

**CHARACTERISATION OF MITES AND *PENICILLIUM*  
SPECIES ASSOCIATED WITH APPLE CORE ROT  
DISEASES**

by

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## DECLARATION

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## SUMMARY

Dry core rot (DCR) and wet core rot (WCR) are among some of the most important post-harvest diseases of apples in South Africa. Mouldy core (MC) is also a symptom associated with the core region of apples, but it is not of economical importance since apple tissue surrounding the core region is not affected as is the case with DCR and WCR. The incidence of core rots in harvested fruits can be as high as 12%, but in general ranges from 3 to 8%. Infections and losses can also occur during fruit handling in pack houses and during storage. Additionally, yield losses also occur prior to harvest within orchards due to premature fruit drop of core rot affected fruits. The incidence of core rot diseases in apples differ among apple cultivars, with most Red Delicious varieties being susceptible to the development of core rots, whereas core rots have rarely been reported in other cultivars such as Granny Smith.

The etiology and epidemiology of WCR and DCR are poorly understood. Although many fungal genera have been associated with the diseases, small-spored *Alternaria* species are mainly associated with DCR, whereas *Penicillium* species including *P. roquefortii*, *P. expansum* and *P. funiculosum* have mainly been associated with WCR. Dry core rot infections have long been known to occur pre-harvest, whereas WCR is primarily known as a post-harvest disease where infections take place during fruit handling in pack houses. Recently, *Tarsonemus* mites have also been indicated as being a potential role player in the etiology of core rot diseases. The mites have been hypothesised to carry pathogen spores into the core region of apples, and they may also possibly cause small wounds that facilitate pathogen entry.

In South Africa, apple growers have recently reported WCR as being present prior to harvest, which has not been reported previously. Therefore, the first aim of the study was to investigate the incidence, as well as the causal agent/s of pre-harvest WCR. The incidence of WCR ranged from 0% to 1.7% in eleven orchards, and was in general lower than that of DCR (0.4% to 6%). Isolation studies from eight internal positions in WCR apples showed that *Penicillium* was the predominant fungal genus in most of the positions, including the lesion area. Morphological and molecular characterisation of *Penicillium* isolates from WCR showed that *P.*

*ramulosum* prov. nom. was the main species isolated from lesions, as well as other isolation positions. However, this species was also the main species isolated from DCR, MC and asymptomatic apples. *Penicillium expansum* was only isolated at low frequencies from WCR and DCR apples. Other *Penicillium* species that were occasionally isolated included *P. glabrum*, *P. chloroloma*, *P. chermisinum* and a putative new species with closest affinity to *P. dendriticum* (*P. species aff. dendriticum*) on a DNA nucleotide sequence basis. Pathogenicity and virulence studies using three different inoculation methods showed that *P. expansum* was the most virulent species, followed by *P. species aff. dendriticum*. The *P. ramulosum* prov. nom. isolates varied in their virulence, but were all considered to have low virulence.

The role of *Tarsonemus* mites in the etiology and epidemiology of core rot diseases is poorly understood, and therefore the second aim of the study was to investigate some of these aspects. The specific aims of the study were to (1) investigate the ecology of *Tarsonemus* mites in Red Delicious and Granny Smith orchards during different apple developmental stages, (2) determine if there is a significant association of *Tarsonemus* mites with diseased (WCR and DCR) fruits and (3) determine if potential core rot pathogenic fungi are associated with the mites. *Tarsonemus* mites were found in all of the investigated apple developmental stages (buds, blossoms, 4cm diameter fruit, mature fruit and mummies), having the highest incidence in mummies and mature fruits from Red Delicious and Granny Smith orchards. In Red Delicious fruits the *Tarsonemus* mites were found within the core and/or calyx tube, whereas in Granny Smith fruits the mites were restricted to the calyx tube. In Red Delicious fruits there was a significant association between dry core rot as well as total core rot (wet- and dry-core rot) with the presence of mites in the core, as well as total mites (mites in core and calyx tubes). Fungal isolation studies from the *Tarsonemus* mites showed that they carried potential core rot fungal pathogens within the genera *Penicillium* and *Alternaria*. The *Penicillium* species isolated from the mites included two of the most virulent WCR species, *P. expansum* and *P. species aff. dendriticum*.

## OPSOMMING

Droë kernvrot and nat kernvrot is van die belangrikste na-oes siektes van appels in Suid-Afrika. Beskimmelde kern word ook met die kern van appels geassosieer, maar hierdie toestand is egter nie van ekonomiese belang nie, aangesien die weefsel rondom die kern nie geaffekteer word soos in die geval van nat- en droë kernvrot nie. Die voorkoms van kernvrot in vrugte na oes, kan vlakke van tot 12% bereik, maar oor die algemeen is die voorkoms tussen 3 en 8%. Infeksie en verliese kan ook voorkom gedurende die hantering en verpakking van vrugte in pakhuse en gedurende storing. Addisionele verliese in opbrengs kan ook voor-oes voorkom in boorde. Dit is te wyte aan voortydige vrugval van appels wat besmet is met kernvrot. Die voorkoms van kernvrot by appels verskil tussen kultivars. Meeste van die “Red Delicious” variëteite is vatbaar vir die ontwikkeling van kernvrot. Die toestand is egter skaars by ander kultivars soos Granny Smith.

Die etiologie en epidemiologie van nat- en droë kernvrot word nie goed verstaan nie. ‘n Groot aantal swamgenera is al met kernvrot geassosieer. Klein-spoor *Alternaria* spesies word hoofsaaklik met droë kernvrot geassosieer en *Penicillium* spesies, insluitende *P. roquefortii*, *P. expansum* en *P. funiculosum*, word meestal met nat kernvrot geassosieer. Dit is lank reeds bekend dat droë kernvrot as voor-oes siekte kan voorkom, maar nat kernvrot is algemeen bekend as na-oes siekte waar infeksie tydens vrughantering en verpakking plaasvind. Daar is onlangs aangedui dat *Tarsonemus* myte potensiële rolspelers in die etiologie van kernvrot is. Hipoteties is die myte in staat om spore van die patogene in die kern van die appels in te dra, asook om klein wonde te veroorsaak wat infeksie deur patogene vergemaklik.

In Suid-Afrika is nat kernvrot wat voor-oes in die boorde ontstaan onlangs deur boere aangemeld; hierdie toestand is nog nie op ‘n vorige geleentheid aangemeld nie. Die eerste doelwit van hierdie studie was dus om die voorkoms en veroorsakende organisme/s van voor-oes nat kernvrot te ondersoek. Die voorkoms van nat kernvrot was tussen 0 en 1.7% in elf boorde en was oor die algemeen laer as die voorkoms van droë kernvrot (0.4 tot 6%). Isolasiestudies uit agt interne posisies van nat kernvrot appels het getoon dat *Penicillium* die dominante swamgenus in die meeste posisies was, insluitend die letsels. Morfologiese en molekulêre karakterisering van

*Penicillium* isolate uit nat kernvrot letsels het aangedui dat *P. ramulosum* prov. nom. die spesie is wat die meeste geïsoleer is vanuit die letsels, asook ander isolasie posisies. Dié spesie was egter ook die mees algemene spesie wat uit nat- en droë kernvrot, asimptomatiese appels en appels wat slegs swamgroei in die kern gehad het, geïsoleer is. *Penicillium expansum* was ook in lae getalle uit nat- en droë kernvrotletsels geïsoleer. Ander *Penicillium* spesies wat ook soms geïsoleer is, sluit *P. glabrum*, *P. chloroloma*, *P. chermisinum*, asook 'n moontlik nuwe spesie wat op DNA volgorde basis die naaste aan *P. dendriticum* (*P. spesie aff. dendriticum*) is. Studies wat patogenseiteit en virulensie van die isolate ondersoek het, is ook uitgevoer deur gebruik te maak van drie verskillende inokulasie metodes. Die studies het aangedui dat *P. expansum* die mees virulente spesie is, gevolg deur *P. spesie aff. dendriticum*. Die *P. ramulosum* prov. nom. isolate het variasie in virulensie getoon maar is oor die algemeen aanvaar om minder virulent te wees.

Die rol van *Tarsonemus* myte in die etiologie en epidemiologie van kernvrot word nie goed verstaan nie en dus was die tweede doelwit van die studie om sommige van dié aspekte te ondersoek. Die spesifieke doelwitte was (1) om die ekologie van die *Tarsonemus* myte in “Red Delicious” en Granny Smith boorde tydens verskillende ontwikkelingstadiums van die appels te ondersoek, (2) om te bepaal of daar 'n betekenisvolle assosiasie van *Tarsonemus* myte met siek (nat- en droë kernvrot) vrugte bestaan en (3) om te bepaal of potensiële kernvrot patogeniese swamme geassosieer is met die myte. *Tarsonemus* myte is gevind in al die ontwikkelingstadiums (knoppies, bloeisels, 4 sentimeter deursnee vrugte, volwasse vrugte en mummies) van appels wat ondersoek is. Die hoogste voorkoms van myte was in die mummies en volwasse vrugte van “Red Delicious”, asook Granny Smith kultivars gevind. In “Red Delicious” vrugte is myte in die kern en/of kaliksbuis gevind, maar in die Granny Smith vrugte was die myte tot die kaliksbuis beperk. In “Red Delicious” vrugte was daar 'n betekenisvolle assosiasie tussen droë kernvrot, asook totale kernvrot (nat en droë kernvrot) met die teenwoordigheid van myte in die kern, asook totale myte (myte in die kern en kaliksbuis). Swam isolasiestudies vanaf die *Tarsonemus* myte het aangetoon dat potensiële kernvrot swampatogene in die genera *Penicillium* en *Alternaria* wel by die myte teenwoordig was. Die *Penicillium* spesies wat vanaf die myte geïsoleer is het twee van die mees virulente nat kernvrot spesies ingesluit, nl. *P. expansum* en *P. spesie aff. dendriticum*.

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# 1. THE ROLE OF *PENICILLIUM* AND MITES IN THE ETIOLOGY OF APPLE CORE ROT DISEASES

## INTRODUCTION

Core rot of apples has been reported world-wide in several countries including Australia (Spotts *et al.*, 1988), U.S.A. (Michailides *et al.* 1994), South Africa (Combrink and Ginsburg, 1973; De Kock *et al.*, 1991, Serdani *et al.*, 2002) and Europe (Teixidò *et al.*, 1999). In South Africa, apple core rot diseases are important post-harvest diseases that can cause losses between 5 to 12% (De Kock *et al.*, 1991; Serdani *et al.*, 1998). Additionally, pre-harvest losses can also occur due to premature fruit drop of affected fruit in orchards (Combrink and Ginsburg, 1973). Red Delicious varieties with an open calyx tube are more prone to the development of core rots, than other cultivars such as Granny Smith that has a closed calyx tube (Combrink and Ginsburg, 1973; Spotts *et al.*, 1988).

