0.000 r-w	0.000 u-w	0.000 r-w	3.651 o-w	2.500 k-s	0.000 v-w	34.345 b-c	4.242 p-w	0.000 n-v	0.000 v-w	1.250 m-u	0.000 v-w

2010

	CA								CB								CC							
	Alternaria		*Penicillium*		*Fusarium*		Other		*Alternaria*		*Penicillium*		*Fusarium*		Other		*Alternaria*		*Penicillium*		*Fusarium*		Other	
	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post
MC	20.685 b-c	33.826 a-c	1.250 e-g	3.095 j-n	0.000 f-h	2.909 k-o	1.417 e-f	2.917 k-o	15.964 c	43.679 a-b	0.500 e-f	2.990 m-p	0.000 e-g	3.699 m-p	2.003 d-f	4.071 l-p	19.544 d-f	47.491 a	3.943 i-k	10.515 g-j	1.518 j-l	3.974 l-p	5.062 h-j	4.088 l-p
DCR	36.208 a	10.747 f-j	0.917 e-g	11.268 f-j	1.250 e-g	0.000 l-p	7.577 d-e	6.733 i-l	27.625 a-b	13.389 f-j	3.417 d-f	4.673 k-o	0.000 e-g	0.000 p	7.360 c-d	18.306 d-f	33.357 b-c	18.793 d-f	4.105 i-j	9.006 i-l	0.000 j-m	0.526 n-p	9.227 f-i	1.357 n-p
WCR	5.673 d-f	0.000 l-p	1.667 e-g	18.428 d-f	3.333 e-f	0.000 l-p	7.524 d-f	10.078 g-j	8.250 d-f	0.417 o-p	7.024 d-f	1.458 n-p	0.417 e-f	0.000 p	7.440 d-f	7.317 j-m	2.688 i-k	0.000 o-p	6.314 g-j	3.481 l-p	0.625 j-l	0.769 n-p	13.616 d-f	0.000 o-p

[a] Means indicated by different letters which differed at the 5% level according to Fischer's least significant difference (LSD) test. The t-values were gained from the LSD test run on the logit transformed data of the causal organisms.

Table 6: Analysis of variance on the total decay incidence of core rot symptoms found on farms in Vyeboom, Elgin for the season 2010.

Source	DF	Mean squares	F	Pr > F
Farm (F)	1	0.040	0.008	0.931
Pre/Post (P/P)	1	2304.000	436.131	< 0.0001
F*P/P	1	0.810	0.153	0.696
Error	96	5.283		
Corrected Total	99			

Table 7: Analysis of variance on the mean incidence[a] of core rot symptoms found on farms in Vyeboom, Elgin for the season 2010.

Source	DF	Mean squares	F	Pr > F
Farm (F)	1	0.014	0.027	0.869
Pre/Post (P/P)	1	12.171	23.252	< 0.0001
Types of Decay (TD)	2	32.539	62.166	< 0.0001
F*P/P	1	0.052	0.099	0.753
F*TD	2	6.553	12.520	< 0.0001
P/P*TD	2	11.845	22.629	< 0.0001
F*P/P*TD	2	1.640	3.134	0.045
Error	288	0.523		
Corrected Total	299			

[a] The ANOVA was run on the logit transformed data to normally distribute the data.

Table 8: The mean[a] percentage incidence of core rot symptoms found during pre- and post-harvest collections from farms in Vyeboom, Elgin for the season 2010.

	VA		**VB**	
	Pre	**Post**	**Pre**	**Post**
MC	8.507 a-b	8.700 a-b	9.263 a	9.000a-b
DCR	4.658 c	6.400 a-b	8.020 a-b	5.800 b-c
WCR	4.763 b-c	0.700 e	0.882 d	0.600 e

[a] Means indicated by different letters which differed at the 5% level according to Fischer's least significant difference (LSD) test. The t-values were gained from the LSD test run on the logit transformed data of the causal organisms.

Table 9: Analysis of variance on the mean incidence[a] of causal organisms obtained from core rot symptoms found on farms in Vyeboom, Elgin for the season 2010.

Source	DF	Mean squares	F	Pr > F
Farm (F)	1	0.673	1.072	0.301
Pre/Post (P/P)	1	145.123	231.186	< 0.0001
Types of Decay (TD)	2	18.368	29.260	< 0.0001
Causal Organism (CO)[b]	3	30.811	49.082	< 0.0001
F*P/P	1	0.560	0.892	0.345
F*TD	2	3.678	5.858	0.003
F*CO	3	3.759	5.988	0.000
P/P*TD	2	6.347	10.112	< 0.0001
P/P*CO	3	9.088	14.477	< 0.0001
TD*CO	6	8.829	14.065	< 0.0001
F*P/P*TD	2	0.673	1.072	0.343
F*P/P*CO	3	0.441	0.702	0.551
F*TD*CO	6	0.974	1.552	0.158
P/P*TD*CO	6	2.278	3.628	0.001
F*P/P*TD*CO	6	1.765	2.812	0.010
Error	1152	0.628		
Corrected Total	1199			

[a] The ANOVA was run on the logit transformed data to normally distribute the data.

[b] The causal organisms consisted of *Alternaria, Penicillium, Fusarium* and other. The other group was the combined data for the following fungi: *Cladosporium, Botrytis, Ulocladium, Stemphylium, Gliocladium, Verticillium, Epicoccum, Phoma, Botryosphaeria* and *Trichoderma.*

Table 10: The mean[a] percentage causal organisms from core rot symptoms found during pre- and post-harvest collections from farms in Vyeboom, Elgin for the season 2010.

	VA								VB							
	Alternaria		*Penicillium*		*Fusarium*		Other		*Alternaria*		*Penicillium*		*Fusarium*		Other	
	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post
MC	18.938 b-c	29.234 a-c	2.465 d-g	6.337 i-m	0.833 e-i	0.769 l-o	10.522 c-d	11.063 f-k	27.216 a-b	40.663 a	6.293 d-f	2.255 l-o	2.927 d-h	1.667 m-o	9.571 d-e	8.426 h-l
DCR	8.670 c-d	27.788 a-c	5.732 d-g	7.484 j-n	0.417 e-j	0.000 n-o	15.465 c-d	10.284 g-k	29.937 a-b	22.524 b-d	1.477 e-i	12.048 f-k	0.714 f-j	0.833 o	18.613 b-c	4.792 k-o
WCR	0.000 f-j	1.250 l-o	3.646 e-h	4.679 k-o	0.833 e-j	0.000 n-o	27.479 a-b	1.111 l-o	0.833 f-j	1.000 o	1.705 e-i	3.333 l-o	0.357 f-j	0.000 o	0.357 f-j	2.458 l-o

[a] Means indicated by different letters which differed at the 5% level according to Fischer's least significant difference (LSD) test. The t-values were gained from the LSD test run on the logit transformed data of the causal organisms.

Table 11: Analysis of variance on the mean incidence[a] of causal organisms of inoculum sources found on farms in the Witzenberg Valley, Cere for the seasons 2009 and 2010.

Source	DF	Mean squares	F	Pr > F
Year (Y)	1	487.359	80.468	< 0.0001
Farm (F)	2	175.034	28.900	< 0.0001
Inoculum source (IS)	2	13614.246	2247.845	< 0.0001
Causal Organism (CO)[b]	3	4555.655	752.183	< 0.0001
Y*F	2	656.072	108.324	< 0.0001
Y*IS	2	189.171	31.234	< 0.0001
Y*CO	3	30.505	5.037	0.002
F*IS	4	65.819	10.867	< 0.0001
F*CO	6	16.248	2.683	0.013
IS*CO	6	1216.994	200.938	< 0.0001
Y*F*IS	3	420.659	69.455	< 0.0001
Y*F*CO	6	16.303	2.692	0.013
Y*IS*CO	6	60.708	10.023	< 0.0001
F*IS*CO	12	7.818	1.291	0.216
Y*F*IS*CO	9	17.673	2.918	0.002
Error	5132	6.057		
Corrected Total	5199			

[a] The ANOVA was run on the logit transformed data to normally distribute the data.

[b] The causal organisms consisted of *Alternaria, Penicillium, Fusarium* and Other. The group other was the combined data for the following fungi: *Cladosporium, Botrytis, Ulocladium, Stemphylium, Gliocladium, Paecilomyces, Verticillium, Epicoccum, Phoma, Botryosphaeria* and *Trichoderma*.

Table 12: The mean[a] percentage of causal organisms from inoculum sources collected from farms in the Witzenberg Valley, Ceres for the seasons 2009 and 2010.

	2009									2010								
	A			B			C			A			B			C		
	Air	Mummy	Mites	Air	Mummy	Mites	Air	Mummy	Mites	Air	Mummy	Mites	Air	Mummy	Mites	Air[b]	Mummy	Mites
Alternaria	74.395 a	0.000 w	0.000 t	47.574 a-e	0.000 w	0.000 r-s	53.266 a-d	0.000 w	0.000 o-p	16.696 a-f	0.000 w	0.000 n-p	35.355 a-f	0.000 u-v	0.000 k-n	-	0.000 w	0.000 q-r
Penicillium	0.875 f-i	80.378 p	56.919 p	0.722 e-i	81.760 m-p	42.846 p	3.008 d-i	83.148 l-p	21.422 g-i	11.131 a-f	93.678 j-m	18.007 j-l	0.801 c-h	89.375 g-j	11.158 h-k	-	98.882 h-k	40.020 l-p
Fusarium	11.521 d-i	0.000 w	0.000 t	3.796 d-i	0.000 w	0.000 r-s	1.756 d-i	0.000 w	0.000 o-p	0.000 b-g	0.000 w	0.000 n-p	0.000 d-h	0.000 u-v	0.997 j-m	-	0.000 w	0.025 q-r
Other	13.210 c-h	10.622 u	18.081 s	47.877 a-e	6.240 u-v	12.154 q	41.970 a-f	13.852 u	10.578 k-n	72.173 a-b	3.322 v-w	6.993 k-o	63.844 a-c	0.625 u	4.845 i-k	-	1.118 v-w	6.955 q

[a] Means indicated by different letters which differed at the 5% level according to Fischer's least significant difference (LSD) test. The t-values were gained from the LSD test run on the logit transformed data of the causal organisms.

[b] The trees in farm C's orchard was extracted and destroyed. Data could not be obtained for the air inoculum source.

Table 13: Analysis of variance on the mean[a] incidence of the treatment of core rot with the fungicide Bellis® on farms in the Witzenberg Valley, Ceres for the season 2008/2009.

Source	DF	Mean squares	F	Pr > F
Farm (F)	2	7.927	16.097	< 0.0001
Pre/Post (P/P)	1	69.685	141.505	< 0.0001
Treatment (TMT)	1	3.308	6.718	0.010
Type of Decay (TD)	2	35.502	72.091	< 0.0001
F*P/P	2	4.786	9.718	< 0.0001
F*TMT	2	0.454	0.922	0.399
F*TD	4	1.658	3.367	0.010
P/P*TMT	1	0.012	0.025	0.875
P/P*TD	2	20.400	41.424	< 0.0001
TMT*TD	2	0.249	0.505	0.604
F*P/P*TMT	2	0.642	1.303	0.273
F*P/P*TD	4	2.763	5.611	0.000
F*TMT*TD	4	0.861	1.749	0.139
P/P*TMT*TD	2	0.061	0.124	0.883
F*P/P*TMT*TD	4	1.782	3.619	0.007
Error	324	0.492		
Corrected Total	359			

[a] The ANOVA was run on the logit transformed data to normally distribute the data.

Table 14: The mean[a] percentage of core rot symptoms from fruit untreated and treated with Bellis® during pre- and post-harvest collections from farms in the Witzenberg Valley, Ceres for the season 2008/2009.

	A				B				C			
	Pre-harvest		Post-harvest		Pre-harvest		Post-harvest		Pre-harvest		Post-harvest	
	Untreated	Treated	Untreated	Treated	Untreated	Treated	Untreated	Treated	Untreated	Treated	Untreated	Treated
MC	3.000 h-k	4.553 g-i	8.189 c-g	6.500 e-h	16.382 a	18.000 a-b	7.900 c-h	10.500 a-f	6.000 d-h	9.056 b-f	10.600 a-e	13.400 a-c
DCR	5.000 g-i	8.000 c-h	2.234 l-o	4.500 h-j	9.625 c-f	11.000 a-d	2.200 l-o	3.100 i-l	6.000 d-h	8.056 c-g	2.100 l-o	1.800 n-o
WCR	1.000 j-m	5.579 d-h	1.613 m-o	1.500 m-o	6.204 c-h	7.500 c-g	2.100 l-n	2.400 k-n	12.500 a-d	5.000 f-h	0.400 p	1.100 o-p

[a] Means indicated by different letters which differed at the 5% level according to Fischer's least significant difference (LSD) test. The t-values were gained from the LSD test run on the logit transformed data of the causal organisms.

Table 15: Analysis of variance on the mean incidence of causal organisms obtained from the core rot symptoms during the fungicide trial of Bellis®. The organisms were found on farms in the Witzenberg Valley, Ceres for the season 2009. The pre- and post-harvest data were compared by a four factorial analysis.

	Pre-harvest				Post-harvest			
Source	DF	Mean squares	F	Pr > F	DF	Mean squares	F	Pr > F
Farm (F)	2	40.301	51.843	< 0.0001	2	0.663	1.573	0.208
Treatment (TMT)	1	4.569	5.878	0.016	1	0.038	0.091	0.763
Type of Decay (TD)	2	4.409	5.672	0.004	2	44.875	106.450	< 0.0001
Causal Organism (CO)[b]	3	19.886	25.582	< 0.0001	3	79.236	187.956	< 0.0001
F*TMT	2	1.275	1.641	0.195	2	1.866	4.425	0.012
F*TD	4	1.965	2.528	0.040	4	2.188	5.190	0.000
F*CO	6	2.419	3.111	0.005	6	1.167	2.769	0.012
TMT*TD	2	0.152	0.195	0.823	2	0.815	1.933	0.146
TMT*CO	3	0.590	0.759	0.517	3	0.475	1.128	0.337
TD*CO	6	5.780	7.435	< 0.0001	6	29.226	69.327	< 0.0001
F*TMT*TD	4	0.707	0.910	0.458	4	1.005	2.383	0.050
F*TMT*CO	6	1.139	1.466	0.187	6	0.758	1.799	0.097
F*TD*CO	12	0.962	1.238	0.252	12	0.754	1.789	0.046
TMT*TD*CO	6	0.382	0.492	0.815	6	0.313	0.742	0.616
F*TMT*TD*CO	12	1.121	1.443	0.142	12	0.795	1.886	0.033
Error	648	0.777			648	0.422		
Corrected Total	719				719			

[a] The ANOVA was run on the logit transformed data to normally distribute the data.

[b] The causal organisms consisted of *Alternaria, Penicillium, Fusarium* and Other. The group other was the combined data for the following fungi: *Cladosporium, Botrytis, Ulocladium, Stemphylium, Verticillium, Epicoccum, Aspergillus, Mucor, Rhizopus* and *Trichoderma*.

Table 16: The mean[a] percentage of causal organisms from core rot symptoms observed in fruit treated and untreated by the fungicide, Bellis®, during pre- and post-harvest collections from farms in the Witzenberg Valley, Ceres for the season 2008/2009.