In literature, the distinction between apple core rots, or symptoms associated with the core region, has not always been clear. This is especially true in the case of dry core rot (DCR) and mouldy core (MC) (Combrink, 1983; Serdani *et al.*, 1998). In general, dry core rot is considered as a dark, dry lesion that spreads from the core region into the fleshy tissue surrounding the core (Fig. 1A). In contrast, MC is usually considered as a symptom where fungal growth is restricted to the core cavity (Fig.1B), not affecting tissue surrounding the core cavity (Combrink and Ginsburg, 1973; Combrink *et al.*, 1985a). However, in literature the term MC has sometimes been used when referring to DCR symptoms (Miller, 1959). In this review DCR specifically refers to a dry rot, whereas MC only refers to fungal hyphae growing within the seed cavity. In addition to MC and DCR, another symptom of the core region is WCR that consists of a straw-coloured decay that is spongy and wet, and which affects the fleshy tissue surrounding the core region (Combrink and Ginsburg, 1973; Combrink *et al.*, 1985a; Fig. 1C).

The etiology of core rot diseases is not well understood, and many different fungal genera have been implicated in causing the symptoms (Miller, 1959). DCR is mainly caused by small-spored *Alternaria* spp., although many other fungi have also been isolated. Infections of DCR take place during the pre-harvest period (Ellis and Barrat, 1983; Spotts *et al.*, 1988). The fungal agents involved in MC have been less well characterised since it is not considered to be economically important. It seems that small-spored *Alternaria* species are most likely also involved (Carpenter, 1942; Ellis and Barrat, 1983). Wet core rot is mainly caused by *Penicillium* species, and infections take place during post-harvest treatment of fruit (Combrink and Ginsburg, 1973; Combrink *et al.*, 1987). Wet core rot (WCR) is often considered as being economically more important than DCR, since it can expand rapidly under cold storage conditions (Combrink and Ginsburg, 1973).

Recently, Michailides *et al.* (1994) reported that a Tarsonemid mite, *Tarsonemus confusus*, may also play a role in the etiology of DCR caused by *Coniothyrium* in California. They hypothesised that the mites may be vectoring pathogen spores into the core region. These mites could also cause small wounds inside the core cavity that facilitate pathogen entry (Michailides *et al.*, 1994). Other than this report of Michailides *et al.* (1994), which has not been published in a peer reviewed journal, no information is available on the role of mites in the etiology of apple core rot diseases, and therefore much remains to be learned.

## WET CORE ROT OF APPLES

**Causative agents.** WCR has been attributed to fungal pathogens such as *Mucor piriformis* E. Fischer, *Penicillium expansum* Link as well as many other fungi (Spotts *et al.*, 1988). According to Combrink and Ginsburg (1973) the main cause of post-harvest WCR in South Africa is *P. expansum* that infects fruits during post-harvest dipping in solutions containing diphenylamine (DPA) emulsion. Additionally, *Penicillium funiculosum* has also been found associated with WCR in South Africa (Combrink *et al.*, 1985a; De Kock *et al.*, 1991; Serdani *et al.*, 1998). This *Penicillium* species has also been associated with storage rot of sugar beet in the USA (Bugbee and Nielsen, 1978), of nuts in Canada (Filtenborg *et al.*, 2004) and pineapple in

Hawaii (Rohrbach and Pfeiffer, 1976; Lim and Rohrbach, 1980). *Penicillium roquefortii* has been isolated from apples with WCR, but its role in disease development could not be proved conclusively (Spotts *et al.*, 1988).

**Symptoms.** In general, WCR consists of an internal rot that affects tissue surrounding the core cavity. The affected tissue exhibits a pale straw- to light brown colour and can be removed from intact tissue, since a very sharp margin exists between decayed and healthy tissue (Combrink *et al.*, 1985a; Fig. 1C). In some instances, WCR can also be manifested as an external symptom when internal lesions expand extensively and reach the surface of fruits. In these cases WCR resembles blue mould, which is a different post-harvest disease that is also caused by *Penicillium* (Combrink *et al.* 1985a; Combrink *et al.*, 1987). Blue mould is caused by *Penicillium* spores that infect the surface of fruits most often through wounds, but sometimes also lenticels, causing a wet, soft rot on the surface of fruits that can be covered by blue to blue-green spore masses (Rosenberger, 1990).

Aside from *Penicillium* species causing visual spoilage of apples, they also pose a threat for food safety. *Penicillium* is of concern for the fresh fruit- and processing industry, since some species such as *P. expansum* produce the mycotoxin patulin. Patulin can reach unacceptable levels and affect the quality of apple juice as well as human health (Sommer *et al.*, 1974; Frisvad and Filtenborg, 1989; Watanabe, 2008).

**Inoculum sources.** Wet core rot infections occur post-harvest during fruit dipping practices (Combrink and Ginsburg, 1973; Spotts *et al.*, 1988). Specific inoculum sources of WCR have not been investigated extensively. It is known that infections occur due to waterborne spores that are present in post-harvest drench solutions and in water flumes that are used for floating fruit on packing lines (Combrink and Ginsburg, 1973; Spotts *et al.*, 1988; Spotts and Cervantes, 1993). Furthermore, inoculum of *Penicillium* can be transferred from infested packing bins onto fruit surfaces and then into the water flumes (Spotts *et al.*, 1988; Sanderson and Spotts, 1995).

**Infection pathways.** The infection pathway for WCR is most likely through the open calyx tube (Miller, 1959), where spores enter the core region during fruit dip solution practices (Combrink and Ginsburg, 1973). Thus, the open core cavity in Red Delicious apples increases the susceptibility of fruit to core rot, as this creates a pathway for pathogen spores to enter the fruit cavity (Spotts *et al.*, 1988).

In South Africa, there are some indications that WCR infections may also occur pre-harvest. De Kock *et al.* (1991) reported WCR in apples harvested directly from trees. In other publications, including Serdani *et al.* (1998) and Combrink *et al.* (1985a), it seems that WCR infections may have also occurred within the orchards, since the fruit were stored directly, and did not receive any pack house treatments.

**Influence of environmental and other factors on disease development.** Several factors can influence the development of WCR including environmental conditions, inoculum concentration and acid content of fruit (Combrink and Ginsburg, 1973; Combrink *et al.*, 1987; Prusky *et al.*, 2004). Environmental conditions such as moisture, ventilation and temperature have a direct influence on WCR decay development (Spotts *et al.*, 1988). Combrink and Ginsburg (1973) found that infection of apple fruit with *Penicillium* takes place during the pre-cooling period before a fruit temperature of -0.5°C is reached. Therefore, an emphasis should be placed on the importance of rapid pre-cooling. It was also shown that the development of WCR decay was slower when apples were stored at temperatures below 15°C (Combrink and Ginsburg, 1973). The incidence of WCR is also influenced by the extent of the spore load on fruits as well as the physiological condition of fruits (Spotts *et al.*, 1988). Low malic and fumaric acid content in apple fruit during the growth and development period may also influence the susceptibility of the fruit to core rot (Combrink, 1983).

Combrink *et al.* (1987) did some interesting studies on the influence of DPA and fungicides on the incidence of WCR. They found that DPA could increase the incidence of WCR. However, when the DPA suspension was chlorinated and thiabendazole was added, the *Penicillium* inoculum decreased markedly. The reduced inoculum levels in the water lowered the risk involved in treating Starking apples with DPA emulsion (Combrink *et al.*, 1987).

**Control Measures.** Although a substantial amount of research has been conducted in order to control post-harvest diseases, very little information is available for the control of WCR specifically. In general for post-harvest diseases the maturity of fruit at harvest is an important factor in the development of rots during ripening and storage of apples. Cultivar, production area and season are all significant factors in this regard (Valiuškaitė *et al.*, 2006). Harvested fruits are often stored for between 6 to 10 months, during which numerous attacks will be launched on the stored fruit by post-harvest pathogens (Rosenberger, 1990). Due to limited information on the control of WCR specifically, the control of blue mould, into which WCR can sometimes develop, will also be discussed in the following section.

*Physical Control.* Sanitation practices form an integral part of all post-harvest disease control. It is important to already start with sanitation of decayed fruit on the orchard floor during the growing season as well as after harvest. Pack house sanitation is important, and decayed fruit should be removed from pack houses daily (Rosenberger, 1990).

The correct use and regulation of packing and storage conditions is important for controlling blue mould and WCR. For blue mould, careful handling and prompt cooling directly after fruit harvest can help to keep bruising of fruit to a minimum, which will assist in the maintenance of low levels of inoculum during packing and storage (Combrink *et al.*, 1987). Controlled atmosphere (CA) storage greatly aids the control of blue mould, since *Penicillium* seldom sporulate under CA conditions, and if sporulation occurs it is much less than in air storage. Consequently, the number of generations of *Penicillium* per storage season is limited (Rosenberger, 1990). Cold storage temperatures that are recommended for apples are between 1 to 4°C (Panhwar, 2006). This would limit progress of WCR, since WCR decay is slower when apples are stored at temperatures below 15°C (Combrink and Ginsburg, 1973). Combrink and Ginsburg (1973) recommended that for WCR control, an emphasis should be placed on the importance of rapid pre-cooling, since infections take place during this period.

Other physical control measures that are less commonly used include hot air treatment, irradiation and the control of vectors. Hot air treatment of fruit after harvest (38°C for 4 days) has been shown to reduce decay development by *P. expansum* in apples for blue mould control (Fallik, *et al.*, 1996; Leverentz *et al.*, 2000). In a study conducted by Conway *et al.* (2005) irradiation as control agent against microbial organisms has been tested and accepted in some countries and can be applied below 1kGy. Apples have a high relative tolerance for this method of control (Panhwar, 2006). In orchards, insects such as mites (Red spider mite, Bryobia mite and European red mite), fruit flies (Engelbrecht, 2002), thrips and aphids could possibly vector plant pathogens. They should therefore be controlled in order to prevent the spread of pathogen spores (Gerson *et al.*, 2003), such as *Penicillium*.

*Chemical Control.* For blue mould and WCR, fungal populations in packing shed water should be reduced to the lowest possible levels with fungicides, disinfectants (chlorine), or frequent water changes (Spotts *et al.*, 1988; Combrink *et al.*, 1987). The fungicide benzimidazole used in pre-harvest sprays, post-harvest dips, drenches, line sprays, or fruit waxes is highly effective against the *Penicillium* spp. that cause blue mould. However, due to the incorrect use of this fungicide, resistant strains have developed in most pack houses (Sholberg *et al.*, 2005). Rosenberger and Meyer (1985) found that diphenylamine, commonly used to control storage scald of apple, is effective against both benzimidazole-resistant and -sensitive *P. expansum* isolates. Unfortunately, although DPA can be useful for controlling blue mould, Combrink *et al.* (1987) found that DPA can increase the incidence of WCR, and it should thus be used with caution. It will be more suitable to control benzimidazole-resistant isolates with some of the new fungicides. Recently, fludioxonil and pyrimethanil have been registered for controlling post-harvest pathogens such as *Penicillium* (Errampalli *et al.*, 2006; Li and Xiao, 2008), which will be more suitable for this purpose.

In South Africa different chemicals are registered for the control of post-harvest decay of apples and pears. According to Nel *et al.* (2003), fruit can be immersed in a suspension of benomyl or it can be sprayed onto harvested fruit before storage. Captab can be applied on empty premises onto walls and floors to aid disinfection. Dimethyl didecyl ammonium chloride, for specific use against *P.*

*expansum*, can be used as dip or drench shortly after harvest or after regular atmosphere storage and for disinfection purposes. A post-harvest dip or drench treatment of iprodione is registered for the control of *Botrytis* and *Penicillium* for local market use only. Apples can also be treated with thiabendazole within 24 hours of harvest and after storage (Nel *et al.*, 2003).

*Biological Control.* The use of antagonistic microorganisms may be effective in reducing the incidence of post-harvest fungal pathogens on different fruits (Lima *et al.*, 1999). Bio-fungicides that are yeast-based are already commercially available in the USA and Israel (Aspire<sup>®</sup>, based on *Candida oleophila*) (Ippolito and Nigro, 2000; Lima *et al.*, 2003). In South Africa, Yield Plus<sup>®</sup> (based on *Cryptococcus albidus*) is available for the control of post-harvest rots of pome- and citrus fruits (Droby *et al.*, 2002; Lima *et al.*, 2003). This method of control relies mainly on efficient competition based on preventative colonisation of fruit wounds. In some cases it has been shown that when yeasts are used with low dosages of fungicide (10% of the commercially recommended dosage) an activity comparable to that of the fungicide applied at full dosage can be obtained (Lima *et al.*, 2003). This may however be problematic with regard to fungicide resistance management.

*Genetic Manipulation.* Cultivars that are resistant to *Penicillium* infections can also be developed through breeding methods (Valiuškaitė *et al.*, 2006). Due to the fact that Red Delicious cultivars with open sinuses are more susceptible to core rot, plant breeding can be used as a tool to manipulate these cultivars. Selections can be made to select trees with fruit that have closed calyx-tubes, which can then be used in specific breeding programs (Spotts *et al.*, 1999).

The development and use of resistant cultivars for controlling post-harvest diseases such as *Penicillium* may be problematic. Spotts *et al.* (1999) found during their evaluation of several apple cultivars for resistance against four decay pathogens, that each cultivar that was the most resistant to one pathogen also was the most susceptible to one of the other pathogens. The cultivar Royal Gala was the most resistant to *P. expansum*, *Mucor piriformis* and *Botrytis cinerea* (Spotts *et al.*, 1999). Because most decay pathogens such as blue mould require a wound to initiate infection, resistance of the epidermis to breakage may be an important factor in



resistance of apple cultivars to decay (Spotts *et al.*, 1999). Many other factors influence resistance of apple cortical tissue decay, including glycoprotein endopolygalacturonase inhibitors (Brown, 1984) and benzoic acid (Seng *et al.*, 1985).

## DRY CORE ROT OF APPLES

**Causative agents.** Several different fungal genera have been isolated from DCR apples in the past and have been shown to be pathogenic (Combrink *et al.*, 1985b). Fungi like *Trichothecium roseum* (Raina *et al.*, 1971), *Pezizula perennans* (Mouat, 1953), *Botryosphaeria obtuse* and *Alternaria* spp. (Taylor, 1955) have been isolated from DCR apples and have been implicated in causing the disease. Combrink *et al.* (1985a) reported the presence of *Pleospora herbarum*, *Coniothyrium* spp., *Epicoccum purpurascence* and *Alternaria alternata* (Fr.) Keissler in DCR apples in South Africa. *Alternaria* spp., more specifically *A. alternata*, has been accepted by several studies as the most important pathogen causing DCR (Combrink *et al.*, 1985b; Fugler, 1990). However, Serdani *et al.* (1998) found that the *Alternaria tenuissima* species-group was the species most frequently associated with DCR of apples and not *A. alternata* as previously thought. The uncertainty as to the specific *Alternaria* species involved is related to controversy and difficulties related to the identification of small spored *Alternaria* species and species groups (Serdani *et al.*, 2002).

**Infection pathways.** The open calyx tube of Delicious apple varieties (Carpenter, 1942) has been implicated as being the passage way through which potential core rot pathogens enter the core cavity (Miller, 1959; Ellis and Barrat, 1983). It was suggested by Miller (1959) that infection takes place shortly before harvest, but studies conducted by Stinson *et al.* (1981) and Combrink *et al.* (1985b) showed that infection primarily takes place shortly after full bloom. The senescent tissue and the calyx tube are only colonised after a lag phase (De Kock, *et al.*, 1991). Fungi may continue to colonise the core cavity through the open calyx tube during the growing season, causing disease symptoms (Combrink *et al.*, 1985b). Cracks in the calyx tube may facilitate pathogen infection (Carpenter, 1942). Apples injured by insects, such as codling moth showed a significantly higher incidence of DCR than

healthy apples, suggesting that wound sites are created through which fungi could infect the fruit (Harrison, 1935).

**Influence of fruit biochemistry and -growth on disease development.** Low fumaric and malic acid content during growth and development of apple fruit are thought to predispose Starking apples to core rot (Combrink, 1983). In contrast, the much higher acid content of Granny Smith and Golden Delicious apples, in which DCR has rarely been reported, seems to inhibit germination and growth of *A. alternata* (Prusky *et al.*, 2004). Niem *et al.* (2007) also hypothesized that mesoderm pH may influence the susceptibility of cultivars to *Alternaria* infection and the development of DCR.

Factors such as fruit size and growth, as well as water availability are thought to also influence the incidence and development of DCR. A lack of water (Carpenter, 1942) and rapid expansion of parenchyma tissue after heavy rains can cause cracks to develop between the core cavity and calyx tube that can facilitate pathogen entry (Harrison, 1935). Fruit size has in the past been linked to MC, where bigger fruit have been shown to have a higher incidence of MC (Harrison, 1935).

Niem *et al.* (2007) hypothesized that the sensitivity of the seed locule to *Alternaria* colonization may be important in determining the susceptibility of cultivars. They found that the seed locule wall of susceptible cultivars is more susceptible to colonization by *Alternaria*, than the seed locules of resistant cultivars. The extensive colonization of the seed locule wall of susceptible cultivars was also followed by a higher incidence of penetration into the mesoderm, than what was observed in resistant cultivars (Niem *et al.*, 2007).

**Influence of environment on disease development.** An important contributing factor for DCR development is storage. After several months of cold storage, an increase in the incidence of DCR has been reported by Mouat (1953) and Stinson (1981). It has also been found that the lesion size increases the longer the fruit are stored (Chaunzen *et al.*, 1993). Temperature and atmosphere composition in storage significantly affects DCR. DCR development is positively correlated with temperature which is the main factor in disease incidence (Chuanzhen *et al.*, 1993).

During the growing season temperature and humidity may influence disease development. When high relative humidity and mild temperatures are prevalent, especially during late spring, infections by many fungi may occur, especially where cultivars with open calyx tubes are concerned. It has previously been reported that disease incidence is higher in apple fruit that have experienced late spring frost and in which seeds do not develop normally (Ellis and Barrat, 1983). It has also been suggested that high relative humidity in the orchard during ripening promotes disease development (Ellis and Barrat, 1983).

**Control.** Because fungi that are already inside the fruit are so well protected from chemicals, control of DCR is very difficult. An integrated pest management (IPM) approach is thus the most effective way of combating DCR (Sugar *et al.*, 2003).

*Genetic and Physical control.* Several aspects can be considered for controlling DCR, including the use of resistant cultivars, orchard management practices as well as storage conditions. The best control option will be the replacement of susceptible with non-susceptible cultivars (Mouat, 1953). Planting of apple varieties with shallow calyx tubes have been suggested by Miller (1959) as fruit of these cultivars are less likely to develop open calyx tubes. Good airflow and light penetration should be kept in mind when orchard pruning is carried out. This promotes fast drying in the orchard (Mouat, 1953). According to Chaunzhen *et al.* (1993), storage conditions may be the solution for controlling DCR. This can be done by using temperatures below 10°C combined with 2 to 4% O<sub>2</sub> and 10 to 14% CO<sub>2</sub> during the initial 40 days of storage.

*Chemical control.* The use of certain pesticides should be avoided close to harvest if open calyx tubes are present, as damage of carpels can take place when the fungicides enter the calyx tube (Miller, 1959). Brown and Hendrix (1978) reported that bloom sprays with fungicides can significantly reduce the incidence of DCR. Reuveni and Prusky (2007) reported that three DMI fungicide sprays during bloom showed promise for controlling DCR. However, controversy still exists on the usefulness of fungicide sprays, and their economic justification. Fugler (1990) came to the conclusion that no cost-effective spraying programs are currently available in

South Africa for the control of DCR or MC, but nonetheless suggested that captab at a rate of 200g/100L water at full bloom and again at 75% petal fall may help to control DCR.

*Biological control.* *Gliocladium roseum* Bainier and *Stachybotrys elegans* Pidopl. have both been shown to act as parasites of *A. alternata*. *Gliocladium roseum* produces antibiotics that cause total collapse of *A. alternata* spores, while the mode of parasitism by other species of fungi was thought to be direct penetration by the hyphal tips (Turhan, 1993). Some bacterial strains may have even greater potential for being biocontrol agents of *A. alternata* isolates involved in core rot. Teixidò *et al.* (1998) reported that *Pseudomonas syringae* (CPA-10) can be used on apples against *A. alternata* with great success. This bacterium strongly inhibits the development of *Alternaria* mould in the field, as well as during cold storage conditions and does not have any pathogenic effects on apples (Teixidò *et al.*, 1998).

## THE GENUS *PENICILLIUM*

*Penicillium* is a common contaminant of various substrates and has a world-wide distribution (Samson *et al.*, 2004). Colonies of *Penicillium* usually grow rapidly and can be distinguished in shades of green, in some cases white, consisting of a dense mass of conidiophores on the substrate (Samson *et al.*, 2004). *Penicillium* species can be identified to the species level using morphological characteristics as well as molecular methods. The use of only cultural characteristics for the classification of *Penicillium* species is not recommended since it can be variable (Raper and Thom, 1949). Therefore molecular methods should support *Penicillium* identifications (Samson *et al.*, 2004).

*Morphological identification.* Growth in pure culture under laboratory conditions has long been used as a standard method for examination of *Penicillium* isolates. Measurement of colony diameter, colony characteristics and microscopic observations are used for this purpose. The three point inoculation method is used to best determine colony texture and colour, along with the appearance of penicilli and conidial chains (Pitt, 1979). This is done on Czapek Yeast autolysate agar (CYA) or

Czapek agar (Cz) and 2% Malt Extract agar (MEA) (Samson and Pitt, 1985). Pitt (1979) suggested that growth rate and water activity with different media and temperatures should be used in addition to colony characteristics (Samson *et al.*, 2004).

*Molecular identification.* Methods based on the polymerase chain reaction (PCR) are frequently used in mycology for diagnostic purposes, and these methods can help with the accurate characterisation of fungi (White *et al.*, 1990). Although the RAPD technique has been shown useful for subspecific analysis in the genus *Penicillium* (Bruns *et al.*, 1991; Williams *et al.*, 1990; Pianzolla *et al.*, 2004), it is not suited for species identification and suffers from a lack of reproducibility between laboratories (Micheli *et al.*, 1994). The use of DNA sequence data is better for investigating species identity and divergence. In *Penicillium*, the sequence of several gene areas including the internal transcribed spacer (ITS) region of the rDNA, cytochrome *c* oxidase 1 (*COI*) and  $\beta$ -tubulin regions have been used to investigate the identity and evolutionary relationships of species. Although the ITS region has been found useful for clarifying subdivisions in this genus (Lobuglio *et al.*, 1993; 1994), certain species within specific *Penicillium* subgenera cannot be distinguished using this region due to insufficient polymorphic sites (Skouboe *et al.*, 1999). The *COI* gene has been shown to be comparable to the ITS region with regards to revealing divergence between species in the subgenus *Penicillium* (Seifert *et al.*, 2007). When compared to the ITS and *COI* areas, the  $\beta$ -tubulin gene is more variable between *Penicillium* species, and is better at revealing divergences between species in the subgenus *Penicillium* (Seifert *et al.*, 2007).