Pre-harvest

	CA								CB								CC							
	Alternaria		*Penicillium*		*Fusarium*		Other		*Alternaria*		*Penicillium*		*Fusarium*		Other		*Alternaria*		*Penicillium*		*Fusarium*		Other	
	U[b]	T[b]	U	T	U	T	U	T	U	T	U	T	U	T	U	T	U	T	U	T	U	T	U	T
MC	16.111 a-d	9.524 b-l	12.222 a-e	7.619 c-m	6.667 a-i	1.429 f-r	0.000 d-n	0.000 g-s	32.963 a-b	13.851 e-p	11.012 f-r	12.725 f-q	3.482 p-u	10.263 i-s	2.857 q-u	10.874 j-t	11.040 f-q	32.307 a	15.706 d-n	4.611 k-t	5.833 j-t	2.222 o-u	0.000 p-u	1.250 p-u
DCR	4.444 a-h	15.833 a-f	3.889 a-g	15.000 c-m	6.111 a-f	9.167 a-j	20.556 a-c	6.786 c-m	16.976 d-o	20.755 b-l	13.832 e-p	11.841 g-s	2.222 q-u	2.111 s-u	1.429 r-u	0.000 u	19.397 b-k	22.584 a-f	5.929 h-s	11.929 f-r	0.000 p-u	0.000 q-u	4.000 m-u	6.286 k-t
WCR	0.000 d-n	2.679 d-p	5.000 a-i	17.381 a-f	2.500 b-k	3.333 f-q	2.500 b-k	1.250 f-r	0.000 s-u	3.088 s-u	15.227 c-m	12.063 d-p	0.000 s-u	1.429 t-u	0.000 s-u	1.000 t-u	2.500 l-t	2.111 n-u	34.345 a	14.449 d-p	0.000 p-u	2.250 n-u	1.250 n-u	0.000 q-u

Post-harvest

	CA								CB								CC							
	Alternaria		*Penicillium*		*Fusarium*		Other		*Alternaria*		*Penicillium*		*Fusarium*		Other		*Alternaria*		*Penicillium*		*Fusarium*		Other	
	U	T	U	T	U	T	U	T	U	T	U	T	U	T	U	T	U	T	U	T	U	T	U	T
MC	58.296 a-b	40.032 c-d	8.834 h-o	4.936 k-s	0.000 v	0.000 s-v	3.982 n-v	5.167 k-s	45.690 b-c	41.461 c-d	9.444 h-n	15.636 f-j	2.937 m-v	0.000 v	2.262 n-v	0.588 t-v	54.221 a-b	67.648 a	21.048 e-f	13.038 f-k	1.538 q-v	0.000 u-v	1.111 s-v	1.538 s-v
DCR	9.960 g-m	27.724 d-e	1.880 s-v	2.269 m-u	0.000 v	0.000 s-v	3.281 o-v	7.500 j-r	11.270 f-k	17.101 f-g	1.667 q-v	3.086 o-v	0.714 r-v	0.000 v	3.222 m-v	4.242 o-v	14.420 f-h	9.974 h-n	3.419 m-v	1.111 t-v	0.000 t-v	0.000 u-v	0.000 t-v	0.000 u-v
WCR	3.587 n-v	0.769 q-v	9.179 h-p	4.019 l-t	0.000 v	0.000 s-v	1.000 t-v	7.583 i-q	1.714 p-v	2.818 q-v	17.429 f-i	10.735 g-l	0.000 t-v	0.000 v	3.651 m-u	4.333 q-v	0.000 t-v	2.020 s-v	4.242 m-v	2.991 q-v	0.000 t-v	0.000 u-v	0.000 t-v	1.678 s-v

[a] Means indicated by different letters which differed at the 5% level according to Fischer's least significant difference (LSD) test. The t-values were gained from the LSD test run on the logit transformed data of the causal organisms.

[b] U = Untreated, T = Treated

Chapter 3: Identification of core rot causal organisms through morphological, molecular and pathogenicity techniques

3.1. Abstract

Core rot is a major contributor to postharvest losses in apples word wide. Identification of fungi to species level through morphological identification can only be done by trained mycologists. The introduction of DNA analysis for species identification and the subsequent molecular techniques established have become a valuable part of scientific research. Pathogens most commonly associated with core rot are *Alternaria* and *Penicillium*. Although both genera show specific morphological characteristic, they can be difficult to identify to species level using morphological characteristics. Genetic loci, such as the internal transcribed spacer (ITS) region, are commonly used to identify fungal species. In this study, *Alternaria* and *Penicillium* isolates, associated with pre- and post-harvest core rot symptomatic apples and inoculum sources, were identified using various molecular techniques. Five pathogenicity tests were compared in order to select a reliable method for determining the pathogenicity of *Alternaria* species isolated from symptomatic fruit and inoculum sources.

Forty-nine *Alternaria* and 97 *Penicillium* isolates were selected from the isolates collected during the trials (chapter 2). *Alternaria* species were identified using the genetic loci ITS and anonymous genomic region (OPA1-3) and the use of inter simple sequence repeats (ISSRs) was also attempted. A possible molecular technique to identify small-spored *Alternaria* species was determined using the anonymous OPA1-3 region. *Penicillium* species were identified through ITS and partial beta-tubulin polymerase chain reaction (PCR) – random fragment length polymorphisms (RFLP) for the samples collected from WCR symptoms.

Phylogenetic analyses separated the *Alternaria* spp. into five clades, including three separate clades for *A. alternata, A. tenuissima* and *A. arborescens,* respectively.

The *Pencillium* phylogeny based on ITS specific primers gave two distinct clades: various *Penicillium* species and *P. ramulosum,* which were the two most identified species, in contrast to what has previously been reported from core rot that *P. funiculosum* was the most frequently isolated species. The same results were found with the partial beta-tubulin PCR-RFLP. From current isolations and molecular identification, *P. ramulosum* and *P. expansum* are the most frequently isolated *Penicillium* species from the three core rot symptoms. The

surface toothpick method described by Serdani *et al.* (2002) is a reliable test to test for pathogenicity against *Alternaria* species causing core rot.

During pathogenicity tests the method using a surface toothpick, where a fungal colonised toothpick was inserted through the surface of the side of the apple, gave the second highest mean lesion diameter and the most variation between *Alternaria* isolates.

3.2. Introduction

Core rot is a post-harvest disease, caused by various pathogenic fungi, including *Alternaria* Nees and *Penicillium* Link. Core rot includes three symptoms, namely mouldy core (MC), dry core rot (DCR) and wet core rot (WCR) (Carpenter, 1942). Core rot symptoms are generally restricted to the core cavity of the fruit and external symptoms are rarely shown (Combrink and Ginsburg, 1973; Reuveni *et al.*, 2003). The causal organisms of MC are restricted to the core cavity of the apple, whereas those of DCR infect the tissue surrounding the core cavity and a dry, corky lesion forms (Carpenter, 1942; McLeod *et al.*, 2008). Wet core rot fungi move quickly through the tissue causing a soft, wet rot (Serdani, 1999). Niem *et al.* (2007) established that core rot causal organisms infect during blossom, especially infecting apple cultivars with an open calyx end (Miller, 1959). Their results show a 100% colonisation of parts of the ovary by *Alternaria.* Non-susceptible fruit do not have a calycine sinus (which later becomes part of the calyx end of the fruit) blocking the penetration of the fungi into the ovary (Niem *et al.*, 2007).

Core rot causal organisms can be identified to genus level through morphological characteristics (Combrink *et al.*, 1985). The fungi are either grown on a general nutrient rich agar, such as potato dextrose agar (PDA), or on a specific media, such as V8 agar, potato carrot agar (PCA) or malt extract agar (MEA), which allows identification of specific characteristics. Some of these characteristics include the colour of the fungus on the media, the growth rate and diameter, as well as the size of the conidia (Andersen *et al.*, 2001).

Based on morphological characteristics, core rot causal organisms have previously been identified as members of the following genera: *Alternaria, Botrytis* P. Micheli ex Pers., *Cladosporium* Link, *Coniothyrium* Corda, *Fusarium* Link, *Aspergillus* P. Micheli ex Link, *Gloesporium* Desm. and Mont., *Epicoccum* Link, *Penicillium, Pestalotia* De Not., *Phoma* Sacc., *Trichoderma* Pers. and *Rhizopus* Ehrenb. (Ellis and Barrat, 1983). The two most prevalent core rot causing pathogens are *Alternaria* spp., which is most frequently isolated from DCR, and MC, and *Penicillium* spp., isolated from WCR (Combrink and Ginsburg, 1973; Combrink *et al.*, 1985; Spotts, 1990). Morphological identification of core rot causing

fungi to species level is not always possible, therefore new methods have to be used, such as molecular characterisation. Some fungi can be identified to species level using one genetic locus, e.g. the beta-tubulin gene for *Penicillium* (Seifert *et al.*, 2007), whereas other species cannot be distinguished using only one gene region (e.g. *Alternaria alternata* (Fr.) Keissler and *A. tenuissima* (Nees and T. Nees: Fr.) Wiltshire) (Peever *et al.*, 2004, Peever *et al.*, 2005).

The four *Alternaria* species that have been most frequently associated with core rot are *A. alternata, A. tenuissima, A. arborescens* E.G. Simmons and *A. infectoria* E.G. Simmons (Kang *et al.*, 2002; Serdani *et al.*, 2002). Of these four species, *A. infectoria* has been distinguished from the other three species using the internal transcribed spacer (ITS) of ribosomal Ribonucleic Acid (rRNA) gene region (de Hoog and Horre, 2002; Berbee *et al.*, 2003; Andersen *et al.*, 2009). Due to the lack of polymorphisms in the ITS region within other small-spored *Alternaria* species, this region cannot be used to distinguish between the other members of this genus (de Hoog and Horre, 2002).

Alternative genetic loci have been used to identify the small-spored species of *Alternaria* with varying levels of success. These gene regions include glyceraldehyde-3-phosphate dehydrogenase (gpd), translocation elongation factor 1α (tef-1α), endopolygalacturonase (endo-PG), mitochondrial small and large subunit (mtSSU and mtLSU), beta-tubulin and anonymous genomic regions (OPA1-3, OPA2-1 and OPA10-2) (Kusaba and Tsuge, 1995; Pryor and Gilbertson, 2000; Pryor and Michailides, 2002; de Hoog and Horre, 2002; Peever *et al.*, 2002, 2004, 2005; Pryor and Bigelow, 2003, Hong *et al.*, 2005, Pavon *et al.*, 2010). These sequences can be used to distinguish between *A. arborescens* and other *Alternaria* species. However, *A. alternata* and *A. tenuissima* could not be separated into distinct clades using these loci (Pryor and Michailides, 2002).

Further methods investigated in previous studies included deoxyribonucleic acid (DNA) fingerprinting methods such as randomly amplified polymorphic DNA (RAPD), nuclear intergenic spacer polymerase chain reaction (PCR) – restriction fragment length polymorphisms (RFLP) and inter simple sequence repeat (ISSR) analysis (Roberts *et al.*, 2000; Pryor and Michailides, 2002; Hong *et al.*, 2006). These methods have shown similar results to phylogenetic analyses of the above mentioned DNA sequences, with *A. infectoria* and *A. arborescens* separating into distinct clades, and *A. alternata* and *A. tenuissima* occurring in the same clade (Roberts *et al.*, 2000; Pryor and Michailides, 2002; Hong *et al.*, 2006).

Penicillium species that have been isolated from apples have been identified as *P. expansum* Link, *P. solitum* Westling, *P. commune* Thom, *P. aurantiogriseum* Dierckx, *P. crustosum* Thom and *P. brevicompactum* Dierckx (Amiri and Bompeix, 2005). These species also cause the postharvest disease, known as "blue mould" on apples and is the main causal organism for the WCR symptom. Initially South African WCR was thought to be a postharvest disease caused by *Penicillium expansum* (Combrink and Ginsburg, 1973). The spores of *Penicillium* species were reported to be washed into the open calyx through the fungi-contaminated water, containing diphenylamine (DPA)-emulsion, in the pack house. The post-harvest treatment for apple scald, DPA, lowers the surface tension of the suspension and allows the water to move through the open calyx (Combrink and Ginsburg, 1973).

Although postharvest WCR can be caused by dipping the fruit in a DPA-emulsion against superficial scald (Combrink *et al*., 1985; Combrink *et al*., 1987; Spotts *et al*., 1988), it has been shown that infection can also take place in the orchard prior to harvest (McLeod *et al*., 2008; Van der Walt *et al*., 2010). De Kock *et al*. (1991) observed pre-harvest WCR in orchards, but this phenomenon was only reported again in the 2005/06 season in Ceres (McLeod *et al*., 2008).

Little research has been done to identify the *Penicillium* species that cause WCR. Sanderson and Spotts (1995) identified the species collected from their symptomatic fruit using the colony colour and morphology of the isolates. Spotts *et al*. (1988) isolated and identified *P. roquefortii* Thom from apples with core rot symptoms, however, this species was not collected from pre-harvest WCR isolations. *Penicillium roquefortii* has been collected from field bins and from decay lesions on apples and pears post-harvest (Sanderson and Spotts, 1995). To verify that *P. roquefortii* occurred only during post-harvest, fruit were inoculated in the orchard prior to harvest with a spore suspension of *P. roquefortii* (Spotts *et al*., 1988). No symptoms were observed during pre-harvest isolations, but isolations from post-harvest symptoms identified *P. roquefortii.* Two other *Penicillium* species, namely, *P. expansum* and *P. funiculosum* Thom were reported to cause WCR in South Africa (Combrink and Ginsburg, 1973; Combrink *et al*., 1985; De Kock *et al*., 1991; Serdani *et al*., 1998).

Van der Walt *et al*. (2010) identified the species of *Penicillium* found in three apple production areas of South Africa that caused pre-harvest WCR, and characterised the virulence and pathogenicity of these isolates. The species that were identified were *P. ramulosum* prov. nom., *P. expansum, Penicillium* species (aff. *P. dendriticum* Pitt), *P.*

glabrum (Wehmer) Westling, *Penicillium* sp. (aff. *P. cecidicola* Seifert, Hoekstra and Frisvad) and *P. chermesinum* Biourge.

The following gene regions have been used to identify *Penicillium* species: ITS, cytochrome c oxidase 1 (CO1) gene and the beta-tubulin gene (Lobuglio *et al*., 1993, 1994; Skouboe *et al*., 1999; Seifert *et al*., 2007). Isolates amplified with ITS can be sequenced to identify the fungi to species level. Other molecular techniques used to identify *Penicillium* species are RAPDs and PCR-RFLP. Pianzolla *et al*. (2004) found that the RAPD technique is useful to separate subspecific genera, but not for identifying species within *Penicillium*. Van der Walt *et al*. (2010) used the beta-tubulin PCR-RFLP technique with restriction enzymes *Hae*III and *Rsa*I to distinguish between *Penicillium* species isolated from core rot symptoms. Different restriction bands distinguished between the *Penicillium* species and were confirmed through sequence analysis (Van der Walt, 2009; Van der Walt *et al*., 2010).

When isolating from symptomatic tissue, various fungi can be grown and identified. To determine which of these is the primary pathogen and which are secondary pathogens, or endophytic organisms, isolated fungi are tested for their pathogenicity on the host plant. Pathogenicity tests used for core rot pathogens include non-wounding techniques such as the injection of *Penicillium expansum* conidial suspension into the core through the open calyx end to test for WCR (Combrink and Ginsburg, 1973; Spotts *et al*., 1988) or wounding the mesoderm with an inoculum-colonized needle or pipette tip to cause a wound infection point (Sommer *et al*., 1974; Niem *et al*., 2007; Reuveni *et al*., 2007). To test for MC the inoculation, of the core of longitudinally cut apple, with a conidial suspension has been described (Niem *et al*., 2007).

Previous pathogenicity studies in South Africa for DCR and WCR were done using halved and whole colonized toothpicks inserted into the apple (Serdani *et al*., 2002; Van der Walt *et al*., 2010). Serdani *et al*. (2002) used halved toothpicks colonized with different *Alternaria* spp., inoculating them into the mesoderm of the apple. Van der Walt *et al*. (2010) used two techniques with toothpicks, a wounding and a non-wounding method. With the wounding technique the colonized toothpick was inserted into the calyx at an angle into the core thus wounding the tissue (Van der Walt *et al*., 2010). In the non-wounding technique the colonized toothpick was inserted through the open calyx end of the susceptible cultivar into the core (Van der Walt *et al*., 2010).

The first objective of this study was to identify the species of *Alternaria* and *Penicillium* sampled from core rot symptomatic fruit and inoculum sources (air, mummies

and mites), using morphological and molecular methods. The second objective was to determine and compare *Penicillium* species isolated from pre- and post-harvest WCR symptomatic fruit, using molecular methods. The final objective was to compare and to select the most reliable pathogenicity test for use in future research.

3.3. Materials and methods

3.3.1. *Alternaria* and *Penicillium* isolates

Alternaria and *Penicillium* isolates collected from a single orchard in both 2009 and 2010 were selected for molecular identification. The isolates selected were from pre- and post-harvest symptomatic fruit, as well as the inoculum sources (air, mummies and mites) collected during the bloom period for both seasons (Chapter 2). The orchard was located in the Witzenberg Valley, Ceres, and had a history of core rot. The 49 *Alternaria* isolates selected included 10 isolates from pre- and post-harvest symptomatic fruit respectively for both seasons, and nine isolates from the air inoculum. *Alternaria* was not isolated from apple mummies, or from mites from these mummies (Chapter 2). The 97 *Penicillium* isolates selected included 10 isolates from pre- and post-harvest symptomatic fruit respectively and 10 isolates from each of the inoculum sources (mummies and mites) for each season. Ten *Penicillium* isolates, collected from air inoculum in this orchard in 2009 were included, however as the trees in this orchard were removed in 2010, seven *Penicillium* isolates from the air inoculum of an alternative orchard in Ceres were included.

3.3.2. Morphological identification

In 2009, 94 and 128 samples were isolated and identified as causal organisms from the pre- and post-harvest sampling respectively from symptomatic core rot apples. From the inoculum sources, mummies and mites, 276 and 221 causal organisms were isolated and identified, respectively. In 2010, 205 and 177 samples were isolated and identified as causal organsism from the pre- and post-harvest sampling respectively from symptomatic core rot apples. From the inoculum sources, air, mummies and mite samples, 147, 273 and 67 causal organisms respectively, were isolated and identified.

The fungi isolated from the core rot symptomatic fruit and inoculum sources were plated out on PDA and MEA. Genera were identified using colour plates (Serdani, 1999; Samson *et al.*, 2002) and microscopy with the aid of keys from the “Illustrated genera of imperfect fungi” (Barnett and Hunter, 1998). The *Alternaria* and *Penicillium* species were

identified using the "*Alternaria* identification manual" (Simmons, 2007) and the "Introduction to food and airborne fungi" (Samson *et al*., 2002). Further confirmation of species identification was done using molecular methods.

3.3.3. Molecular identification of *Alternaria* isolates

3.3.3.1. DNA extraction, PCR amplification and sequencing

Isolates were grown on Potato Dextrose Agar (PDA, Becton Dickinson and Company, Sparks, MD, USA) at room temperature for 4 to 7 days. DNA was extracted from mycelia using the UltraClean® Microbial DNA Isolation kit (MoBio laboratories, Inc., Carlsbad, CA, USA) following the manufacturer's instructions.

To identify individual *Alternaria* species, five genetic loci (endoPG, ITS, OPA1-3, OPA2-1, and OPA10-2) were used for sequence analysis and were amplified as described in the corresponding literature (White *et al*., 1990; de Hoog and van den Ende, 1998; Isshiki *et al*., 2001; Peever *et al*., 2004; Peever *et al*., 2005; Andrew *et al*., 2009; Table 1). Samples were amplified in an Applied Biosystems 2720 Thermal cycler and sequenced with the same primers as used for PCR amplification, using 1µl PCR product in a 10 µl reaction, consisting of distilled water, 10 µM primer, 1x sequence mix (BigDye) and 5x buffer. The sequence program followed an initial denaturation of 1min at 95°C, 30 cycles of denaturation for 10s at 95°C, and annealing for 5 sec at 50°C and 4 min extension at 60°C. The sequencing product was filtered through a Sephadex filled filter plate and centrifuged at 910 rpm. Samples were analyzed on an ABI Prism 3700 genetic analyzer (Applied Biosystems, Foster City, CA), and forward and reverse sequences were assembled with the program Geneious Pro 5.3.3 (Biomatters Ltd., Auckland, New Zealand).

3.3.3.2. Phylogenetic analysis and Partition homogeneity test

ITS sequences of *Alternaria* reference strains and representative isolates from the trials were aligned using the software program CLUSTAL W (Thompson *et al*. 1994). *Alternaria* sequences from the datasets endoPG, OPA1-3 and OPA2-1 were aligned using MAFFT sequence alignment program version 6 (Katoh and Toh, 2008) followed by manual adjustments of the alignments in Sequence Alignment Editor v2.0a11 (Rambaut, 2002). Maximum parsimony analysis as well as Bayesian analysis was conducted on the *Alternaria* sequence alignments using PAUP* (Phylogenetic Analysis Using Parsimony) v.4.0b10. The analysis was performed using the heuristic search option with 100 random taxon additions. Tree bisection and reconstruction (TBR) was used as the branch swapping algorithm with the

option of saving no more than 10 trees with a score greater or equal to five (Harrison and Langdale, 2006). Bootstrap support values were calculated from 1000 heurisitic search replicates and 100 random taxon additions. Bootstrap values below 60% were not included for each genetic locus' phylogenetic analysis. Other measures calculated for the parsimony analysis include tree length (TL), consistency index (CI), retention index (RI) and rescaled consistency index (RC) values.

Bayesian analysis was conducted using MrBayes v3.1.2 (Ronquist and Huelsehbeck, 2003). The program MrModeltest (J.J.A. Nylander, available from the internet: www.ebc.uu.se/systzoo/staff/nylander.html) was used for selecting the optimal model of sequence evolution for each clade alignment. Markov chains were initiated from a random tree and run for 600 000 generations at a temperature set for 0.2. Convergence among chains was monitored by examining plots of log-likelihood values and observing when the values of the four chains have reached a plateau. The first 30 000 generations (burn-in) were discarded for the analysis. The remaining samples were used to calculate the 50% majority-rule tree and the posterior probability for the individual branches.

Topologies of the resulting phylograms were compared using incongruence length difference (ILD) tests (Farris *et al.*, 1994) to determine the suitability of combining the endoPG, OPA1-3 and OPA2-1 data sets. ILD tests were implemented in PAUP* (referred to as "partition homogeneity tests" in PAUP*) with invariant characters removed and 1000 randomized partitions. Tested data partitions included: (i) endoPG, OPA1-3 and OPA2-1; (ii) endoPG and OPA1-3, (iii) endoPG and OPA2-1 and (iv) OPA1-3 and OPA2-1. Data partitions were considered significantly different at $P<0.05$ (Swofford, 2002).

3.3.3.3. ISSR analysis

Six inter simple sequence repeats (ISSRs) were used to amplify the different *Alternaria* species, aiming to distinguish between *A. alternata, A. tenuissima, A. infectoria, A. arborescens*. The ISSRs used were 5'-DHB$(CGA)_5$ (Hantula *et al.*, 1996), 5'-DDB$(CCA)_5$ (Hantula *et al.*, 1997), 5'-HVH$(GTG)_5$ (Pina *et al.*, 2005), 5'-NDB$(CA)_7$C (Mahuku *et al.*, 2002), 5'-YHY$(GT)_7$G (Hantula *et al.*, 1996) and 5'-NDV$(CT)_8$ (Mahuku *et al.*, 2002). Amplification reactions were performed using a thermal cycler (2700 Applied Biosystems) in volumes of 25 µl containing approximately 20 ng template DNA, 0.4 µM primer, 0.4 µM dNTP, 5 mM $MgCl_2$, 1 x PCR buffer, bovine serum albumin (BSA) Fraction V and 0.5 U BIOTAQ DNA polymerase. The program consisted of an initial step of 5 min at 95°C, followed by 34 cycles of denaturation at 95°C for 1 min, annealing at 61°C $(CGA)_5$, 64°C

$(CCA)_5$, 56°C $(GTG)_5$, 41°C $(CA)_7C$, 58°C $(GT)_7G$ or 41°C $(CT)_8$ for 1 min, and an elongation step at 72°C for 2 min. A final extension was performed at 72°C for 10 min. PCR products were separated on 3% agarose gels (Seakem®, Lonza, Rockland, ME USA), stained with ethidium bromide (0.01%) and visualised under UV light.

3.3.4. Molecular identification of *Penicillium* isolates

3.3.4.1. DNA extraction, PCR amplification and sequencing

The isolates were grown on malt extract agar (MEA, Oxoid, Basingstoke, United Kingdom) and incubated at 25°C for 4 to 7 days. The DNA was extracted from the mycelial cells using the UltraClean® Microbial DNA Isolation kit (MoBio laboratories, Inc.) following the manufacturer's instructions.

The ITS region was amplified using the primers V9G and Ls266 (Table 1). The reaction mixture contained 1.5 mM $MgCl_2$, 0.05 mg DMSO, 1x NH buffer, 1 mM dNTP, 10 µM of each primer (V9G and Ls266), 5 U *Taq* polymerase and 1 µl of fungal DNA in a final volume of 25 µl. Amplifications were performed in a Biorad® iCycler PCR machine. The programme consisted of an initial denaturation step for 5 min at 94°C, 5 cycles of 30 s at 94°C, 40 s at 55°C and 1 min at 72°C, 5 cycles of 30 s at 94°C, 40 s at 54°C and 1 min at 72°C, 25 cycles of 30 s at 94°C, 40 s at 53°C and 1 min at 72°C, and extension for 5 min at 72°C. After PCR amplification the products were confirmed by separation on a 1% agarose gel (Seakem®, Lonza, Rockland, ME USA). Sequence analysis was performed as described previously with primers V9G and Ls266. Sequence alignments and phylogenetic analysis was performed as described earlier.

3.3.5. Pre- and Post-harvest WCR

3.3.5.1. Morphological identification of *Penicillium* isolates

In 2009, 46 and 39 samples were identified as WCR from pre- and post-harvest sampling respectively. In 2010, the pre-harvest WCR collection consisted of 234 samples and the post-harvest collection of 102 samples. All the species isolated from the WCR symptoms were identified after being plated onto PDA and MEA media. The genera were identified from their colony colour from above and beneath the plate as well as microscopically. *Penicillium* isolates were identified with the aid of colour plates as described in Samson *et al.* (2002). Species identification through morphological identification proved to be difficult and further identification was performed using molecular methods.

3.3.5.2. Collection and isolation of *Penicillium* isolates

Fruit was collected pre-harvest (fallen fruit) and post-harvest for the seasons 2009 and 2010. Tissue was isolated from fruit infected with WCR symptoms. All the *Penicillium* samples collected for both seasons and the various farms (Ceres and Vyeboom) were compared with one another. Twenty-nine and 32 samples were collected pre-harvest and 27 and 36 samples were collected post-harvest in 2009 and 2010 respectively.

3.3.5.3. Characterisation of *Penicillium* isolates

The *Penicillium* isolates obtained from the WCR affected apples were characterised at the molecular level. Genomic DNA was extracted from single-spored *Penicillium* isolates (N=124), using a slightly modified method of Lee and Taylor (1990). Subsequently, the beta-tubulin PCR-RFLP group of each isolate was determined by PCR amplification of a partial region of the beta-tubulin gene using primers Bt2a (5'-GGTAACCAAATCGGTGCTGCTTTC-3') (Glass and Donaldson, 1995) and PentubR (5'-GACGGACGACATCGAGAACCTG-3') (Van der Walt, 2009). The PCR reaction consisted of 0.2 µM of each primer, 0.2 mM of each dNTP, 1x PCR buffer, 0.5 U BIOTAQ DNA polymerase, 0.2 mg bovine serum albumin (BSA) Fraction V, 2 µl DNA and 3 mM $MgCl_2$ in a final volume of 40 µl. Amplifications were conducted in a 2720 Applied Biosystems thermocycler, starting with an initial denaturation cycle of 5 min at 94°C, followed by 40 cycles of 45 s at 94°C, 45 s at 55°C and 60s at 72°C and a final extension cycle of 7 min at 72°C. Successfully amplified PCR products were digested using the restriction enzymes *Hae*III (Fermentas Inc., Glen Burnie, MD, USA) and *Rsa*I (Fermentas Inc., Glen Burnie, MD, USA). The *Hae*III digest reaction consisted of 1x enzyme buffer, 15 U/µl *Hae*III and 10 µl PCR product in a total volume of 20 µl. The *Rsa*I digestion reaction consisted of 1x enzyme buffer, 5 U/µl *Rsa*I and 10 µl PCR product in a total volume of 20 µl. Digests were incubated overnight at 37°C, and 15 µl of the restriction digest products were seperated along with a 50 bp DNA standard on a 3% agarose gel (Seakem®, Lonza, Rockland, ME USA) containing 0.001% of ethidium bromide. Isolates that exhibited the same restriction pattern for both enzymes were classified into the same beta-tubulin PCR-RFLP group using Van der Walt *et al.* (2010) restriction patterns as control.

3.3.6. Selection of a reliable pathogenicity test for *Alternaria* species associated with apple core rot

The apple cultivar, 'Red Delicious' was used in the pathogenicity tests due to the high susceptibility of this cultivar to core rot infection (Combrink and Ginsburg, 1973; Combrink *et al.*, 1985; Serdani *et al.* 1998, 2002). The fruit was collected from a pack house in Ceres, Western Cape following short term cold storage. The apples were similar in size and at the same physiological stage of maturity. The trial was repeated twice during 2009 and once during 2010.

3.3.6.1. *Alternaria* cultures

For the pathogenicity test nine *Alternaria* isolates were randomly selected from the Stellenbosch University's fungal collection. These samples had been collected and identified from previous studies on core rot. The nine isolates selected were drawn from four *Alternaria* species complexes, *Alternaria tenuissima* (STE-U 7629, 7630, 7631), *A. alternata* (STE-U 7632, 7633), *A. infectoria* (STE-U 7634, 7635) and *A. arborescens* (STE-U 7636, 7637) and grown on PDA at 20°C for seven days.