***Penicillium* as post-harvest fruit pathogen.** In apples, *Penicillium* species can cause both WCR (discussed above) and blue mould. Blue mould is the most important post-harvest pathogen of apples (Rosenberger, 1990). In the USA losses in storage have gradually increased during the 1990s and can be as much as 15% (Spotts *et al.*, 1999; Rosenberger *et al.*, 2006). The *Penicillium* species causing blue mould are well characterised and *P. expansum*, as well as *P. solitum* are considered the predominant species associated with this disease. Species that are less frequently isolated are *P. commune*, *P. aurantiogriseum*, *P. crustosum* and *P. brevicompactum* (Sanderson and Spotts, 1995; Sholberg and Haag, 1996; Amiri and Bompeix, 2005;

Sholberg *et al.*, 2005). *Penicillium verrucosum* is considered as an important component of blue mould in New South Wales (Penrose *et al.*, 1984).

*Penicillium* is also known to cause post-harvest diseases in several other fruits including citrus, nuts and tomatoes. In citrus (lemons, oranges, mandarins, tangerine, cumquats, tangelo, limes, pomelos and grapefruit), three *Penicillium* species, *P. digitatum*, *P. italicum* and *P. ulaiense* are of paramount importance in post-harvest decay (Filtenborg *et al.*, 2004; Holmes *et al.*, 1994). The *Penicillium* species associated with post-harvest rot of nuts include *P. commune*, *P. crustosum*, *P. discolor*, *P. solitum*, *P. funiculosum*, *P. oxalicum* and *P. citrinum* (Filtenborg *et al.*, 2004). *Penicillium olsonii* has been consistently isolated from tomatoes and can grow on commercial tomatoes, but fortunately does not produce any mycotoxin (Samson *et al.*, 2004).

***Penicillium* as pre-harvest fruit pathogen.** The genus *Penicillium* is not usually associated with pre-harvest diseases. It has only been reported as a pre-harvest disease on corn in Spain by Jaminez *et al.* (1985) and on apples (WCR) by De Kock *et al.* (1991). *Penicillium* species are seldom present on apple surfaces in the orchard, except for fruit that has fallen to the ground (Amiri and Bompeix, 2005). However, in pear orchards it has been found that *Penicillium* spores are on fruit surfaces as well as in the orchard air (Lennox *et al.*, 2003). The low incidence of pre-harvest *Penicillium* diseases can to some degree be attributed to the fact that *Penicillium* requires wounds for entry and subsequent colonisation (Spotts *et al.*, 1985a; Spotts *et al.*, 1999).

## THE ACARI

**Introduction.** Mites have a worldwide distribution and inhabit a wide range of habitats, including salt and fresh water, organic debris, plants and animals. Mites fall in the subclass Acari along with ticks, and form an important part of the arthropodan class Arachnida (Jeppson, 1982).

Mites all have a general morphology in common, of which some components are important for identifying major groups. The presence or absence of stigmata,

spiracular openings, as well as the relative position of stigmata are used as a diagnostic tool in the separation of orders (Krantz, 1978, Jeppson, 1982). Stigmata, when present, open internally into a tracheal system that branches throughout the body to various organ systems. Some groups have no apparent stigmata or tracheal system, and oxygen exchange occurs through the integument (Jeppson, 1982). Mites also have many sensory receptors, most of which are setiform, and are situated on the idiosoma. One or two pairs of eyes are located laterally on the prodosoma in most groups. Some of the major groups of mites have paired sensory organs located ventrally between coxae I and II (Jeppson, 1982). Four pairs of segmented legs are usually present in adult and nymphal mites, except the Eriophyoidae, whereas larvae only have three pairs of legs. Primary segments in the legs can be discerned, namely the coxa, trochanter, femur, patella, tibia and tarsus. The tarsus usually terminates in a claw-like or sucker-like appendage, consisting of paired claws and a median pad-like or claw-like empodium (Jeppson, 1982).

Many factors can influence the population ecology of mites, their life cycle and outbreaks. Some of the factors include biotic potential of the species, reproduction mechanisms, influence of meteorological factors, availability and susceptibility of hosts, competition between species, structural and chemical adaptations, as well as pathogens and predators of mites (Jeppson, 1982). Some tetranychid species show diverse responses to changing environmental conditions, as well as overcrowding (Boudreaux, 1963; Jeppson, 1982). Competition within and between species also causes changes in populations to occur. Spider mites respond to light, as well as humidity (Suski and Naegele, 1968; Jeppson, 1982). In mites, reproduction generally follows the usual pattern of fertilisation and subsequent production of male and female progeny. Facultative parthenogenesis occurs throughout the Acari and arrhenotoky (production of males from unfertilised eggs) sometimes occur in the Mesostigmata and Prostigmata. The production of females from unfertilised eggs (thelytoky) is also commonly found in certain Prostigmata and other groups (Jeppson, 1982).

The mites associated with apple orchards have not been well studied other than phytophagous mites such as *Tetranychus urticae* Koch and *Panonychus ulmi* Koch that cause economical damage in orchards, and the mites *Neoseiulus californicus*

McGregor and *Euseius addoensis* Van der Merwe and Ryke that are used to control them (Pringle and Heunis, 2006). The report of Oatman (1963) is the only study where a substantial investigation into all mite families within apple orchards in Wisconsin was made. The study investigated mites on foliage, and revealed the presence of 14 mite families of which seven families were classified as phytophagous, eight as predacious and three as general organic feeders. The phytophagous mites belonged to the families Tetranychidae, Tenuipalpidae and Eriophyidae, whereas the predacious mites included the Phytoseiidae, Stigmaeidae, Tydeidae, Hemisarcoptidae, Raphignathidae, Anystidae, Bdellidae and Acaridae. The other three families consisted of the general organic feeders Tarsonemidae, Saprogllyphidae and Ceratozetidae. According to the study of Oatman (1963), the Tarsonemidae was the third most numerous family on apple leaves.

Recently, another study conducted in the United States reported that *Tarsonemus* mites are not only associated with apple leaves, but can also be found within apple fruits (Michailides *et al.*, 1994). The discovery of the mites within the core and calyx tube of fruit was due to an investigation that was initiated on the causal agent of apple dry core rot that was reported by growers as being very problematic. The study found that a Tarsonemid mite, *Tarsonemus confusus*, had a high incidence (50 to 86%) within the core region of apples with dry core rot caused by *Coniothyrium*. In contrast, healthy apples (83%) only contained the mite among the floral parts in the calyx region, but not within the core region (Michailides *et al.*, 1994). Michailides *et al.* (1994) hypothesised that the *Tarsonemus* mites may play an important role in the epidemiology and etiology of the disease, since they may be vectoring pathogenic fungi into the core region. Furthermore, preliminary co-inoculation studies of *Coniothyrium* alone and *Coniothyrium* along with mite adults and eggs, showed that co-inoculation of the fungus with mites caused 65% of inoculated fruit to rot, whereas inoculation with the fungus alone only caused 28% of the fruit to rot. This suggests that the mites could possibly also cause small wounds in the core region that facilitates pathogen entry, although more research is required to confirm this (Michailides *et al.*, 1994).

There are over 500 known mite families, of which only a few will be discussed in the following section. Some of the mite families that are discussed have been



associated with apple orchards in the United States (Oatman *et al.*, 1963) as well as in a more recent study in apple orchards in South Africa (Chapter 3). Special emphasis will be placed on the family Tarsonemidae, since this family may play a role in apple core rot diseases (Michailidis *et al.*, 1994; Chapter 3).

**Phytoseiidae (Mesostigmata).** Phytodeiids are present in the upper soil layers, as well as on plants. These mites are predatory and are able to move very fast. They feed mainly on mites, but are also known to feed on smaller insects, nematodes, fungi, and may feed on plants or plant exudates. This family of mites has been studied extensively, since they have been used very successfully for controlling spider mites, other mites and thrips (Thysanoptera) (Gerson *et al.*, 2003) particularly on orchard and vine crops (Krantz, 1978).

Within the Phytoseiidae family, several genera and species have varying feeding habits, although they are mainly predatory. *Phytoseiulus persimilis* feed on spider mites, especially *Tetranychus* spp. and rarely on *Oligonychus* spp. (Gerson *et al.*, 2003). This mite currently is used in South Africa in integrated pest management (IPM) programmes (Pringle and Heunis, 2006). *Galendromus occidentalis* also feeds on spider mites but is not restricted to *Tetranychus* spp. It feeds on spider mites that produce little webbing, such as *Panonychus ulmi* (Koch) and the European red mite (ERM), as well as other mites and even plant exudates. Generalist predators such as *Neoseiulus californicus* (McGregor) prefer prey other than spider mites like tarsonemid mites and thrips. *Eusius addoensis* mainly feeds on citrus red mites, and plays an important role in the control of this mite pest (Gerson *et al.*, 2003).

Phytoseiids survive unfavourable winter conditions either by undergoing a reproductive diapause or resistance to low temperatures (Zhang, 2003). Prevailing light conditions seems to affect their behaviour as they don't possess eyes. For dispersion, the Phytoseiidae relies on wind, but over short distances they run on leaves or crawl on the soil surface, or walk along spider mite webs. Wind dispersal is usually associated with the depletion of prey, and mites that are ready to disperse move onto open surfaces and position themselves to be lifted by air currents. Phoresy (dispersal on insects) is rare, the only known case is the dissemination of

*Kampimodromus aberrans* (Oudemans) by the aphid (Hemiptera: Aphidoidea) (Gerson *et al.*, 2003).

**Tetranychidae (Acari: Prostigmata).** The Tetranychidae (spider mites) is a large family and has a worldwide distribution. Most members of this family feed on the foliage of plants, and they are considered to be the most important mites attacking plants (Zhang, 2003). Spider mites have specialised mouthparts for piercing plant cells and extracting the contents (McDaniel, 1979). Feeding on leaf cells results in white patches on the leaves and heavy infestation leads to curling of leaves. Some members of this family spin webs that cover the foliage, and then live and lay eggs underneath the web (McDaniel, 1979).

A large number of species are major pests of agricultural crops, where populations rapidly increase when their natural enemies are eliminated by agricultural chemical applications (Helle, 1965; Heunis, 1992). Spider mites are also prone to the development of resistance against certain acaricides, which makes their control difficult (Helle, 1965). The European red mite, *Panonychus ulmi*, and the red spider mite, *Tetranychus cinnabarinus*, are major pests of deciduous fruit trees in many countries around the world. The red spider mite is polyphagous and can be found on a wide range of crops in South Africa (Annecke and Moran, 1982).