3.3.6.2. Preparation of trial

Colonised toothpicks were used as inoculum vectors. The toothpicks were cut in half for one test with the remaining kept whole for the other tests. The toothpicks were autoclaved five times in distilled water to remove impurities and autoclaved once in Potato Dextrose Broth (Serdani *et al.*, 2002). The toothpicks were placed onto colonized PDA plates and allowed to grow for two weeks.

All the fruit were surface-sterilised in 70% ethanol for 30 s and left to air dry before inoculation. After inoculation the apples were placed into sterilised moisture chambers and incubated for 5 to 10 days at 20°C. The following five inoculation methods were used:

3.3.6.3. Method 1: Surface wounding of apple with colonized toothpicks (Serdani *et al.*, 2002)

The sterilised apples were inoculated with colonised halved toothpicks. The toothpick was inserted into the surface of the apple. Ten apples were inoculated for each of the nine isolates and replicated three times, with another apple inoculated with a non-colonised toothpick as a control. After incubation the apple was cut longitudinally through the lesion, and the lesion diameter was measured.

3.3.6.4. Method 2: Surface wound inoculated with pipette (Reuveni *et al.*, 2007)

A spore suspension of 10^6 spores/ml, was prepared for each isolate. The number of spores in the suspension was determined using a haemocytometer. Using a sterilised pipette tip a wound of approximately 2 mm was made in the surface of the apple. The wound was inoculated with 15 µl of spore suspension. Ten apples were inoculated for each of the nine isolates and replicated three times. One apple was inoculated with distilled, deionised water, which served as the control. After the incubation period the fruit was cut longitudinally through the wound and the lesion diameter was measured.

3.3.6.5. Method 3: Inoculation of open mesoderm core cavity (Niem *et al.*, 2007)

A spore suspension of 10^6 spores/ml was prepared for each isolate. The sterilised apples were cut in half longitudinally, and inoculated with 20 µl of spore suspension into the core region of the apple. Ten apple halves were inoculated for each of the nine isolates and replicated three times. A control apple half was inoculated with sterilised water. After the fruit was inoculated it was placed in a black plastic bag and incubated for five days. The lesion diameter was measured after the incubation period.

3.3.6.6. Method 4: Deep wounding of apple fruit with colonized toothpicks (Van der Walt *et al.*, 2010)

Ten sterilised apples were inoculated with colonised toothpicks from each of the nine isolates and repeated three times. A control apple was inoculated with a non-colonised toothpick. The colonised toothpick was inserted off-centre from the calyx into the core. After incubation the apples were cut longitudinally through the lesion and the lesion diameter was measured.

3.3.6.7. Method 5: Non-wounding of apple fruit with colonized toothpicks (Van der Walt, 2009; Van der Walt *et al.*, 2010)

Ten sterilised apples were inoculated with colonised toothpicks from each of the nine isolates and repeated three times. A control apple was inoculated with a non-colonised toothpick. The toothpick was inserted through the calyx into the core. After incubation the apples were cut longitudinally through the lesion and the lesion diameter was measured.

3.4. Results

3.4.1. Morphological identification

Genera identified from the isolations of core rot symptomatic fruit and inoculum sources were *Alternaria, Penicillium, Fusarium, Cladosporium, Botrytis, Gloeosporium, Aspergillus, Trichoderma, Epicoccum, Ulocladium, Stemphylium* and *Verticillium.*

3.4.2. Molecular identification of *Alternaria* and *Penicillium* species from core rot symptomatic fruit and inoculum sources

3.4.2.1. *Alternaria* species identification

The aligned sequence data set of the ITS region spanned 595 base pairs. The multiple alignment of some *Alternaria* spp. and the ITS phylogenetic tree contained *A. alternata* EGS 34-016, *A. tenuissima* EGS 34-015, *A. arborescens* EGS 39-128, *A. infectoria* EGS 27-193 and *Embellisia eureka* (JN383490.1 and AF392989.1) as reference sequences (Fig. 1, 2). Of the samples collected, one sample (Alt 10, pre-harvest collection 2009) was identified through a Genbank Basic Local Alignment Search Tool (BLAST) search for nucleotides, phylogenetic analysis and multiple-alignment as *A. infectoria* and one isolate as *E. eureka* (Alt16, post-harvest collection 2009) (Fig. 1, 2). As previously reported, the other *Alternaria* species, *A. alternata, A. arborescens* and *A. tenuissima* could not be separated using sequence analysis of this genetic region (Fig. 1, 2).

Parsimony analysis was further conducted on each of the following genetic loci: endoPG, OPA1-3 and OPA2-1 (single locus and combined). One further locus, the anonymous region OPA10-2, resulted in weak clades and several reference sequences, which were used for the other loci, were not available on Genbank. This region could therefore not be included in the combined phylogeny (data not shown). The sequences obtained from Genbank (Table 2) are representative type strains of *A. alternata* EGS34-016 and *A. perangusta* EGS44-160 being ex-type strains and *A. arborescens* EGS39-128 and *A. dumosa* EGS45-007 being holotype strains (Simmons, 2007).

The length of the endoPG sequence alignment was 381 bp with 13 parsimony-informative characters. One parsimonious tree was recovered and tree scores were TL = 24, CI = 1.000, RI = 1.000 and RC = 1.000. Eight clades were observed from this phylogeny with *A. longipes* (Clade 7) and *A. gaisen* (Clade 8) as the outgroup references. *Alternaria*

tenuissima clustered separately from the *A. alternata* reference isolates, but fell within the clade containing the *A. dumosa* reference isolate (Fig. 3).

The length of the OPA1-3 sequence alignment was 499 bp with 63 parsimony-informative characters. Seven equally parsimonious trees were recovered and tree scores were TL = 133, CI = 0.850, RI = 0.959 and RC = 0.814. Seven clades were observed from phylogeny with *A. longipes* (Clade 6) and *A. gaisen* (Clade 7) as the outgroup reference sequences. Clade 4 contained the *A. tenuissima* with the reference isolate *A. alternata* EGS 45-062 (Fig. 4).

The length of the OPA2-1 sequence was 532 bp with 11 parsimony-informative characters. Two equally parsimonious trees were recovered and tree scores were TL = 19, CI = 0.947, RI = 0.978 and RC = 0.926. Seven clades were observed from this phylogeny with *A. longipes* and *A. alternata* EGS 45-062 as the outgroup reference sequences (Clade 7). *Alternaria tenuissima* combined with various reference isolates including *A. gaisen, A. dumosa* and multiple *A. alternata* isolates (Clade 6, Fig. 5).

A partition homogeneity test (PHT) was performed on the invariant characters of the three combined datasets (endoPG, OPA1-3 and OPA2-1) as well as the combination of individual genetic loci with another locus (endoPG and OPA1-3, endoPG and OPA2-1, and OPA1-3 and OP2-1). Isolates from each source were compared with the reference strain and identical isolates were removed from the clades. Nine isolates from Clade 2 and two isolates from Clade 7 were removed as their sequences were identical to other isolates from the same source. Each of the combinations gave a P-value of 0.001 in the PHT and parsimony and Bayesian analysis were conducted on the combined dataset.

The phylogeny of the combined datasets resulted in ten clades, each containing the sequence of a Genbank reference isolate (Fig. 6). The length of the combined sequence alignment was 1412 bp with 87 parsimony-informative characters. Two equally parsimonious trees were recovered and tree scores were TL = 204, CI = 0.760, RI = 0.918 and RC = 0.697. Significant (>60%) Bayesian probability values are shown together with the most likelihood (bootstrap) values of the parsimony analysis. Clade 1 consists of the reference strains *A. turkisafria* EGS 44-159 and *A. perangusta* EGS 44-160. Clade 2 contains the reference strain *A. dumosa* EGS 45-007, Clade 3 contains the reference isolate *A. alternata* EGS 45-002, and Clade 4 contains the ex-type reference strain *A. alternata* EGS 34-016 as well as the reference isolates *A. alternata* EGS 34-039 and EGS 45-008. Clade 5, 6 and 7 contained the reference strains *A. alternata* EGS 45-062, *A. tenuissima* EGS 34-015 and *A. alternata* EGS

45-010, respectively. Clade 8 contains the reference strain *A. arborescens* EGS 39-128. The outgroup sequences were *A. gaisen* EGS 37-1321 (Clade 9) and *A. longipes* EGS 30-033 (Clade 10). The representative strains and the isolates identified as *A. infectoria* and *Embellisia eureka* could not be sequenced for the endoPG region or for the anonymous region datasets, OPA1-3 and OPA2-1, due to lack of PCR amplification with the primers used.

The isolates, the source they were collected from and the identification for each isolate according to the phylogenetic analyses of each genetic region is presented in Table 3. The genetic region, endoPG, separated the isolates into three species-groups, *A. alternata, A. arborescens* and *A. dumosa / A. tenuissima*. For the pre-harvest seasons and the air inoculum the species-groups *A. dumosa / A. tenuissima* had the highest percentage population, with the *A. arborescens* species-group having the highest population for both post-harvest seasons (Table 4).

The genetic region, OPA1-3, could not distinguish between *A. alternata* and *A. tenuissima* nor could *A. alternata* be distinguished between various other reference sequences. *Alternaria arborescens* was the only species-group that could be distinguished from the other sequences (Table 4). The genetic region, OPA2-1, distinguished *A. arborescens* from the other reference sequences. It occurred most frequently post-harvest. *Alternaria alternata* combined with other reference sequences such as *A. tenuissima, A. dumosa* and *A. gaisen.* Isolates could not be separated from one another in this clade. Some of the isolates could not be identified beyond genus level as they did not group with any of the reference sequences (Table 4).

The three genetic regions were combined and this combined phylogenetic analysis could distinguish between the various *Alternaria* species. *Alternaria arborescens* occurred frequently post-harvest (50% of post-harvest isolates) and *A. dumosa* occurred with high incidences (above 30%) in each of the sources (Table 4). *Alternaria alternata* and *A. tenuissima* could be separated, with *A. alternata* occurring in all the sources more frequently during pre-harvest and in the air inoculum. Only one isolate from the 2010 pre-harvest collection was identified as *A. tenuissima.*

3.4.2.2. ISSR analysis

Repeated amplification attempts resulted in no distinguishing bands for each of the four *Alternaria* species (*A. alternata, A. arborescens, A. infectoria* and *A. tenuissima*) present in core rot isolations. The primers $(CGA)_5$, $(CCA)_5$ and $(GTG)_5$ amplified as single or only

few amplicons for some of the *Alternaria* isolates without association to species identified through sequence analysis (data not shown). This implies variation within the species, which should further be investigated using fluorescently labelled primers and a larger set of samples. The primers $(CA)_7C$, $(GT)_7G$ and $(CT)_8$ did not amplify after optimisation attempts (data not shown). Species identification using ISSRs could not be verified.

3.4.2.3. *Penicillium* species identification

The aligned sequence data set of the ITS region spanned 525 base pairs with 228 parsimony-informative characters. Eight hundred and thirty parsimonious trees (PT) were produced through parsimony analysis of the alignment with a 100 random sequence input orders. A consensus tree was selected and evaluated with 1000 bootstrap replications in a heuristic search for clade stability (Fig. 7). Reference sequences from Genbank were included in the parsimony analysis to aid in the identification of the *Penicillium* species. The accession numbers of the reference sequences can be found in Table 5. The tree scores for the parsimonious tree were TL = 632, CI = 0.600, RI = 0.912 and RC = 0.547. The ITS gene tree divided the references and 8 *Penicillium* isolate sequences into 13 clades. The bulk of the *Penicillium* isolates were identified as *P. ramulosum* (Clade 1) and *P. expansum* (Clade 4). Clade 4 consisted of 42 *Penicillium* isolates as well as the *P. expansum* reference sequence, with only ten isolates used in the phylogenetic tree (Fig. 7). Isolates were also identified as *P.* sp. (aff. *cecidicola*), *P. cecidicola, P. paneum, P. solitum, P. crustosum, P. brevicompactum, P. novae-zeelandiae, P. glabrum* and *P. rugulosum.* One isolate had been incorrectly identified morphologically as *Penicillium*, but was molecularly identified, with very good bootstrap support (100%), as *Clonostachys rogersoniana* (Clade 12). *Talaromyces helicus* was used to root the phylogenetic tree (Fig. 7).

The population distribution of *Penicillium* was determined from each collection pre- and post-harvest as well as for each of the inoculum sources, mummies, mites and air (Table 6). *Penicillium expansum* was the only species that occurred throughout the distribution area and *P. ramulosum* occurred only from the symptomatic fruit pre- and post-harvest (Table 6). Small percentages of the other species occurred throughout the collection sources (Table 6).

3.4.3. *Penicillium* species associated with pre-and post-harvest wet core rot

3.4.3.1. Morphological identification

From the WCR isolations, 60 samples from pre-harvest and 63 samples from post-harvest were identified as the genus *Penicillium.* The other WCR isolations were

morphologically identified as the following genera: *Alternaria, Botryosphaeria, Botrytis, Epicoccum, Fusarium, Gliocladium, Phoma, Phomopsis, Ulocladium* and *Verticillium.*

3.4.3.2. Molecular identification

The partial beta-tubulin PCR-RFLP distinguishes between the various *Penicillium* spp. giving each a specific band pattern (Van der Walt *et al.*, 2010). The following *Pencillium* species were identified from WCR isolations: *P. expansum, P.* species (aff. *dendriticum*), *P. ramulosum, P. rugulosum, P. chermesinum, P. glabrum* and *P.* species (aff. *cecidicola*). Of the 60 samples collected pre-harvest and the 63 samples post-harvest, 13.33% and 7.94% could not be identified to species level using the beta-tubulin PCR-RFLP (Table 7).

The two most frequently isolated *Penicillium* species from WCR were *P. expansum* and *P. ramulosum,* where *P. expansum* had the highest incidence post-harvest and *P. ramulosum* the highest incidence pre-harvest. *Penicillium expansum* increased from 15% pre-harvest to 52.38% post-harvest, whereas *P. ramulosum* decreased from 50% to 28.57% from pre- to post-harvest. *Penicillium glabrum* was only isolated pre-harvest and *P. rugulosum* occurred only during post-harvest. The other species, *P.* species (aff. *dendriticum*), *P. chermesinum* and *P.* species (aff. *cecidicola*) occur at low frequencies both pre- and post-harvest (Table 7).

3.4.4. Pathogenicity testing

Methods one (surface toothpick method), two (surface pipette method) and four (calyx end wound inoculation) resulted in cone shaped lesions with the widest diameter at the surface of the apple. The lesion was soft and spongy, light brown streaked with darker colour or dark brown. Method five (calyx end non-wounding inoculation) showed symptoms extending from the core of the apple into the flesh towards the surface of the apple. In method three (core inoculation) little to no growth was observed in either the locules or as a substantial infection of the surrounding flesh.

Analysis of variance (ANOVA) for the influence of method, isolate and repetition on the lesion diameter of the pathogenicity testing indicated no significant interaction between the different methods, isolates and repetitions (Table 8). ANOVA indicated a significant difference between the different methods and isolates as well as between the different methods and between the different isolates. ANOVA for the influence of isolates and repetitions for each method was done and compared with another. No significant interaction

occurred between the isolates and repetitions, confirming the stability of the method. There was a significant difference between the isolates for all of the methods (Table 8), except for the deep wounding of the apple with a colonized toothpick method.