**Tydeidae.** The Tydeidae is cosmopolitan and more than 400 species have already been described. Tydeids are small, soft-bodied (Zhang, 2003), fast-moving soil and plant inhabitants. Their feeding habits range from carnivory and omnivory to phytophagy (Baker, 1965; Jeppson, 1982; Gerson *et al.*, 2003). The mycophagous habits (feeding on fungi) of tydeids have been shown to indirectly reduce damage caused by certain pests and they can thus be seen as a sanitising agent in fruit orchards (English-Loeb *et al.*, 2007; Gerson *et al.*, 2003; Mendel and Gerson, 1982). These mites also feed on some nematodes and could thus regulate the number of nematodes that graze on bacteria in decomposing litter, promoting litter decomposition (Gerson *et al.*, 2003). Some species like *Tydeus californicus*, *T. caudatus* Duges, *T. praefatus* and *Lorryia formosa* feed on leaves of plants and are known to cause significant damage to host plants (Zhang, 2003).

**Bdellidae.** This family of mites consists of about 100 species in 15 genera which can range in colour from red-brown to green and can be up to 4mm long. The Bdellidae, which are predators, occur commonly on plants, in the soil and in stored products (Krantz, 1978; Gerson *et al.*, 2003). They are active hunters, locating prey such as springtails or other mites with their palpi. A salivary fluid is deposited onto the prey, hardening on contact into silken lines, after which the body contents of the prey is sucked out. These mites can be effectively used in pest control and were one of the first mite families shown to have pest control capabilities (Gerson *et al.*, 2003).

**Acaridae (Acari: Astigmata).** The Acaridae are slow-moving, whitish mites that have long setae. They occur in leaf litter and upper strata of soil with high organic matter content. They can also be found in decomposing animal droppings, on plants, as well as in laboratory fungal cultures (Gerson *et al.*, 2003). The Acaridae are omnivorous and can survive on anything from animal food, aphids, stored grains, cheese, mushrooms and even fungal cultures (Kevan and Sharma, 1963; Gerson *et al.*, 2003).

Acaridae can be major pests of stored foods, especially *Rhizoglyphus*, *Acarus* and *Tyrophagous* (Sinha, 1963; Zhang, 2003). Several species of the genus *Rhizoglyphus* are pests of root crops, especially when bulbs are not completely cured or dried (Jeppson, 1982; Zhang, 2003). Economic damage can also be caused by *Tyrophagous* on ornamental flowers and greenhouse grown vegetables (Zhang, 2003). However, some species in this family may be more useful, since these mites may serve, in some instances, as control agents for nematodes, grape phylloxera and plant pathogenic soil fungi, preventing injury by these fungi on seedlings (Gerson *et al.*, 2003). Interestingly, compounds emitted by Acarids that serve as sex and alarm pheromones, may have adverse effects on fungi and also may attract predators (Jeppson, 1982).

**Cunaxidae.** Included in this family are 200 species in less than 20 genera (Gerson *et al.*, 2003). The Cunaxidae are cosmopolitan and can be found on leaf litter, soil, compost, moss, plants and stored products (Zhang, 2003). These generalist predators are fast-running and feed on small arthropods and nematodes in a diversity of crops (Zhang, 2003). Rootknot nematodes (*Meloidogyne* spp.) are also prey for

these mites. Silken threads are excreted through the mouthparts to fasten hunted prey, but these mites also snare or ambush their prey. They are also cannibalistic, making them unsuitable for biological control or mass rearing (Gerson *et al.*, 2003; Zhang, 2003).

**Ascidae.** This family of slow-moving, predacious mites, has been collected from plants in the tropics, but is also commonly found in dead leaves, upper layers of soil, in moss and in animal waste products. They prey on small arthropods and their eggs, as well as nematodes and can at times be cannibalistic (Krantz, 1978).

**Stigmaeidae.** Stigmaeids reside in the soil and on plants and are often found feeding on eggs and sessile forms of tenuipalpid, tetranychid and other mites (Krantz, 1978; Gerson *et al.*, 2003). Some species parasitise flies or prey on scale insects. This family, especially in the genera *Zetzellia* and *Agistemus*, is believed to be the second most important group of predators on plant mites after the Phytoseiidae. Little is known about their dispersal (Gerson *et al.*, 2003).

**Iolionidae.** This family consists of less than six tiny (<500µm) species of mites. Some species have been recovered from the wings of cockroaches (Pritchard, 1956), as well as on locusts in Java and the Galapagos Islands (Fain and Evans, 1966). The relationship with their hosts is not clear (Krantz, 1978).

**Pyemotidae.** Some species are known to be pests of grasses and cereals. More than 30 species of grasses, wheat, barley, as well as rye serve as hosts for these mites. Retarded growth, due to feeding of the mite within sheaths of the upper part of the stem, can cause the symptom known as silver tip (Jeppson, 1982). Some pyemotid mites have the ability to attach themselves to their food source. The abdomen balloons into a globular brood chamber where the young are protected (Jeppson, 1982).

**Oribatidae.** Oribatid mites are some of the most common soil and litter inhabitants. Some oribatid species are associated with plants, but their economic importance is unknown (Zhang, 2003). Some of the main food sources of oribatids

are plant organic matter, as well as living or dead organisms like algae, lichens, fungi and mosses (Woodring, 1963).

**Dolichocybidae.** This family is considered as one of the most primitive pyemotoids. Some species of *Dolichocybe* are found under bark of trees, whereas other members are generally encountered on insects (Krantz, 1978).

**Tarsonemidae (Acari: Prostigmata).** Until recently, only approximately 500 species belonging to 40 genera have been identified, but after recent revision the family now includes 700 species (Gerson *et al.*, 2003; Lindquist, 1986). The family consists of three subfamilies, namely Pseudotarsonemoidinae, Acarapinae and Tarsoneminae. The Tarsoneminae has the largest number of described species, which include two large genera namely *Steneotarsonemus* Beer and *Tarsonemus* Canestrini and Fanzago (Lindquist, 1986). Members of the family Tarsonemidae are phytophagous, fungivorous, insectophilus and parasitic.

*Geographical Distribution.* Tarsonemid mites are known from most major regions and biotopes of the world, excluding Antarctica, major deserts and aquatic habitats. They are thought to be best represented in warm-temperate and tropical regions, but are also well represented in cool-temperate and mountainous parts of the northern hemisphere. Collection data are not sufficiently available to obtain an accurate idea of the distribution of most of this family's genera (Lindquist, 1986).

*Diagnostic characters.* Mites belonging to this family are small (*c.* 0.2-0.3mm in length) with broad to elongated bodies that are covered by a shiny integument (Gerson *et al.*, 2003). The bodies of Tarsonemids are divided into two well-defined portions. Pronounced sexual dimorphism occurs, with the males being much smaller than the females. Both sexes are dorsoventrally depressed, which aids them in living between sheaths and stems of grass hosts and the integument of insects. The development of the apodemes is used in characterisation (McDaniel, 1979). Females have prodorsal, clavate sensilli (pseudostigmatic organs), which are lacking in males. Furthermore, the 4<sup>th</sup> pair of legs differs from the other pairs in male and females, since they end with apical and subapical whip-like setae in females and in males they

terminate with a large claw (Gerson *et al.*, 2003). In males this modification aids in copulation (McDaniel, 1979).

*Economic importance.* The Tarsonemidae are of great economic importance to agriculture. Numerous greenhouse crops can suffer damage caused by the cyclamen mite, *Steneotarsonemus pallidus* Banks. One of the symptoms of damage is chlorosis of leaves, but necrosis does not occur, except within buds or on young tissues (Jeppson, 1982). Another *Steneotarsonemus* species, *S. spirifex* attacks maize, oats and other grain crops. *Steneotarsonemus bancrofti* feeds on newly planted stocks of strawberries and sugar cane cuttings (McDaniel, 1979). Other members of this family that are also of economic importance include *Tarsonemus randsi*, an important pest in commercial mushroom production and fungal cultures in laboratories.

*General biology.* The eggs are laid one at a time and are large relative to the idiosoma of the adult female. Larvae are active and do not remain quiescent, but actively emerge and rid themselves of the egg chorion. They move about and feed in most cases as the adults do, but exceptions do exist. All larvae of the Tarsonemidae pass into an inactive, turgid state, without moulting, before hatching to adults (Jeppson, 1982; Lindquist, 1986). It has been shown that unfertilised eggs of several genera usually give rise to males and fertilised eggs mostly to females. This is an indication of arrhenotokous parthenogenesis and that a haplo-diploid sex mechanism is generally involved. The female:male sex ratio in the progeny of mated females varies considerably intraspecifically as well as between species (Lindquist, 1986). Tarsonemids disperse as adult females. Species that are parasitic are dispersed by their hosts. Long distance dispersal can be achieved by phoretic association with flying insects (Zhang, 2003).

*Tarsonemus sp.* Almost all species of the genus *Tarsonemus* are considered to be fungivorous (McDaniel, 1979), although some species attack plants (Lindquist, 1986). *Tarsonemus confusus* Ewing occasionally occurs as a minor pest in greenhouses and as a pest in mushroom cultures. Although *Tarsonemus floricolus* Canestrini and Fanzago was recorded in the Brooklyn Botanical Garden in New York, on decaying buds of *Iris kaempferi*, it is known to be fungivorous. This species along with *T. myceliophagus* Hussey, are known pests in mushroom houses, and are

therefore unlikely to be plant pests. Another widespread species is *Tarsonemus bilobatus* Suski, which like many other *Tarsonemus* species, is primarily fungivorous but has been collected from many plant species, fungal and bacterial cultures, stored food and products, litter, and soil (Zhang, 2003).

## **THE ROLE OF ACARI IN THE EPIDEMIOLOGY AND ECOLOGY OF PLANT PATHOGENIC AND NON-PATHOGENIC FUNGI**

There are not many examples of the role of acari in the epidemiology and ecology of pathogenic and non-pathogenic fungi. The best studied example is that of the vectoring of Ophistomatoid fungi by mites phoretic on beetles. There are a few other examples where mites are also thought to play a role in the spread and development of plant diseases, but none of these involve *Tarsonemus* mites. Central bud rot of carnations caused by *Fusarium poae* (Peck) Wollenweber is spread by *Siteroptes cerealium* Kirchner. The eriophyoid mite *Eriophyes tulipae* Keifer is thought to be involved in the spread of the fungus-causing rot of garlic bulbs (Jeppson, 1982). Another *Siteroptes* species, *Siteroptes avenae*, is possibly involved in the spread of *Fusarium* glume spot of wheat caused by *Fusarium poae* (Kemp *et al.*, 1996). *Aceria mangiferae* (mango bud mite) plays a role in mango malformation disease caused by *Fusarium mangiferae* (Gamliel-Atinsky *et al.*, 2005). Species of the genus *Rhizoglyphus* are associated with the spread of various bulb diseases caused by *Fusarium* and *Pseudomonas* (Jeppson, 1982; Zhang, 2003).

On the other hand, mites may actually be involved in suppressing disease development of some plant diseases. English-Loeb *et al.* (2007) showed that the mycophagous tydeid mite, *Orthotydeus lambi* (Baker), may have potential for biological control of grape powdery mildew. They were able to show that on some grape genotypes, treatments with *O. lambi* alone were as effective as fungicides in controlling the disease. Also on citrus, a reduction in the population of the tydeid mite *Lorryia Formosa* Cooreman resulted in an increase of sooty mould (Mendel and Gerson, 1982), suggesting that these mites may limit disease development.