The deep wounding of the apple with a colonised toothpick (method 4) had the highest lesion diameter, with the non-wounding with a colonised toothpick (method 5) and inoculation of the core cavity (method 3) having the lowest lesion diameter (Table 9). The t-values showed little variation between the isolates for the deep wounding of the apples with a colonised toothpick (method 4, Table 10) and with ANOVA analysis (Table 11) showing no significant difference between the isolates. The other methods were all significant for the different isolates, and no significant interaction occurred between the isolates and repetitions (Table 11). The surface wounding of apple with colonized toothpicks (method 1) was significant between the isolates, had the second highest mean lesion diameter and no variation occurred between the repetitions or between the isolates and repetitions (Table 10 and 11). Significant differences occurred between the different *Alternaria* species and within the species the isolates varied, except between the isolates of *A. infectoria* where no difference occurred.

3.5. Discussion

Fungi isolated from pre- and post-harvest core rot symptomatic fruit and inoculum sources could be identified to genus level, using morphological characteristics. These included the two most frequently isolated fungi from core rot symptomatic fruit, *Alternaria* and *Penicillium.* It was not possible to identify these fungi to species level using only morphological characteristics, thus molecular tools were implemented to assist in the identification of the *Alternaria* and *Penicillium* species.

In this study, small spored *Alternaria* spp. were identified as *A. infectoria, Embellisia eureka, A. dumosa* and the other three common core rot *Alternaria* species (*A. alternata, A. tenuissima* and *A. arborescens*) were also present. Only the first two were identified using the ITS locus. The combined genetic loci endoPG, OPA1-3 and OPA2-1 further enabled species identification of *A. arborescens, A. alternata, A. dumosa* and *A. tenuissima.*

Small-spored *Alternaria* species are difficult to separate using genetic regions. During this trial the results obtained from the ITS sequence analysis were consistent with what has previously been reported (de Hoog and Horre, 2002). *Alternaria infectoria* can easily be identified using ITS sequence analysis, but the other three core rot causing *Alternaria* species, namely *A. alternata, A. arborescens* and *A. tenuissima,* cannot be

distinguished using the ITS locus. *A. infectoria* could be identified with ITS, due to an insert of 26 bp, that does not occur within the *A. alternata* sequence (de Hoog and Horre, 2002). *Alternaria infectoria* is one of the few *Alternaria* species that has a teleomorph, *Lewia infectoria* (Simmons, 2007). It is possible that due to sexual recombination this insert occurred within *A. infectoria*, but to date no data has been published to support this hypothesis.

Embellisia eureka, a species not previously identified as a core rot pathogen, was identified from the 2009 post-harvest collection in this study. Further pathogenicity tests will have to be conducted to confirm this species' association with apple core rot.

Previous research done by Peever *et al*. (2004 and 2005) combined the various genetic loci (endoPG, OPA1-3 and OPA2-1) aiming to distinguish between the species *A. alternata*, *A. tenuissima* and *A. arborescens* isolates from citrus hosts. The group successfully used phylogenetic analysis to distinguish *A. arborescens* from the other two species, but *A. tenuissima* combined with the *A. alternata* reference sequences. A partition homogeneity test (PHT) performed on the combination of the three datasets or the endoPG and OPA1-3 resulted in a significant P-value of 0.002 (Peever *et al*. 2004). The current study revealed a P-value of 0.001 for the same combination of genetic loci.

Combination of the datasets is therefore controversial, since Cunningham (1997) stated that the accuracy of the phylogenetic analysis increases with an increase in P-value (above 0.01), and if the P-value of the PHT is lower than 0.001 the combined data suffers. This statement contradicts Peever *et al*. (2004) conclusion to combine the genetic loci, due to the low P-value observed in this study.

Due to this controversy the datasets were also presented separately to establish whether each of the genetic loci could distinguish between the *Alternaria* species observed from apple core rot as well as other small-spored *Alternaria* species used as reference isolates. The separate datasets, endoPG and OPA1-3 gave well defined clades, separating *A. arborescens* from *A. alternata*. In the endoPG dataset *A. tenuissima* combined with the reference isolate *A. dumosa*, a species occurring on citrus. Various *A. alternata* reference sequences separated into different clades of the phylogenetic tree. Both pre- and post-harvest isolates as well as air inoculum isolates were identified as the following *Alternaria* species or combination of species, namely *A. alternata*, *A. arborescens*, and the combination of *A. dumosa* and *A. tenuissima* (Table 3, Figure 3). This indicates that the same species occur during pre- and post-harvest as well as from the air inoculum. In the OPA1-3 dataset the *A.*

tenuissima reference isolate combined with the *A. alternata* EGS45-062 in a well supported clade. Both the post-harvest isolates and air inoculum isolates were identified as the following species or combination of species, namely *A. arborescens, A. alternata* combined with *A. tenuissima* and the combination of *A. turkisafria, A. perangusta, A. dumosa* and *A. alternata.* The pre-harvest isolates included all these species as well as *A. alternata* on its own (Table 3, Figure 4). The OPA2-1 dataset distinguished between *A. arborescens* and *A. tenuissima / A. alternata* but the bootstrap values were not well supported and the reference isolates *A. gaisen, A. dumosa, A. tenuissima* and various *A. alternata* isolates grouped together to form one clade. The pre- and post-harvest isolates were identified as the following species or combination of species, namely *A. species, A. dumosa, A. tenuissima* and *A. gaisen.* The air inoculum isolates consisted of *A. arborescens* and the combination of *A. alternata, A. dumosa, A. tenuissima* and *A. gaisen* (Table 3, Figure 5).

Alternaria arborescens is consistent between the three analyses for each of the collection sources. The endoPG analysis gave a more consistent distribution of the species between each of the collection points. The combination of species makes identifying the population difficult.

The phylogenetic results of endoPG and OPA1-3 can be compared with one another to establish which genetic loci distinguishes more between *A. arborescens, A. tenuissima* and *A. alternata.* EndoPG had higher values for the consistency, retention and rescaled consistency indexes, but had lower tree length values and parsimony-informative characters compared to OPA1-3. EndoPG is a gene of known function most likely under negative selection, whereas OPA1-3 is an anonymous region. OPA1-3 has more variation between the species, which increases its chance to separate the species from one another, but variation within the species also occurs. This results in isolates from the same species grouping in different clades of the phylogenetic tree. The combined dataset has low bootstrap values causing certain clades to collapse. This decreases the ability of the phylogenetic tree to distinguish between specific *Alternaria* species except for *A. arborescens.* The isolated *Alternaria* species were distributed equally among the pre- and post-harvest collections as well as from air inoculum, except for *A. arborescens* that occurred more frequently post-harvest than pre-harvest.

Previous research done to distinguish between small-spored *Alternaria* species such as *A. alternata, A. tenuissima* or *A. arborescens* within new genetic regions resulted in no

specific distinction between *A. alternata* and *A. tenuissima* (Peever *et al*. 2004; Peever *et al*. 2005).

Alternaria species have been difficult to separate using DNA fingerprinting. While other studies, that used *Alternaria* isolates ISSR fingerprints of 22 to 34 bands, reported that *Alternaria infectoria* and *A. arborescens* can be separated into two distinct clades, with *A. alternata* and *A. tenuissima* forming one clade, these results could not be repeated in this study (Roberts *et al*., 2000; Pryor and Michailides, 2002; Hong *et al*., 2006). ISSR genotyping resulted in no more than three bands, with no consistency between isolates of the same species (data not shown). Optimising the PCR protocol did not improve the amount of amplification seen, while ITS PCR of the same DNA was successful. Establishing a quick, reliable identification method for *Alternaria* species can be economically important. South Africa has quarantine requirements against *A. mali*, as this species does not occur in the country. Screening of quarantine organisms from imported plant material can reduce the risk of quarantine organisms causing major losses if not contained.

The ITS region for *Penicillium* resulted in thirteen clades, including various Genbank entries. The isolates were identified as *P. ramulosum*, *P.* sp (aff. *cecidicola*), *P.* sp (aff. *dendriticum*), *P. expansum*, *P. paneum*, *P. solitum*, *P. crustosum*, *P. brevicompactum*, *P. novae-zeelandiae*, *P. glabrum* and *P. rugulosum*. *Penicillium expansum* and *P. ramulosum* were the two species most often identified, were *P. expansum* (N=42) and *P. ramulosum* (N=30). *Penicillium expansum* is distributed through all of the collection sources, with high incidences in each of the inoculum sources. *Penicillium ramulosum* occurred during pre- and post-harvest collection, but did not occur in any of the inoculum sources. The remaining isolates were distributed between the other species. *Penicillium novae-zeelandiae* was isolated from the air inoculum. There has been no previous mention of this species in literature of core rot. Pathogenicity tests were not done using this species thus presently we do not know if this species is pathogenic on apples.

Previously, *P. funiculosum* was identified as the main *Penicillium* species isolated from core rot symptoms (Combrink *et al*., 1985), yet none of the isolates were identified as *P. funiculosum* through sequence BLAST analysis. This could be due to a too small sample size, the fungus not occurring in the orchard or isolates previously identified as *P. funiculosum* morphologically are now, using molecular techniques, identified as other or new *Penicillium* species.

Wet core rot (WCR) is an economically important symptom of core rot as it progresses quickly through the tissue of the fruit causing a wet rot. In South Africa, WCR was initially thought to be a post-harvest symptom due to DPA treatment for physical scald as the DPA reduced the water tension and allowed spores to freely move into the open calyx of susceptible fruit (Combrink and Ginsburg, 1973; Combrink *et al*., 1985; Spotts *et al*., 1988a, 1988b). Wet core rot was detected prior to harvest by De Kock *et al*. (1991) and McLeod *et al*. (2008) from fruit picked from the trees. Previously WCR isolates were identified as *Penicillium expansum* and *P. funiculosum* (Combrink and Ginsburg, 1973; Combrink *et al*., 1985). Newer techniques either through morphological identification using colour plates and fungal growth (Samson *et al*., 2002) or molecular techniques, such as the genetic loci, beta-tubulin (Seifert *et al*., 2007) gives as a wider range of species being isolated from WCR symptoms. Van der Walt *et al*. (2010) used the partial beta-tubulin PCR-RFLP to identify species of *Penicillium* isolated from pre-harvest (fruit picked from trees) WCR. This resulted in specific band patterns for each of the following species: *P. expansum, P. sp.* (aff. *dendriticum*), *P. ramulosum, P. chermesinum, P. glabrum,* and *P.*sp. (aff. *cecidicola*).

The identification of *Penicillium* species from pre-harvest WCR (fallen fruit) and post-harvest WCR were done using the partial beta-tubulin PCR-RFLP method and comparing the isolates' band patterns with the identified species of Van der Walt *et al*. (2010). The two main species isolated from both pre- and post-harvest were *P. expansum* and *P. ramulosum. Penicillium expansum* occurred more frequently post-harvest and *P. ramulosum* more frequently pre-harvest. The other isolates were distributed between the other specified species. These isolates occurred in low distributions both in pre- and post-harvest WCR. Van der Walt *et al*. (2010) isolated *P. ramulosum* frequently from pre-harvest WCR symptoms, followed by *P. expansum.* The results suggest that prior to harvest the fruit is infected with *P. ramulosum* and that some of the fruit drop prematurely due to the infection of the fungi. The infected fruit that does not drop prematurely ripens normally and is harvested with the rest of the orchard. These fungi stay latent until after storage, when favourable conditions increases the fungi's infection rate. *Penicillium expansum* is an opportunistic fungus infecting fruit through small openings. *Penicillium expansum* is also the main causal organism of 'blue mould' and it produces the toxin patulin in apples (Sanderson and Spotts, 1995; Pitt and Hocking, 1997).

Previous work done on WCR established that DPA used to prevent superficial scald reduced the water tension of the flume water (Combrink and Ginsburg, 1973; Combrink *et*

al., 1985; Spotts *et al*., 1988). Consequently contaminated water flowed into the core regions of susceptible cultivars and infection took place. Currently DPA is being phased out and replaced with 1-MCP, a gaseous ethylene inhibitor (Kim and Xiao, 2008). 1-MCP does not protect against fungi and can increase the decay incidence of fungi (Janisiewicz *et al*., 2003; Leverentz *et al*., 2003; Kim and Xiao, 2008).

A high incidence of *P. expansum* was found post-harvest. The fruit was treated with 1-MCP, but was not subjected to the flume water or subsequently DPA. It is possible that the drench water was contaminated with *P. expansum* increasing the incidence, from prior to harvest infection with a secondary infection post-harvest. Some of the isolates for both pre- and post-harvest could not be identified using the specific species' band patterns of the partial beta-tubulin genetic loci. It is possible that these isolates are other *Penicillium* species that were not tested for, such as *P. crustosum, P. solitum,* and *P. funiculosum* that occur frequently in other apple diseases (Combrink *et al*., 1985; Sanderson and Spotts, 1995).

The need for a reliable pathogenicity test for core rot is required to differentiate between the isolates which are non-pathogenic (endophytes or latent) and pathogic. Five inoculation methods from previous publications were compared to select the most reliable method. The most reliable test should be able to distinguish between the virulence of the pathogenic isolates and to indicate stability between the different replications of the isolates used. The deep wounding method gave the highest mean lesion diameter of the five methods tested, but no significant interaction was observed between the different isolates. This result confirms what Van der Walt *et al*. (2010) observed with this technique for the pathogenicity and virulence of *Penicillium* species. A reason indicated for the lack of virulence is due to the wounding, where endogenous fungi occurring in the core can cause infections along with the inoculated isolate (Van der Walt *et al*., 2010).

The non-wounding method and core inoculations resulted in the smallest lesion diameters of the five methods. The non-wounding method showed little consistency in the size of the lesions while some of the isolates were more virulent than others, resulting in larger lesions. Van der Walt *et al*. (2010) observed similar trends with isolates being able to infect without artificial wounding. The core inoculation method was inconsistent between the isolates in causing lesions, although variation did occur between the isolates. Due to the open mesoderm, contamination with opportunistic fungi occurred frequently.

The surface wounding method resulted in the second highest lesion diameter with significant interactions between the isolates. No significant variations occurred between the

repetitions or between the isolate and repetition interaction. The surface method is reliable, due to its stability between the repetitions, its lesion size and the variation in virulence between the isolates.

Serdani *et al*. (2002) found three lesion types that correlated well the different *Alternaria* species. During this trial the predominant lesion found was a soft brown lesion with or without brown streaks, which occurred in all the *Alternaria* species. According to Serdani *et al*. (2002) *A. infectoria* was non-pathogenic, causing no lesions and *A. tenuissima* had the highest virulence and was the main *Alternaria* species that caused DCR. The current results show *A. infectoria* producing moderate lesion diameters. It is possible that due to isolate variation occurring within this method, that different isolates of the same species can be pathogenic or endophytic. The results show similar trends with *A. tenuissima* having the highest mean lesion diameter but was not significantly different than some of the isolates of the other species.

In conclusion although *A. alternata* and *A. tenuissima* could not be separated using the molecular techniques, *A. infectoria, A. dumosa* and *A. arborescens* could be separated and identified. This confirms previous research done by Peever *et al*. (2004). More gene regions need to be tested apart and in combination to distinguish between these small-spored species. Although the inter simple sequence repeats (ISSRs) gave no conclusive results more combinations should be looked at as this could possibly be a useful tool for identifying small-spore *Alternaria* species.

Wet core rot is not just caused by *Penicillium expansum* but through a host of different species, including *P. ramulosum* pre-harvest. Although the incidence of the *Penicillium* species that occurred in both pre- and post-harvest isolations differed, a link could be established between the species that occurred in both pre- and post-harvest WCR. Managing this link between pre- and post-harvest can be problematic. An integrated management strategy should be implemented where chemical control should be used in combination with sanitation practices, both in the orchard and in the pack house, to reduce the inoculum build up of the same species.

A reliable pathogenicity test is required to differentiate between isolates which are non-pathogenic and pathogenic and should include parameter such as stability between replications and virulence of the pathogenic isolates. Of the five methods tested the surface toothpick method resulted in good lesion diameter, variation between the various *Alternaria*

isolates and no variation between the replications. The inoculation of the open mesoderm core cavity resulted in the least reliable method to distinguish pathogenicity.

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Table 1: Identification of *Alternaria* and *Penicillium* isolates using genetic loci.

Locus	Primers	Sequence	Reference	Initial	Cycles	Denaturation	Annealing	Elongation	Extension
ITS	V9G	5'-TTACGTCCCTGCCCTTTGTA-3'	de Hoog and Gerrits van den Ende, 1998	5 min @ 94°C	40	45s @ 94°C	45s @ 55°C	1 min @ 72°C	7 min @ 72°C
	Ls266	5'-AATGTGCGTTCAAAGATTCG-3'							
	ITS4	5'-TCCTCCGCTTATTGATATGC-3'	White *et al.* 1990						
OPA1-3	OPA1-3L	5'-AGGCCCTTCCAATCCAT-3'	Peever *et al.*, 2005	1 min @ 94°C	35	20s @ 94°C	20s @ 56°C	20s @ 72°C	
	OPA1-3Rb	5'-AGCCACATGCTCTGGTTAGC-3'	Peever *et al.*, 2005						
OPA2-1	OPA2-1L	5'-TGCCGAGCTGTCAGATAATTG-3'	Peever *et al.*, 2005						
	OPA2-1R	5'-GCCGAGCTGGTGGAGAGAGT-3'	Peever *et al.*, 2005						
OPA10-2	OPA10-2L	5'-TCGCAGTAAGACACATTCT(G)ACG-3'	Andrew *et al.* 2009	1 min @ 95°C		30s @ 95°C	30s @ 62°C	30s @ 72°C	
	OPA10-2R	5'-GATTCGCAGCAGGGAAACTA-3'	Andrew *et al*. 2009						
endoPG	PG3	5'-TACCATGGTTCTTTCCGA-3'	Isshiki *et al.* 2001	2 min @ 95°C		1 min @ 95°C	1 min @ 50°C	1 min @ 72°C	5 min @ 72°C
	PG2b	5'-GAGAATTCRCARTCRTCYTGRTT-3'	Isshiki *et al.* 2001						

Table 2: Accession numbers of *Alternaria* reference isolates for the genetic loci endoPG, OPA1-3 and OPA2-1 from Genbank.

			Genbank accession number		
Name	**Isolate**	**ITS**	**endoPG**	**OPA1-3**	**OPA2-1**
A. alternata	EGS 34-016		AY295024.1	AY295043.1	AY295065.1
A. alternata	EGS 34-039		AY295025.1	AY295044.1	AY295064.1
A. alternata	EGS 45-002		AY629224.1	AY631442.1	AY631467.1
A. alternata	EGS 45-008		AY629226.1	AY631444.1	AY631469.1
A. alternata	EGS 45-010		AY629227.1	AY631445.1	AY631470.1
A. alternata	EGS 45-062	AY751455.1	AY629228.1	AY631446.1	AY631471.1
A. arborescens	EGS 39-128	AF347033.1	AY295028.1	AY295035.1	AY295071.1
A. dumosa	EGS 45-007		AY629225.1	AY631443.1	AY631468.1
A. gaisen	EGS 37-1321		AY629231.1	AY631439.1	AY631464.1
A. longipes	EGS 30-033		AY629232.1	AY295038.1	AY295068.1
A. perangusta	EGS 44-160		AY295023.1	AY295053.1	AY295055.1
A. tenuissima	EGS 34-015	AY751455.1	AY629223.1	AY631438.1	AY631463.1
A. turkisafria	EGS 44-159		AY295022.1	AY295051.1	AY295054.1
A. infectoria	EGS27-193	AF347034.1			
E. eureka	EGS 36-103	AF392989.1			
E. eureka		JN383490.1			

Table 3: The identification of *Alternaria* isolates collected from pre- and post-harvest samples and air inoculum, using the genetic loci, endoPG, OPA1-3, OPA2-1 and OPA10-2 as well as the combination of the first three.

Isolate*	Source	endoPG**	OPA1-3**	OPA2-1**	OPA10-2**	Combination**
Alt2	Pre-harvest 09	*d / te*	*tu / p / d / al*	*al / d / te / g*	*al / g*	*d*
Alt3	Pre-harvest 09	*al*	*tu / p / d / al*	*al / d / te / g*	*al / g*	*al*
Alt8	Pre-harvest 09	*ar*	*ar*	*ar*	*ar*	*ar*
Alt9	Pre-harvest 09	*d / te*	*tu / p / d / al*	*al / d / te / g*	*al / g*	*d*
Alt10	Pre-harvest 09			*A. infectoria*		
Alt11	Post-harvest 09	*al*	*al / te*	species	*al / g*	*al*
Alt12	Post-harvest 09	*d / te*	*tu / p / d / al*	*al / d / te / g*	*al / g / tu / p*	*d*
Alt14	Post-harvest 09	*d / te*	*tu / p / d / al*	*al / d / te / g*	*al / g / tu / p*	*d*
Alt15	Post-harvest 09	*ar*	*ar*	*ar*	*ar*	*ar*
Alt16	Post-harvest 09			*Embellisia* spp.		
Alt19	Post-harvest 09	*ar*	*ar*	*ar*	*ar*	*ar*
Alt20	Post-harvest 09	*ar*	*ar*	*ar*	*ar*	*ar*
Alt21	Pre-harvest 10	*d / te*	*tu / p / d / al*	*al / d / te / g*	species	*d*
Alt22	Pre-harvest 10	*d / te*	*al / te*	*ar*	*al / g / tu / p*	*te*
Alt23	Pre-harvest 10	*d / te*	*tu / p / d / al*	*al / d / te / g*	*al / g / tu / p*	*d*
Alt24	Pre-harvest 10	*d / te*	*al*	species	*al / g*	*al*
Alt25	Pre-harvest 10	*d / te*	*al*	species	*ar*	*al*
Alt26	Pre-harvest 10	*ar*	*ar*	*ar*	*ar*	*ar*
Alt27	Pre-harvest 10	*al*	*tu / p / d / al*	*al / d / te / g*	*al / g*	*al*
Alt28	Pre-harvest 10	*d / te*	*tu / p / d / al*	*al / d / te / g*	species	*d*
Alt29	Pre-harvest 10	*d / te*	*al*	species	no sequence	*al*
Alt30	Pre-harvest 10	*al*	*tu / p / d / al*	*al*	*al / g*	*al*
Alt32	Post-harvest 10	*ar*	*ar*	*ar*	*ar*	*ar*
Alt33	Post-harvest 10	*d / te*	*tu / p / d / al*	*species*	*al / g*	*d*
Alt34	Post-harvest 10	*d / te*	*tu / p / d / al*	*al / d / te / g*	*al / g / tu / p*	*d*
Alt35	Post-harvest 10	*ar*	*ar*	*ar*	*ar*	*ar*
Alt36	Post-harvest 10	*ar*	*ar*	*al / d / te / g*	*ar*	*ar*
Alt38	Post-harvest 10	*ar*	*ar*	*ar*	*ar*	*ar*
Alt39	Post-harvest 10	*al*	*al / te*	*al*	species	*al*
Alt40	Post-harvest 10	*ar*	*ar*	*ar*	*ar*	*ar*
Alt41	Air inoculum 10	*d / te*	*tu / p / d / al*	*al / d / te / g*	*al / g / tu / p*	*d*
Alt42	Air inoculum 10	*ar*	*ar*	*ar*	*ar*	*ar*
Alt44	Air inoculum 10	*d / te*	*tu / p / d / al*	*al / d / te / g*	*al / g*	*d*
Alt45	Air inoculum 10	*al*	*al / te*	*al / d / te / g*	*al / g / tu / p*	*al*
Alt46	Air inoculum 10	*d / te*	*tu / p / d / al*	*al / d / te / g*	species	*d*
Alt48	Air inoculum 10	*d / te*	*tu / p / d / al*	*al / d / te / g*	*al / g / tu / p*	*d*
Alt49	Air inoculum 10	*al*	*tu / p / d / al*	*al / d / te / g*	*al / g*	*al*
Alt50	Post-harvest 10	*d / te*	*tu / p / d / al*	*al / d / te / g*	species	*d*
Alt51	Post-harvest 10	*d / te*	*tu / p / d / al*	*al / d / te / g*	*al / g / tu / p*	*d*

* Isolates not virulent: Alt1, 4, 5, 6, 7 (pre-harvest 2009), 13, 17, 18 (post-harvest 2009), 31, 37 (post-harvest 2010), 43, 47 (Air inoculum 2010)

** Abbreviation of reference *Alternaria* species used to identify *Alternaria* isolates collected: *al: A. alternata, ar: A. arborescens, d: A. dumosa, g: A. gaisen, p: A. perangusta, te: A. tenuissima, tu: A. turkisafria* and species: *Alternaria* spp. not identified.

Table 4: The population distribution (%) of the identified *Alternaria* isolates for each collection source.

	Pre-harvest 2009	**Post-harvest 2009**	**Pre-harvest 2010**	**Post-harvest 2010**	**Air inoculum 2010**
endoPG analysis					
n	4	6	10	10	7
A. alternata	25.00	16.67	20.00	10.00	28.57
A. arborescens	25.00	50.00	10.00	50.00	14.29
A. dumosa / A. tenuissima	50.00	33.33	70.00	40.00	57.14
OPA1-3 analysis					
n	4	6	10	10	7
A. alternata	0.00	0.00	30.00	0.00	0.00
A. alternata / A. tenuissima	0.00	16.67	10.00	10.00	14.29
A. arborescens	25.00	50.00	10.00	50.00	14.29
A. turkisafria / perangusta / dumosa / alternata	75.00	33.33	50.00	40.00	71.43
OPA2-1 analysis					
n	4	6	10	10	7
A. alternata	0.00	0.00	10.00	10.00	0.00
A. alternata / A. dumosa / A. tenuissima / A. gaisen	75.00	33.33	40.00	40.00	85.71
A. arborescens	25.00	50.00	20.00	40.00	14.29
A. species	0.00	16.67	30.00	10.00	0.00
Combination analysis					
n	4	6	10	10	7
A. alternata	25.00	16.67	50.00	10.00	28.57
A. arborescens	25.00	50.00	10.00	50.00	14.29
A. dumosa	50.00	33.33	30.00	40.00	57.14
A. tenuissima	0.00	0.00	10.00	0.00	0.00

Table 5: Genbank accession numbers of *Penicillium* reference sequences of the gene ITS, used in *Penicillium* phylogenetic tree.

Name	**Isolate**	**Genbank accession number**
Clonostachys rogersoniana	CBS 582.89	AF 210 691
P. brevicompactum	EPA462	AY 373 899
P. cecidicola	DAOM 233329	AY 787 844
P. chermesinum	STE-U6583	FJ 491 802
P. chermesinum	NRRL 2048	AY 742 693
P. crustosum	DAOM 215345	JN 942 857
P. dendriticum	DAOM 226674	AY 787 842
P. expansum	CBS 325.48	AB 479 309
P. funiculosum	DAOM 221148	JN 942 854
P. glabrum	STE-U6577	FJ 491 804
P. novae-zeelandiae	NRRL 35618	EF 200 078
P. paneum	CBS 465.95	HQ 442 349
P. ramulosum	STE-U6584	FJ 491 788
P. ramulosum	STE-U6579	FJ 491 800
P. ramulosum	STE-U6571	FJ 491 794
P. rugulosum	DAOM 216317	JN 942 851
P. solitum	CBS 14786	HQ 225 713
P. sp.	CMV-2008 cv330	EU 795 707
P. sp.	CMV-2008 cv318	EU 795 708
P. sp. *(*aff. *cecidicola)*	STE-U6582	FJ 491 803
P. sp. *(*aff. *dendriticum)*	STE-U6575	FJ 491 806
Talaromyces helicus	NRRL 2106	AF 033 396

Table 6: The population distribution (%) of the identified *Penicillium* isolates for each collection source.

Name	Pre-harvest 2009	Pre-harvest 10	Post-harvest 2009	Post-harvest 2010	Mummies 2009	Mummies 2010	Mites 2009	Mites 2010	Air A 2010	Air B 2010
n	11	10	11	10	10	10	10	10	11	8
C. rogersoniana	9.09	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
P. brevicompactum	0.00	0.00	0.00	0.00	0.00	0.00	0.00	10.00	0.00	0.00
P. cecidicola	9.09	0.00	0.00	0.00	0.00	0.00	10.00	0.00	9.09	12.50
P. crustosum	0.00	0.00	9.09	0.00	0.00	0.00	0.00	0.00	0.00	0.00
P. expansum	27.27	10.00	18.18	30.00	70.00	60.00	40.00	40.00	45.45	87.50
P. glabrum	0.00	0.00	0.00	0.00	0.00	0.00	10.00	0.00	18.18	0.00
P. novae-zeelandiae	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	9.09	0.00
P. paneum	0.00	0.00	0.00	0.00	10.00	0.00	0.00	0.00	0.00	0.00
P. ramulosum	45.45	60.00	72.73	70.00	0.00	0.00	0.00	0.00	0.00	0.00
P. rugulosum	0.00	0.00	0.00	0.00	0.00	20.00	0.00	0.00	0.00	0.00
P. solitum	0.00	10.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
P. sp. (aff. *cecidicola*)	0.00	10.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
unidentified	9.09	10.00	0.00	0.00	20.00	20.00	40.00	50.00	18.18	0.00

Table 7: The percentage of isolates from pre- and post-harvest WCR identified as various *Penicillium* species through partial beta-tubulin PCR-RFLP.

Species	Pre-harvest	Post-harvest
P. expansum	15.00	52.38
P. species (aff. dendriticum)	5.00	6.35
P. ramulosum	50.00	28.57
P. rugulosum	0.00	1.59
P. chermesinum	5.00	1.59
P. glabrum	10.00	0.00
P. sp. (aff. *cecidicola*)	1.67	3.17
not identified	13.33	7.94
n[a]	60	63

[a] Total number of samples

Table 8: Analysis of variance of the mean lesion diameter of *A. alternata*, *A. tenuissima*, *A. infectoria* and *A. arborescens* isolates from pathogenicity tests.