**Dispersal of Ophistomatoid fungi by *Tarsonemus* mites phoretic on beetles.**

The ophistomatoid group of fungi, that include plant pathogens and non-pathogens, consists of diverse genera such as *Gondwanamyces*, *Ceratocystis*, *Ophiostoma* and their asexual states (Wingfield *et al.*, 1999). The adaptation of the ophistomatoid fungi to arthropod dispersal is evident from their sticky spores that are carried on stalked fruiting structures (Malloch and Blackwell, 1993). Hetrick (1949) was the first to hypothesise that the fungus *Ceratocystis minor* could be transmitted between trees by mites that sometimes occur on the southern pine beetle. Subsequently, Roton (1978) found that Tarsonemid mites feed on *Ceratocystis minor* and then move from areas of bark colonised by the fungus to cracks in the outer bark. The mites will then attach to mature adult beetles, usually after beetles have left their pupal cells (Roton, 1978). Knowledge on the dispersal of ophistomatoid fungi by mites phoretic on bark beetles (Coleoptera: Scoltinae) and picnic beetles (Coleoptera: Nitidulidae) has since become quite substantial (Gibbs and French, 1980; Juzwik, 2001).

The best example of the dispersal of ophistomatoid fungi by *Tarsonemus* mites is the dispersal of the blue stain pathogen, *Ophiostoma minus*, by mites that are phoretic on the southern pine beetle (Wingfield *et al.*, 1993; Cassar and Blackwell, 1996; Paine *et al.*, 1997; Klepzig *et al.*, 2001a; Klepzig and Six, 2004). Within the genus *Ophiostoma*, the association and vectoring of non-pathogenic *Ophiostoma* species by *Tarsonemus* and other mites, also phoretic on beetles in *Protea* spp., have recently been investigated (Lévieux *et al.*, 1989; Klepzig *et al.*, 2001a, 2001b; Klepzig and Six, 2004; Roets, 2007).

*Dispersal of the bluestain fungal pathogen, Ophiostoma minus, by Tarsonemus mites phoretic on beetles.* The fungal pathogen *Ophiostoma minus*, is of great economic importance and causes 'bluestain' in infected wood. Initially, it was known that a symbiotic relationship exists between the bluestain fungus and the southern pine bark beetle, *Dendroctonus frontalis* Zimmermann (Coleoptera: Scolytidae) (Dowding, 1969). However, it was later discovered that mites phoretic on the beetles also form part of the symbiotic relationship in a very complex manner. In this complex system, pathogen spores are dispersed between various coniferous trees by *D. frontalis*, as well as by mites (Moser and Roton, 1971).



More than 90 mite species have been associated with the southern pine beetle *D. frontalis*, of which 15 are phoretic on the beetle (Kinn, 1967; Moser and Roton, 1971; Moser, 1976a). The mites that have most often been found associated within *D. frontalis* are *Tarsonemus ips* Lindquist, *T. krantzii* Smiley and Moser, and *T. fusarii* Cooreman (Moser and Roton, 1971; Moser and Bridges, 1986). It has also been found that some of the mites possess specialised spore-carrying structures (sporothecae) that can be filled with spores of ophiostomatoid fungi (Bridges and Moser, 1983; Moser, 1985; Moser *et al.*, 1995). A mutualistic association may thus exist between these mites and the fungi (Klepzig *et al.*, 2001b).

*The role of mites phoretic on beetles in the dispersal of ophiostomatoid fungi in Protea infrutescences.* The role of mites in the dispersal of ophiostomatoid fungi in *Protea* spp. has recently been investigated in South Africa (Roets, 2007). Ophiostomatoid fungi were first detected in the infrutescences of some *Protea* spp. in 1988 (Wingfield *et al.*, 1988). Subsequently, studies were initiated to investigate the role of insects and mites in the dispersal of the fungi (Roets, 2002; Roets, 2007). It was found that few insects carried DNA of *Ophiostoma*, whereas various mite species frequently carried propagules of this fungus, suggesting that the mites may be the primary vectors of *Ophiostoma* (Roets, 2007).

Mites are now considered as primary vectors of *Ophiostoma* associated with infrutescences of *Protea* in South Africa (Roets *et al.*, 2007, 2008). Four mite species were identified as carriers of the fungi, namely *Proctolaelaps vandenbergi* Ryke, two unidentified *Tarsonemus* spp. (A and B), as well as a species of *Trichouropoda* (Roets *et al.*, 2007). The frequency (percentage) with which *Ophiostoma* was isolated from these mites ranged between 2 to 27.3%. Specifically, the *Tarsonemus* spp. had a frequency of 4% for species A and 15.8% for species B (Roets *et al.*, 2007). It was found that the number of mites carried on an individual beetle varied considerably, but *Tarsonemus* sp. A, was recorded at up to 108 per beetle (Roets *et al.*, 2007). Specialised structures which frequently contained spores of *Ophiostoma* (Roets *et al.*, 2007) were observed in *Trichouropoda* and one of the *Tarsonemus* species with light and scanning electron microscopy.

Dispersal of the mites by beetles is important in dispersal of *Ophiostoma*, although the mites carrying *Ophiostoma* can also self-disperse. Roets *et al.* (2008) found that mites could self-disperse to fresh, moist infrutescences as infrutescences desiccate. However, long-range dispersal of the mites by beetles is achieved through their phoretic association with three beetle species namely *Genuchus hottentotus* (F), *Trichostetha fascicularis* L. and *T. capensis* L. (Roets *et al.*, 2008). The long-range, hyperphoretic dispersal of *Ophiostoma splendens* G.J. Marais and M.J. Wingf. and *O. phasma* Roets *et al.* are thought to be effective since host-colonisation was achieved during the first flowering season 3 to 4 years after fire (Roets *et al.*, 2008).

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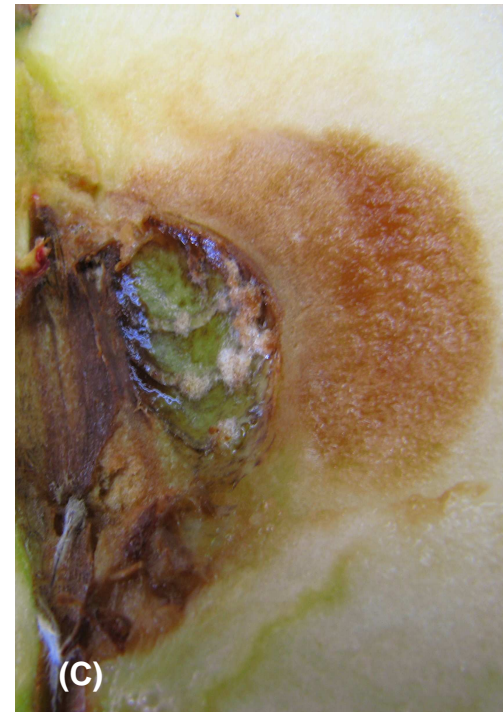
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**Fig. 1.** Different symptoms associated with the core region of apples. (A) Dry Core Rot (DCR), (B) Mouldy Core (MC) and (C) Wet Core Rot (WCR).

## 2. CHARACTERISATION OF *PENICILLIUM* ISOLATES ASSOCIATED WITH PRE-HARVEST WET CORE ROT IN SOUTH AFRICA, AND THEIR ASSOCIATION WITH ASYMPTOMATIC, DRY CORE ROT AND MOULDY CORE APPLES

### ABSTRACT

Symptoms associated with the core region of apples can be divided into mouldy core (MC), wet core rot (WCR) and dry core rot (DCR). Wet core rot is primarily known as a post-harvest disease with infections taking place during fruit handling in pack houses. In South Africa, however, apple growers recently reported WCR as being a pre-harvest disease. Therefore, the aim of this study was to investigate the incidence and causal agent/s of this disease. The incidence of WCR ranged from 0% to 1.7%, and was in general lower than that of DCR (0.4% to 6%) when investigated in one orchard in the 2005/06 season, as well as 11 orchards in the 2006/07 season. Isolation studies from eight internal positions in WCR apples showed that *Penicillium* was the predominant fungal genus in most of the positions, including the lesion area. Morphological and molecular characterisation of *Penicillium* isolates obtained from WCR apples showed that *P. ramulosum* prov. nom. was the main species isolated from lesions, as well as other isolation positions of apples sampled in both seasons. However, this species was also one of the main species isolated from DCR, MC and asymptomatic apples but at a much lower incidence than in WCR fruit. *Penicillium expansum* was only isolated from WCR lesions in the 2005/06 season, as well as DCR lesions in both seasons. Other *Penicillium* species that were occasionally isolated included *P. glabrum*, *P. chloroloma*, *P. chermisinum* and a putative new species with closest affinity to *P. dendriticum* (aff. *dendriticum*). The pathogenicity and virulence of *Penicillium* isolates were investigated using colonised toothpicks inserted (A) into the surface of apples to a depth of 8 mm, (B) through the calyx tube not causing wounding and (C) off-centre from the calyx tube through to the core region causing wounding. Eighty-four *Penicillium* isolates were tested using inoculation method A, and 10 isolates with

methods B and C. Three main lesion types, based on colour and texture, were observed in the inoculation studies. Lesion type 1 was light brown and very wet, lesions type 2 was medium brown and spongy, and lesion type 3 was medium brown, dry and small. The inoculation studies showed that *P. expansum* was the most virulent species, causing lesion type L1 and the largest lesions (41 to 85.8 mm) in method A, as well as complete rotting of apples in methods B and C. The *P. ramulosum* prov. nom. isolates varied in their virulence causing lesion types L2 and L3, and were all considered to have low virulence, causing only small lesions (0 to 18 mm) in wounding experiments. The *P.* species (aff. *dendriticum*) caused a significantly larger lesion type L2 (14 to 20.5mm) than *P. ramulosum* prov. nom. isolates in inoculation method A. This species was also the only other *Penicillium* species aside from *P. expansum*, which could cause a substantial lesion in inoculation method B.

## INTRODUCTION

In South Africa, core rots are among some of the most important post-harvest diseases of apples and are mainly associated with susceptible Red Delicious varieties (Combrink and Ginsburg, 1973; Serdani *et al.*, 1998). Post-harvest losses can range between 5 to 8% (Serdani *et al.*, 1998), with some additional losses occurring prior to harvest due to premature fruit drop in orchards (Combrink and Ginsburg, 1973). Core rots can be divided into dry core rot (DCR) and wet core rot (WCR), based on whether the rot that spreads from the core region into the surrounding fleshy tissue has a dry- or wet appearance respectively. Another symptom that is associated with apple core regions is mouldy core (MC), which consists of fungal hyphal growth that is restricted to the seed locule (Combrink *et al.*, 1985; Spotts *et al.*, 1988). Although many fungi have been found associated with DCR and WCR, the main fungal genera that are considered to be the causative agents are *Alternaria* and *Penicillium* respectively (Combrink *et al.*, 1985; De Kock *et al.*, 1991).

*Penicillium* species not only cause WCR of apples, but can also cause an external rot known as blue mould (Rosenberger, 1990; Spotts *et al.*, 1988). Blue mould is the most important post-harvest disease of stored apples world-wide

(Rosenberger, 1990). Wet core rot (WCR) is in general considered less important than blue mould, although it can also expand rapidly under cold storage conditions and is thus more damaging than DCR (Combrink and Ginsburg, 1973).