Source	DF	Mean squares	F	Pr > F
Method	4	22778.427	342.265	< 0.0001
Isolate	8	782.141	11.752	< 0.0001
Rep	2	8.841	0.133	0.876
Method*Isolate	32	223.996	3.366	< 0.0001
Method*Rep	8	44.562	0.670	0.719
Isolate*Rep	16	42.992	0.646	0.848
Method*Isolate*Rep	64	61.416	0.923	0.649
Error	1349	66.552		
Corrected Total	1483			

Table 9: Mean[a] lesion diameter for the five pathogenicity tests.

Methods	LS means	Groups			
4	25.145	A			
1	15.035		B		
2	7.099			C	
5	5.155				D
3	4.513				D

[a]Means indicated by different letters which differed significantly at the 5% level according to Fischer's least significant difference (LSD) test.

Table 10: Mean[a] lesion diameter of the nine *Alternaria* isolates for the five pathogenicity tests.

	Method 1	**Method 2**	**Method 3**	**Method 4**	**Method 5**
A. tenuissima (7629)	21.758 c-e	9.379 g-l	10.848 g-j	25.288 a-c	7.470 i-n
A. infectoria (7635)	10.439 g-k	5.561 l-q	1.227 r	25.818 a-b	4.803 m-r
A. infectoria (7634)	11.121 g-i	4.015 n-r	1.121 r	28.318 a	2.197 q-r
A. arborescens (7637)	16.485 i	8.000 h-m	4.136 m-r	27.318 a-b	6.364 l-p
A. arborescens (7636)	20.197 d-f	11.621 g-h	4.697 m-r	23.576 b-d	6.788 k-p
A. alternata (7632)	12.348 g	9.318 g-l	3.697 n-r	24.591 a-c	5.242 m-q
A. alternata (7633)	18.303 e-f	7.091 j-o	7.303 i-o	24.848 a-c	6.136 l-p
A. tenuissima (7631)	18.606 e-f	4.379 m-r	2.953 p-r	24.970 a-c	3.500 o-r
A. tenuissima (7630)	6.061 l-q	4.530 m-r	4.667 m-r	21.576 c-e	3.894 n-r

[a]Means indicated by different letters which differed significantly at the 5% level according to Fischer's least significant difference (LSD) tests.

Table 11: Analysis of variance for each of the five pathogenicity methods to indicate variation between isolates, but not between repetitions or between the isolates and the repetitions.

	Method 1				Method 2				Method 3				Method 4				Method 5			
Source	**DF**	**Mean squares**	**F**	**Pr > F**	**DF**	**Mean squares**	**F**	**Pr > F**	**DF**	**Mean squares**	**F**	**Pr > F**	**DF**	**Mean squares**	**F**	**Pr > F**	**DF**	**Mean squares**	**F**	**Pr > F**
Isolate	8	913.968	15.878	<0.0001	8	236.193	5.845	<0.0001	8	304.459	4.071	0.000	8	127.434	1.138	0.338	8	97.592	2.041	0.042
Rep	2	28.495	0.495	0.610	2	77.304	1.913	0.150	2	19.607	0.262	0.770	2	57.551	0.514	0.599	2	2.316	0.048	0.953
Isolate*Rep	16	47.109	0.818	0.664	16	33.641	0.832	0.648	16	43.532	0.582	0.896	16	99.172	0.886	0.586	16	65.759	1.375	0.153
Error	270	57.561			270	40.411			270	74.791			270	111.950			270	47.812		
Corrected Total	296				296				296				296				296			

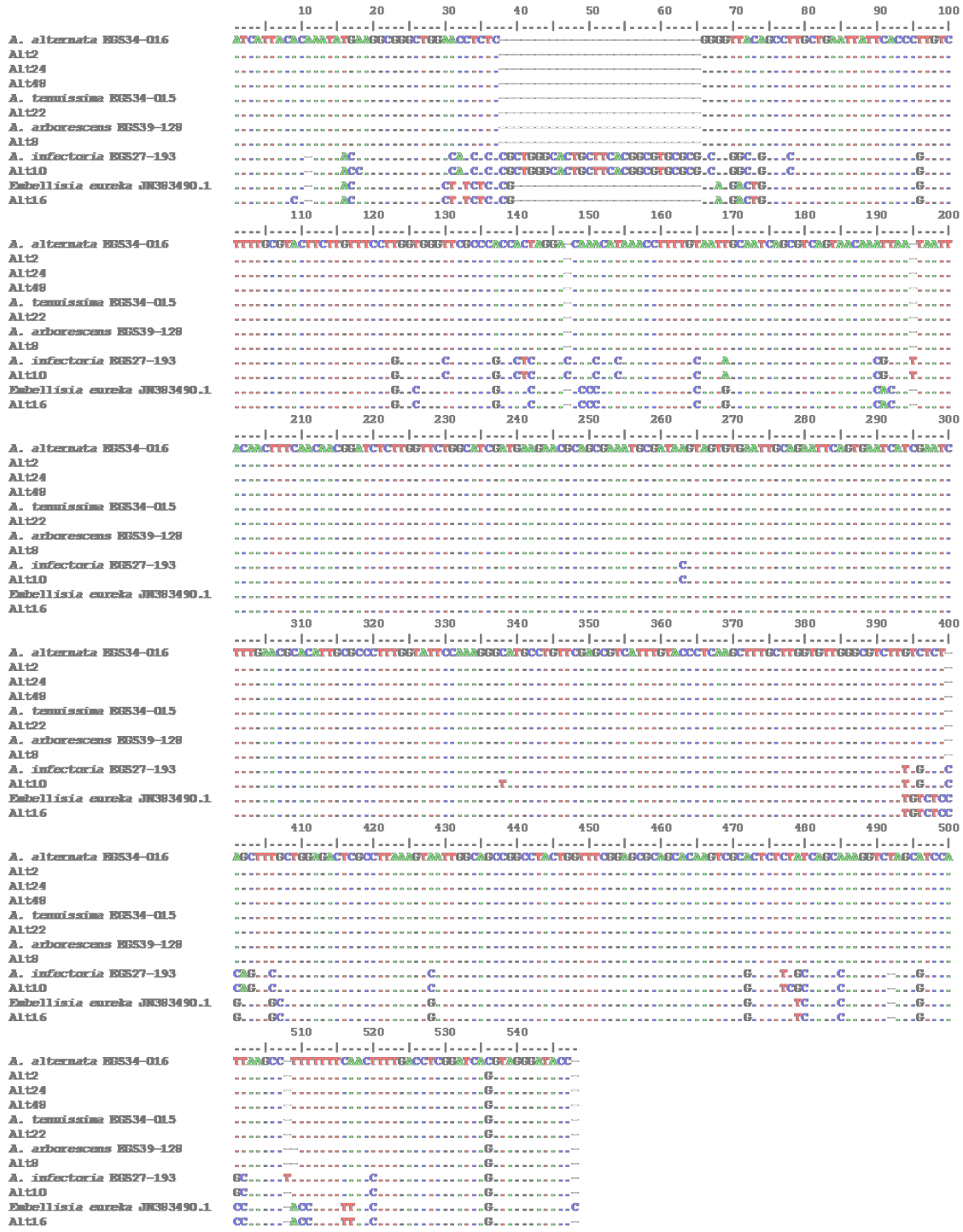


Figure 1: Multiple alignments of internal transcribed spacer region sequences of *Alternaria* and *Embellisia eureka* reference strains and SA apple core rot isolates (Alt2-Alt48). A dash (-) indicates insertion/deletion events and dots (.) indicate the same nucleotide as the chosen representative strain, ex-type *A. alternata* EGS34-016. Nucleotide changes are indicated in the alignment.

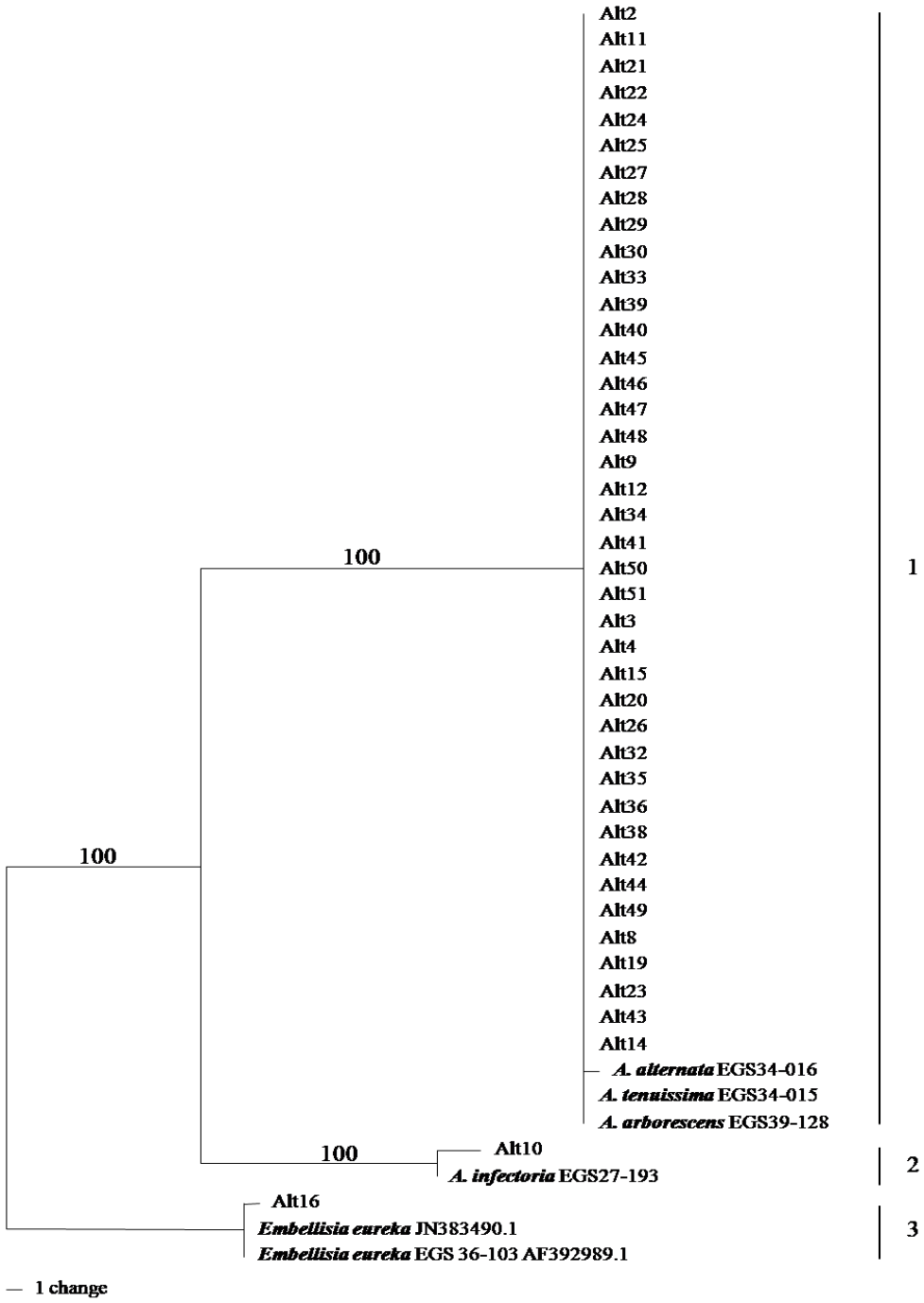


Figure 2: Phylogeny of *Alternaria* species based on the ITS region. The tree presents one parsimonious tree of a heuristic search. The reference isolates *A. alternata, A. tenuissima* and *A. arborescens* grouped together in clade one. The phylogenetic tree was rooted with the reference isolate *A. infectoria.* The genus *Embellisia* grouped with one isolate to form clade two.

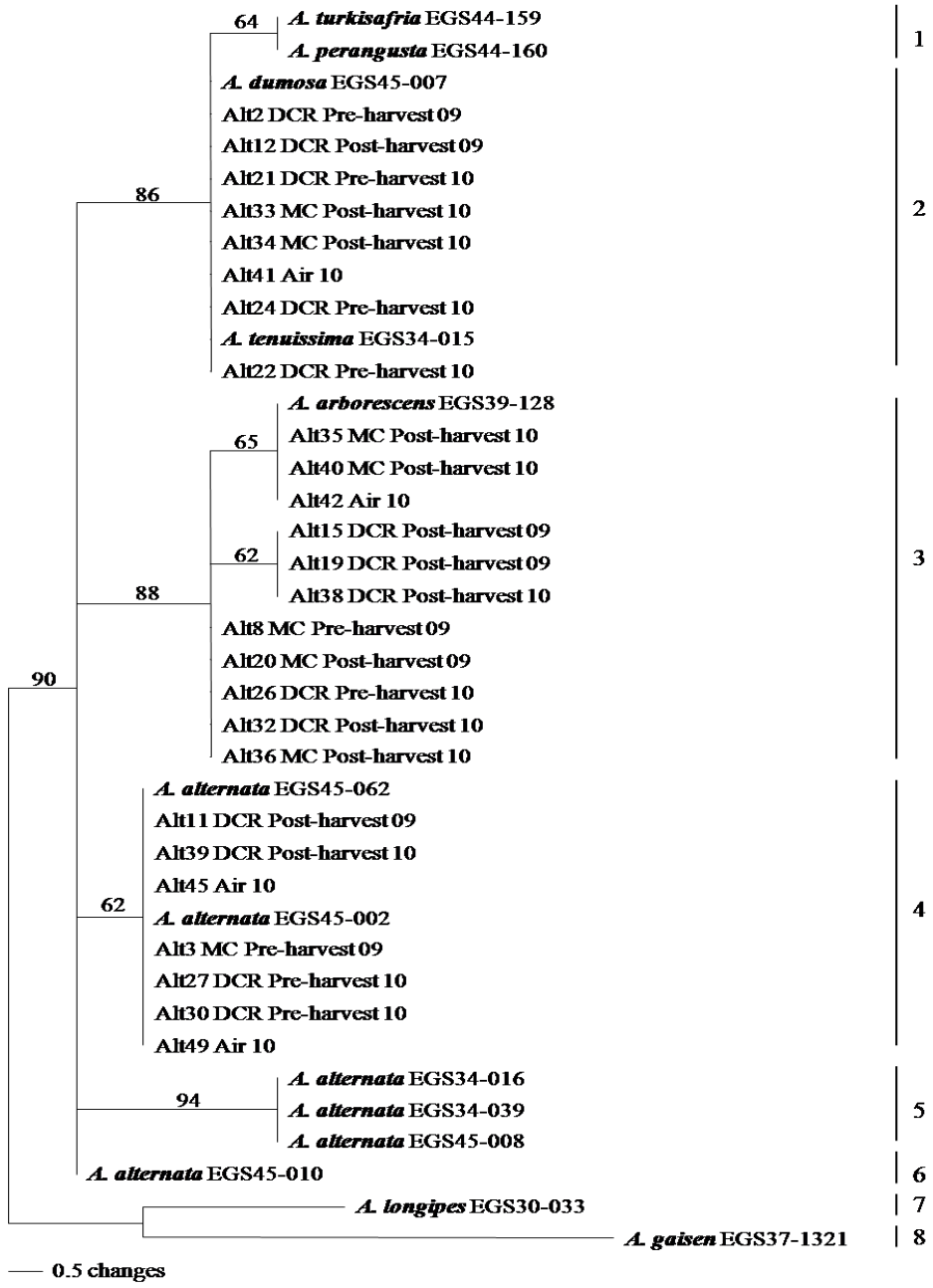


Figure 3: Phylogeny of *Alternaria* species based on the endoPG region. The tree presents one parsimonious tree of a heuristic search. Numbers within the tree represent the bootstrap values with values lower than 60% not shown. A representative sample for each of the seasons of pre- and post-harvest, inoculum source, air and the various core rot symptoms were used in the phylogeny. Sequences of *Alternaria* species identical to the chosen representative isolates were removed. Reference sequences (EGS) represent type strains and isolates as published by Peever *et al*. (2004 and 2005).