Wet core rot infections occur when pathogen spores enter the apple core region during dipping in pathogen-contaminated water in the pack house (Combrink and Ginsburg, 1973; Spotts *et al.*, 1988). In South Africa, WCR was first reported as a serious post-harvest disease of Starking apples in 1973 (Combrink and Ginsburg, 1973). However, the disease was mostly associated with apples that were dipped in diphenylamine (DPA) emulsions, which resulted in a decrease in surface tension of the suspension and thus causing the suspension to penetrate the core more readily. The DPA emulsion was also able to stimulate spore germination and thus infection (Combrink and Ginsburg, 1973).

The *Penicillium* species causing blue mould and wet core rot may be comprised of the same species, or different species may be involved. The blue mould- causing *Penicillium* species have been well characterised and the predominant species have been identified as *P. expansum* and *P. solitum*. Other species such as *P. commune*, *P. aurantiogriseum*, *P. crustosum* and *P. brevicompactum* have also been isolated, but less frequently (Sanderson and Spotts, 1995; Sholberg and Haag, 1996; Amiri and Bompeix, 2005; Sholberg *et al.*, 2005). Furthermore, in New South Wales, *P. verrucosum* is considered as an important component of blue mould (Penrose *et al.*, 1984). Less is known about the *Penicillium* species causing WCR. Spotts *et al.* (1988) isolated *Penicillium roquefortii* from apples with core rot, but later showed that *P. roquefortii* inoculated during the pre-harvest period did not cause WCR. However, when *P. roquefortii* was inoculated during the pre-harvest period followed by post-harvest inoculation with *P. expansum*, or if only *P. expansum* was inoculated in the post-harvest period, extensive WCR developed (Spotts *et al.*, 1988). In South Africa, *P. expansum* (Combrink and Ginsburg, 1973) as well as *Penicillium funiculosum* (Combrink *et al.*, 1985; De Kock *et al.* 1991; Serdani *et al.*, 1998) have been reported as causing WCR.

The identification of *Penicillium* isolates to species level is important in etiological studies, as well as for the identification of inoculum sources. In earlier

years, *Penicillium* isolates were identified to species level using only morphological characteristics, such as measurements of the colony diameter, colony characteristics and microscopic observations. Colony characteristics are assessed by determining the colony texture and colour, as well as the appearance of penicilli and conidial chains (Pitt, 1979). Colony characteristics are determined best by using the three point inoculation method of isolates onto Czapek Yeast autolysate agar (CYA) or Czapek agar (Cz) and 2% Malt Extract Agar (MEA), as suggested by Samson and Pitt (1985). Pitt (1979) suggested that growth rate and water activity with different media and temperatures should be used in addition to colony characteristics (Samson *et al.*, 2004). Classification of *Penicillium* species by their cultural characteristics alone is not recommended as these can be very variable (Raper and Thom, 1949).

More recently, molecular techniques have been found very useful for identifying *Penicillium* species, and several gene regions have been investigated for their usefulness. The noncoding internal transcribed spacer (ITS) region of the rDNA unit has been investigated to clarify subdivisions in the genus (Lobuglio *et al.*, 1993; 1994). The ITS region, however, does not always contain enough variability to distinguish between all *Penicillium* species, especially some species within specific *Penicillium* subgenera (Skouboe *et al.*, 1999). The cytochrome c oxidase 1 (*COI*) gene has also been found variable in *Penicillium*, and was shown to be comparable to the ITS region in showing divergences between species in the subgenus *Penicillium* (Seifert *et al.*, 2007). Glass and Donaldson (1995) were among the first to show that the  $\beta$ -tubulin gene is highly polymorphic in fungi, and this region has subsequently also been found suitable for identifying *Penicillium* species (Samson *et al.*, 2004). The  $\beta$ -tubulin gene is highly variable between *Penicillium* species. Seifert *et al.* (2007) found that sequence divergences between species in the subgenus *Penicillium* were higher for the  $\beta$ -tubulin gene, than for the ITS and *COI* gene regions (Seifert *et al.*, 2007).

Apple growers in South Africa have recently reported that apples already have WCR symptoms when harvested, and that symptoms do not only develop after fruit dipping and storage. Therefore, the first aim of the study was to determine the incidence of WCR in apples harvested from trees in 11 orchards situated in the Ceres, Grabouw and Ermelo production regions, mainly in the 2006/07 season. The

incidence of dry core rot (DCR) and mouldy core (MC) was also recorded in order to determine the relative contribution of each core rot symptom to disease. The second aim of the study was to determine the main fungal genera associated with eight internal positions of WCR apples. *Penicillium* was predominantly isolated from WCR apples, and it was therefore also investigated whether the high incidence of this genus is restricted to WCR apples, or whether it could also be isolated from asymptomatic, DCR and MC apples. The *Penicillium* isolates obtained from all the isolation studies were identified to the species level using morphological and molecular methods. The isolates were also characterised with regard to their pathogenicity and virulence using colonised toothpicks inserted (A) into the surface of apples to a depth of 8 mm, (B) through the calyx tube not causing wounding and (C) off-centre from the calyx tube through to the core region causing wounding.

## MATERIALS AND METHODS

**Orchards surveyed and evaluation of DCR, WCR and MC symptoms.** In total, 11 orchards that were situated in the Ceres, Grabouw and Ermelo production regions of South Africa were surveyed (Fig. 1; Table 1). One orchard was sampled in the 2005/06 season, whereas 11 orchards were sampled in the 2006/07 season (Table 1). Apples were picked one to three weeks before harvest. The cultivars, Oregon Spur and Top Red, which were included in the survey, are known to be susceptible to core rot diseases and contain an open calyx tube (Combrink *et al.*, 1985). The apples were picked at random from at least 50 trees within each orchard, with not more than five apples sampled per tree. The harvested apples, 238 to 300 per orchard (Table 1), were stored in cardboard boxes in a cold room at 4°C for 2 to 4 weeks prior to evaluation.

The incidence of DCR, WCR and MC in apples was determined by cutting apples longitudinally through the core, and again 1cm from the core on each side with a sterile knife. The apples were cut, starting at the stem end region in order to prevent fungal growth from the blossom end being transferred to the internal surface of the apples, since the apples were not surface sterilised. The different core rot symptoms were classified according to their appearance with WCR being a wet, spongy rot

extending from the core into the fleshy region of the apple, whereas DCR is a dry, corky rot that extends from the core region into the fleshy tissue. Mouldy core consists of fungal hyphae, usually greyish, that are restricted to the core region, with no apparent rot symptoms.

**Isolations for fungi from WCR apples.** The apples that were cut open and evaluated for core symptoms, were also used for isolation studies. The specific orchards from which apples were obtained for WCR isolations, are indicated in Table 1. In the 2005/06 season and 2006/07 season, isolations were made from 4 and 24 WCR apples respectively. Isolations were made from 8 internal positions (Fig. 2), after surface sterilisation of the cut apples. The isolations were made by plating small tissue pieces (1 to 3mm) onto 90mm-plates containing potato dextrose agar with 0.04g streptomycin/L (PDA<sup>+</sup>). Plates were incubated at 25°C ± 4°C on the laboratory bench and inspected regularly for fungal growth. Sub-culturing of fungal growth was conducted onto smaller (65mm diam.) PDA<sup>+</sup> plates, which were used to identify fungi to the genus level. All *Penicillium* isolates were single spored, and stored at 4°C on PDA slants as well as in 20ml bottles containing sterile water.

**Isolations for *Penicillium* from asymptomatic, DCR and MC apples.** Isolations from asymptomatic, DCR and MC apples were made as described for WCR apples, except that after sub-culturing onto 65mm-PDA-plates, only *Penicillium* colonies were analysed further. Isolations were made from 25 asymptomatic, 9 MC and 1 DCR apple in the 2005/06 season, and from 48 asymptomatic, 27 MC and 52 DCR apples in the 2006/07 season.

**Molecular characterisation of *Penicillium* isolates.** Eighty-seven *Penicillium* isolates obtained from WCR, MC, DCR and asymptomatic apples were characterised at the molecular level. Genomic DNA was extracted from each of the single spored *Penicillium* isolates, using a slightly modified method of Lee and Taylor (1990). Subsequently, the  $\beta$ -tubulin polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP) group of each isolate was determined by first PCR amplifying a partial region of the  $\beta$ -tubulin gene. PCR amplification of the partial  $\beta$ -tubulin gene was conducted using primers Bt2a



(GGTAACCAAATCGGTGCTGCTTTC) (Glass and Donaldson, 1995) and PentubR (GACGGACGACATCGAGAACCTG). The PCR reaction mixture consisted of 0.2  $\mu$ M of each primer (Bt2a and PentubR), 0.2 mM of each dNTP, 1x PCR buffer (Bioline USA Inc., Taunton, MA), 0.7 U BIOTAQ DNA polymerase (Bioline), 0.2 mg bovine serum albumin (BSA) Fraction V (Roche Diagnostics, South Africa, Randburg), 1.3  $\mu$ L DNA and 3 mM  $MgCl_2$  in a final volume of 50  $\mu$ L. Amplifications were conducted in a 2700 Applied Biosystems (Foster City, CA) PCR machine, starting with an initial denaturation cycle of 5 min. at 94 °C, followed by 40 cycles of 45s at 94 °C, 45s at 55 °C and 60s at 72 °C and a final extension cycle of 7 min. at 72 °C. Successful amplification was checked on a 1% agarose gel containing ethidium bromide. Subsequently, the PCR products were restriction digested using the restriction enzymes *HaeIII* and *RsaI*. The *HaeIII* digest reaction consisted of 1x enzyme buffer (Fermentas Inc., Glen Burnie, MD), 1.5  $\mu$ L *HaeIII* (Fermentas) and 8  $\mu$ L PCR product in a total volume of 25  $\mu$ L. The *RsaI* digestion reaction consisted of 1x enzyme buffer (Fermentas), 0.5  $\mu$ L *RsaI* (Fermentas) and 8  $\mu$ L PCR product in a total volume of 25  $\mu$ L. Reactions were conducted overnight at 37°C, and 15  $\mu$ L of the restriction digest products were run along with a 50 bp DNA standard (Fermentas) on a 3% agarose gel (MS-8 agarose, Molecular screen, Hispanagar, Burgos, Spain) containing ethidium bromide. Isolates that exhibited the same restriction pattern for both enzymes were classified into the same  $\beta$ -tubulin PCR-RFLP group.

A subset of the isolates that represented the different  $\beta$ -tubulin PCR-RFLP groups were selected for sequencing of the ITS and  $\beta$ -tubulin gene areas (Table 2). ITS amplification was conducted using the same PCR reaction conditions as for the  $\beta$ -tubulin gene, except that the ITS1 and ITS4 primers (White *et al.*, 1990) were used. Amplification conditions of the ITS region was also similar to those used for the  $\beta$ -tubulin gene, except that the annealing temperature was 52°C. The partial  $\beta$ -tubulin gene was amplified as described above. ITS and  $\beta$ -tubulin PCR products were cleaned using the Invitex kit (Invitex, Berlin), according to manufacturer's instructions. Sequence analyses were conducted by the Central Analytical Sequencing Facility at Stellenbosch University using the BigDye system (version 3.1 dye terminators, Applied Biosystems) and an ABI 3130XL Genetic Analyzer. Geneious Pro (Biokmatters Ltd., Auckland, New Zealand) was used to view ABI trace files, and

to obtain consensus double strand sequences for each isolate, which were submitted to Genbank (Table 2).