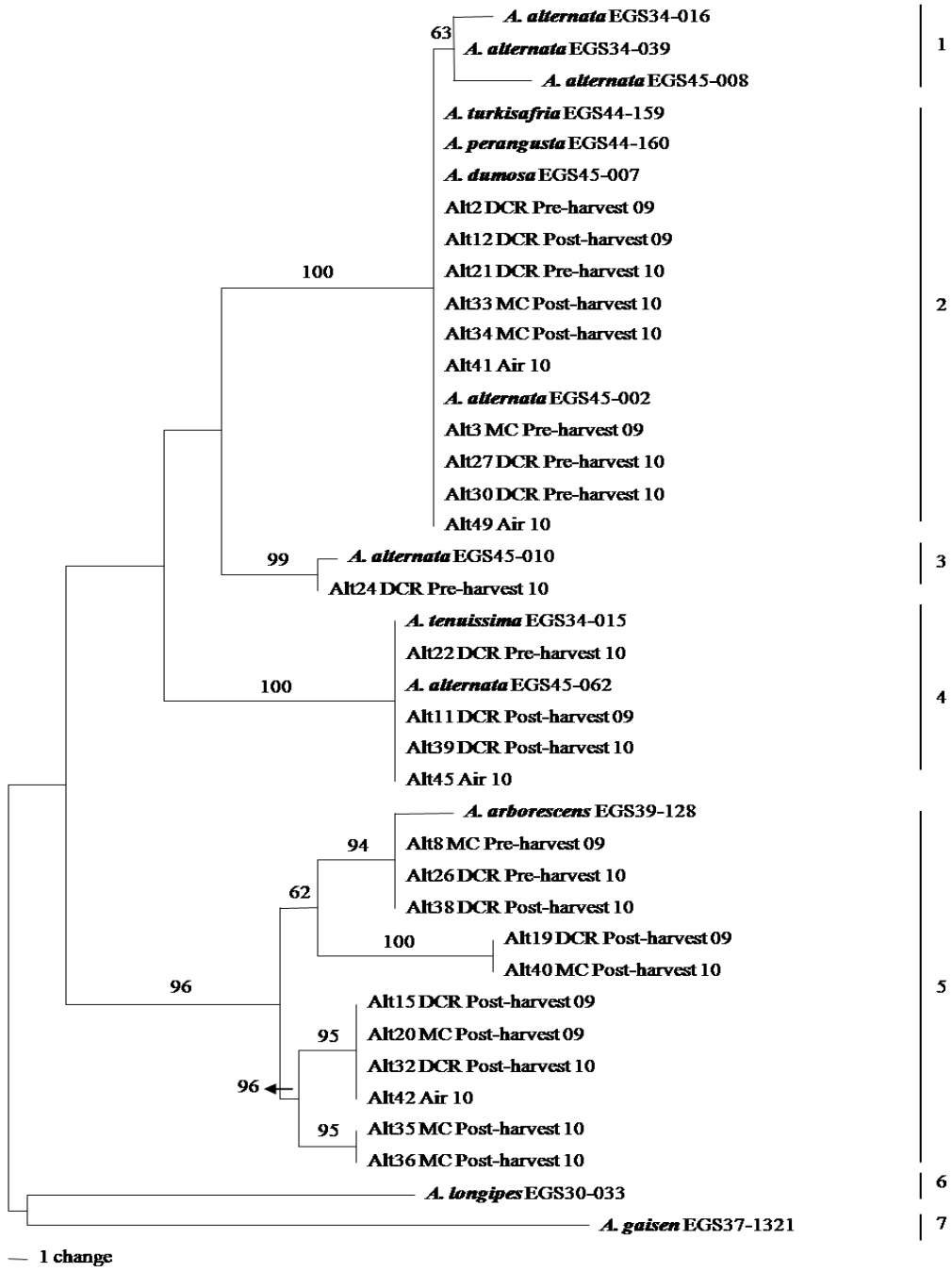


Figure 4: Phylogeny of *Alternaria* species based on the OPA1-3 region. The tree presents one of seven parsimonious trees of a heuristic search. Numbers within the tree represents the bootstrap values with values lower than 60% not shown. A representative sample for each of the seasons of pre- and post-harvest, inoculum source, air and the various core rot symptoms were used in the phylogeny. Sequences of *Alternaria* species identical to the chosen representative isolates were removed. Reference sequences (EGS) represent type strains and isolates as published by Peever *et al*. (2004 and 2005).

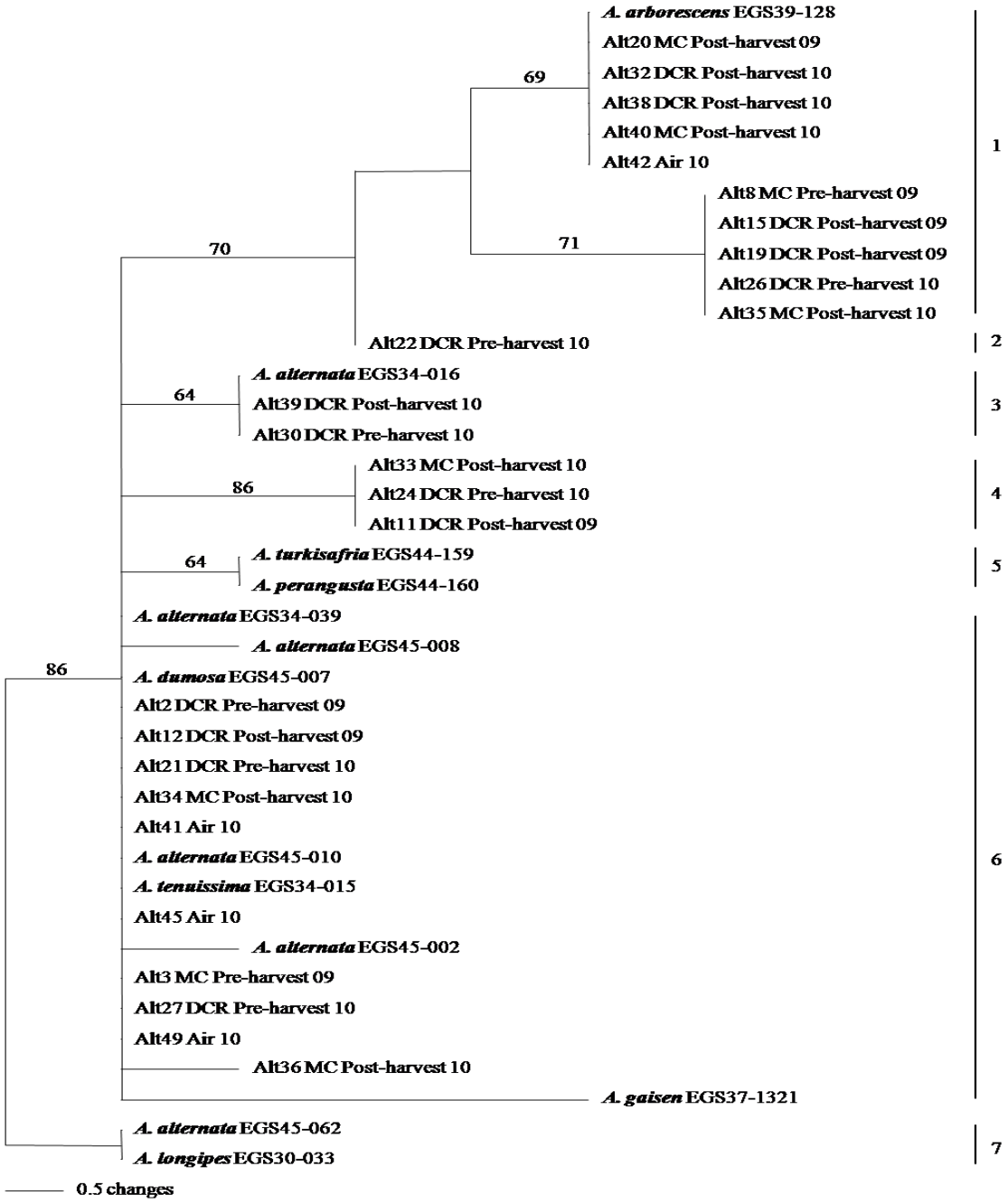
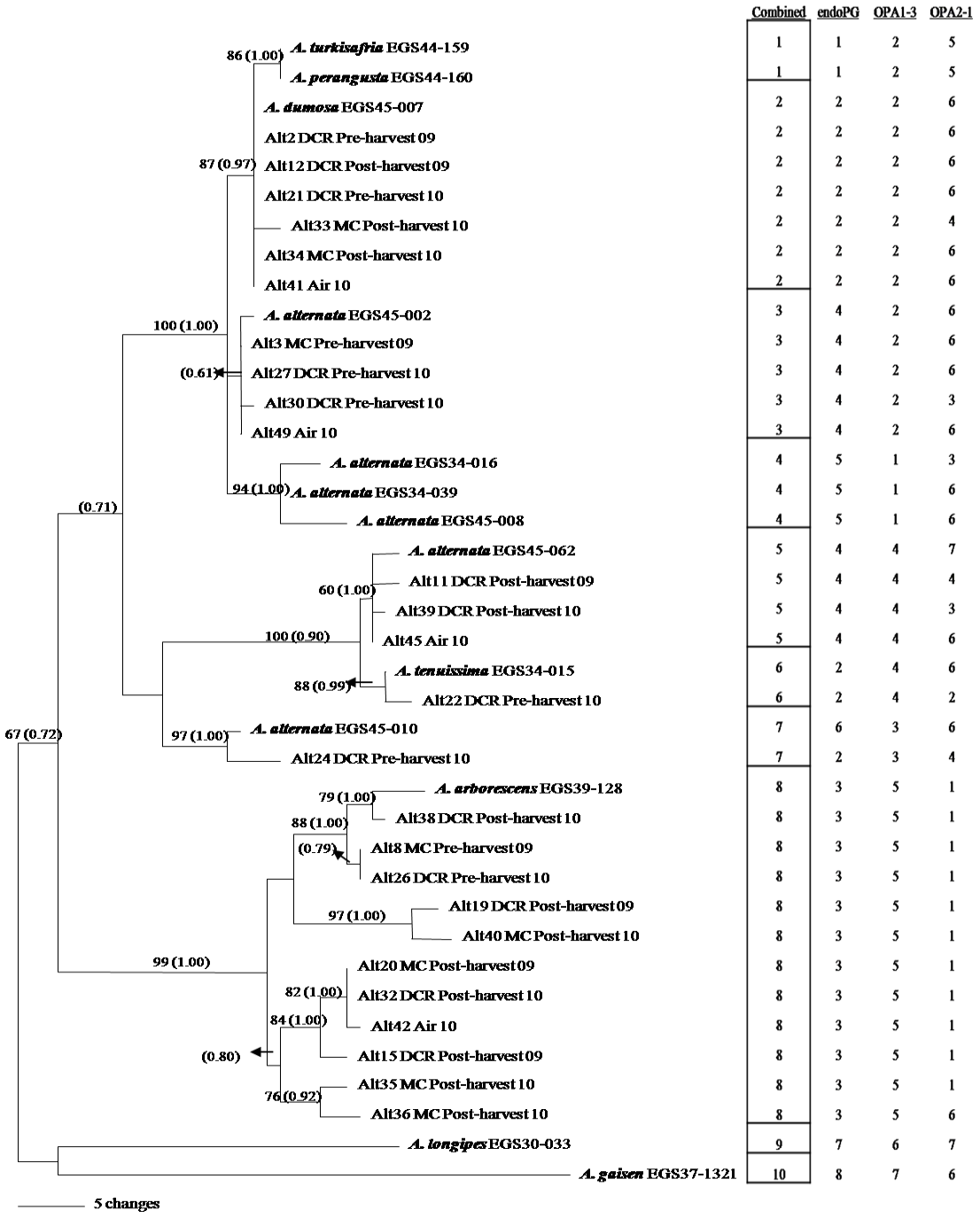


Figure 5: Phylogeny of *Alternaria* species based on the OPA2-1 region. The tree presents one of two parsimonious trees of a heuristic search. Numbers within the tree represents the bootstrap values with values lower than 60% not shown. A representative sample for each of the seasons of pre- and post-harvest, inoculum source, air and the various core rot symptoms were used in the phylogeny. Sequences of *Alternaria* species identical to the chosen representative isolates were removed.Reference sequences (EGS) represent type strains and isolates as published by Peever *et al*. (2004 and 2005).



Combined	endoPG	OPA1-3	OPA2-1
1	1	2	5
1	1	2	5
2	2	2	6
2	2	2	6
2	2	2	6
2	2	2	6
2	2	2	4
2	2	2	6
2	2	2	6
3	4	2	6
3	4	2	6
3	4	2	6
3	4	2	3
3	4	2	6
4	5	1	3
4	5	1	6
4	5	1	6
5	4	4	7
5	4	4	4
5	4	4	3
5	4	4	6
6	2	4	6
6	2	4	2
7	6	3	6
7	2	3	4
8	3	5	1
8	3	5	1
8	3	5	1
8	3	5	1
8	3	5	1
8	3	5	1
8	3	5	1
8	3	5	1
8	3	5	1
8	3	5	1
8	3	5	1
8	3	5	6
9	7	6	7
10	8	7	6

Figure 6: Phylogeny of *Alternaria* species based on the endoPG, OPA1-3 and OPA2-1 regions. The tree presents one of two equally parsimonious trees of a heuristic search. Numbers within the tree represents the bootstrap values followed by probability values in brackets. Bootstrap values lower than 60% are not shown. TL = 204, CI = 0.760, RI = 0.918 and RC = 0.697. Sequences of *Alternaria* species that are ex-type or representative strains (Peever *et al.*, 2004 and 2005) are indicated in bold. Reference sequences (EGS) represent type strains and isolates as published by Peever *et al*. (2004 and 2005).

Figure 7: The phylogenetic tree obtained from one of 830 parsimony analysis trees from inter transcribed spacer (including Genbank entries of the same gene from *Penicillium expansum, P. cecidicola, P. funiculosum, P. dendriticum, P. chermesinum, P.* sp. (aff. *dendriticum* and *cecidicola*), *P. paneum, P. solitum, P. crustosum, P. brevicompactum, P. novae-zeelandiae, P. glabrum, P. rugulosum, P. ramulosum* and *Clonostachys rogersoniana*). The scale bar indicates the number of base changes per 100 nucleotide positions in the parsimony analysis, with parsimony bootstrap values of 1000 replicates indicated above and below the branches respectively. Bootstrap values lower than 60% are not shown.