**Morphological characterisation of *Penicillium* isolates.** A subset of the isolates representing the different  $\beta$ -tubulin PCR-RFLP groups was also selected for morphological characterisation (Table 2). *Penicillium* isolates were morphologically characterised by preparing spore suspensions of isolates in semi-solid agar (0.2% agar, 0.05% Tween). The spore suspension of each isolate was inoculated on Czapek Yeast Agar (CYA), Malt Extract Agar (MEA) and 25% Glycerol Nitrate Agar (G25N) using the three point inoculation method (Pitt, 1979; Samson and Pitt, 1985). The inoculated 90mm-Petri-plates were incubated at 25°C (CYA, MEA, G25N), 5°C (CYA) and 37°C (CYA). Plates were incubated in the dark and were left unwrapped to allow sufficient aeration (Okuda *et al.*, 2000). Additional CYA and MEA plates were inoculated and incubated at room temperature ( $\pm 23^\circ\text{C}$ ) on the laboratory bench for 7 to 21 days. Characterisation and descriptions were made according to the methods of Pitt (1979), Samson and Pitt (1985) and Okuda *et al.* (2000). Codes and colour names for the isolates were assigned according to Kornerup and Wanscher (1967).

**Evaluation of the pathogenicity of 84 *Penicillium* isolates using a toothpick surface inoculation method.** The pathogenicity of 84 *Penicillium* isolates that were identified to the species level was determined using a surface inoculation method with colonised toothpicks. Colonised toothpicks were obtained by first cutting toothpicks in half, followed by five consecutive rounds of autoclaving in deionised water, with the water being replaced after each autoclave session. The toothpicks were then transferred to potato dextrose broth (Difco Laboratories, Detroit) and autoclaved again (Serdani *et al.*, 2002). The toothpicks were placed aseptically onto 90mm-PDA-plates, and each plate was inoculated with one agar plug of an approximately 7-day-old *Penicillium* culture growing on PDA<sup>+</sup>. Top Red apples were surface sterilised prior to inoculation by immersing the apples for 30 seconds in 70% ethanol, 2 minutes in 1% NaOCl and 15 seconds in 70% ethanol. The apples were air dry in a laminar flow cabinet. Toothpicks colonised with the *Penicillium* isolates, as well as control toothpicks consisting of toothpicks that were placed on uninoculated PDA<sup>+</sup>-plates,

were inserted  $\pm$  8 mm into the apples. Each apple contained 6 toothpicks of which five were each colonised with a different isolate, with the sixth toothpick being a control uncolonised toothpick. Four replicates of each isolate were inoculated into apples, and the experiment was repeated. Inoculated apples were incubated in moisture chambers at high relative humidity (85 to 90%) for 10 days at  $25^{\circ}\text{C} \pm 4^{\circ}\text{C}$ . At the end of the incubation period, toothpicks were removed from apples, and apples were split longitudinally across the inoculation points with a sterile knife. Lesions were characterised with regard to diameter (mm), colour (medium brown or light brown) and texture (dry, very wet or spongy). The diameter of the lesions formed on the surface (skin) of the apple was measured. Isolations were made from a sub-set of the apples onto PDA<sup>+</sup> to fulfil Koch's postulates.

*Statistical analysis.* Data of the lesion sizes produced by each *Penicillium* isolate in the surface inoculation experiment, as well as lesion sizes produced by the different *Penicillium* species was analysed using Statistica version 8.0 [Statsoft Inc. 2008, STATISTICA (data analysis software system), version 8.0. www.statsoft.com]. A repeated measures analysis of variance (RM ANOVA) was done between the 2007 (Rep 1) and 2008 (Rep 2) external lesion diameters (two repeats of experiments) over *Penicillium* species. The lesion diameters were also compared to lesion types per year with ANOVA. Appropriate Bonferroni multiple comparisons were done to investigate where significant differences (interactions) occurred. Where the residuals were not normally distributed, non-parametric tests like the Kruskal-Wallis' analysis of variance test or a bootstrap test was done to confirm the results of ANOVA's. All analyses were done at the 5% significance level.

**Evaluation of the pathogenicity of 10 *Penicillium* isolates using two calyx end toothpick inoculation methods.** The pathogenicity and virulence of 10 *Penicillium* isolates was tested by using two different calyx end-based colonised toothpick inoculation methods that either resulted in wounding, or non-wounding of apple tissue during the inoculation. The 10 isolates that were selected represented the main *Penicillium* species identified in the study (Table 3). The experiments were conducted twice.

The colonised toothpicks were prepared as described above for the surface inoculation experiment, except that toothpicks were not cut in half. The non-wounding technique consisted of the toothpick being inserted directly into the calyx tube and core region. The wounding technique consisted of a toothpick inserted off-centre from the calyx tube at an angle into the core region, thus wounding the apple fleshy tissue. For each isolate, 5 apples were inoculated using the non-wounding techniques, as well as 5 apples for the wounding technique. Controls for the non-wounding and wounding experiments consisted of 5 apples each being inoculated with an uncolonised toothpick either causing wounding or non-wounding. Top Red apples were incubated at  $25^{\circ}\text{C} \pm 4^{\circ}\text{C}$  for 2 to 4 weeks (range due to the difference in virulence of isolates) under high relative humidity (85 to 90%). Evaluation of apples was conducted by cutting each apple longitudinally through the core region and calyx tube, and recording the type of lesion formed, i.e, colour (medium brown or light brown) and texture (dry, very wet or spongy).

## RESULTS

### **Orchards surveyed and evaluation of DCR, WCR and MC symptoms.**

Orchard CSC1 was the only orchard in which the survey for different core rot symptoms was conducted in two consecutive seasons. The incidence of WCR in this orchard was similar in both seasons, being 1.7% in the 2005/06 season and 1.3% in the 2006/07 season (Table 1). WCR symptoms typically consisted of medium brown, spongy to wet lesions that varied somewhat in appearance in different apples (Fig. 3).

Nine of the ten other orchards also contained apples with WCR (Table 1), with the symptoms being similar to that observed in orchard CSC1 (Fig. 3). The only orchard that did not contain WCR was one orchard in the Grabouw region (CSG2). The incidence of WCR in the ten orchards ranged from 0% to 1.7% (Table 1). In general the incidence of WCR was higher in the Ceres area (1% to 1.7%) where Oregon Spur apples were analysed, than in the Grabouw area (0% to 0.7%) (Table 1) where Top Red apples were analysed. Sampling in the Ermelo region was only done in one orchard and generalisations could therefore not be made.

Almost all of the 11 orchards that were surveyed contained apples with DCR and MC, the exception being one orchard in the Grabouw area that did not contain any MC apples. In most of the orchards, the incidence of DCR was higher than that of WCR, whereas the incidence of MC was higher than DCR and WCR in all the orchards. The exception was orchard CSC1 that had a higher incidence of WCR than DCR in the 2005/06 season. The average incidence of WCR, DCR and MC in all the orchards was 0.8%, 3.5% and 7.6% respectively (Table 1).

**Isolations of fungi from WCR apples.** *Penicillium* was the main fungal genus that was isolated from WCR apples. It was isolated from 25 of the 28 WCR apples, in at least one of the eight isolation positions shown in Fig. 2. In most of the isolation positions *Penicillium* was the main fungal genus, except in the stem end (position 7), flower parts (position 8) and calyx tube (position 6), in both seasons (Fig. 4). Isolations from the edge of the lesion (position 1) showed that *Penicillium* was isolated from 75% and 57% of the apples in the 2005/06 and 2006/07 seasons respectively (Fig. 4). Isolations 1 cm away from the lesion (position 2) yielded *Penicillium* in 75% and 52.2% of the apples in the 2005/06 and 2006/07 seasons respectively (Fig. 4). *Penicillium* was also isolated at moderate levels (> 35%) in both seasons from the calyx tube (position 6), in tissue next to the calyx tube (position 3), 1 cm away from the calyx tube (position 4) as well as the core region (position 5) (Fig. 4).

The other fungal genera that were also isolated from WCR apples included *Alternaria*, *Aureobasidium*, *Cladosporium* and *Epicoccum*. Several fungi from other genera were also isolated but were not represented by more than 3 or 4 isolates, and were grouped under “other fungi” (Fig. 4). In the 2005/06 season, when isolations from only 4 WCR apples were made, only *Aureobasium* was isolated at frequencies similar to *Penicillium* in the core (position 5) and the regions surrounding the calyx tube (positions 3 and 4) and the core regions (positions 1 and 2) (Fig. 4). However, in the 2006/07 season, all of the main fungal genera were isolated from positions 1 to 4 at frequencies much lower than *Penicillium*, with *Aureobasium* having the highest isolation frequency (Fig. 4). In all of the WCR apples in both seasons the incidence of *Alternaria* increased progressively from the calyx tube (position 6) to the flower parts (position 8) (Fig. 4).

### **Isolations for *Penicillium* from asymptomatic, DCR and MC apples.**

*Penicillium* was also isolated from healthy apples, DCR and MC apples, although the incidence was much lower than in WCR apples for most of the isolation positions (Fig. 5). In the 2006/07 season, the incidence of *Penicillium* in DCR apples in all of the isolation positions was low, and did not exceed 9.6%. This was low considering that isolations were made from 52 DCR apples and only 24 WCR apples. Similarly, in the 2006/07 season in MC and asymptomatic apples, the incidence of *Penicillium* in all the isolation positions was low, with MC apples having the highest incidence in the calyx tube and surrounding tissue (positions 3, 4 and 6) (Fig. 5).

### **Molecular and morphological characterisation of *Penicillium* isolates.**

Eighty-seven *Penicillium* isolates were obtained from isolations made from apples (7 DCR apples, 25 WCR apples, 3 MC apples and 6 asymptomatic apples). Since *Penicillium* isolates were obtained from more than one isolation position within an apple, the total number of isolates that were obtained totalled 87, of which 60 were WCR isolates, 13 DCR isolates, 6 MC isolates and 8 were isolates from asymptomatic apples.

Eight  $\beta$ -tubulin PCR-RFLP groups were identified among the 87 *Penicillium* isolates collected from both seasons (Fig. 6). Sequencing of the ITS and  $\beta$ -tubulin regions of isolates representing the different PCR-RFLP groups showed that PCR-RFLP Group 1 represents *P. expansum*, Group 2, 5 and 8 represents *P. ramulosum* prov. nom. which is a new species that is in the process of being described (personal communication, K. Jacobs). Group 3 represents a putative new *Penicillium* species of which the closest known relative is *P. dendriticum*, and these isolates are therefore here referred to as *P. species* (aff. *P. dendriticum*). Group 4 represents *P. glabrum*, Group 6 represents *P. chloroloma* and Group 7 represents *P. chermisinum*.

Morphological analyses of a subset of the isolates representing the different PCR-RFLP groups confirmed the molecular identifications. The morphology of isolates in PCR-RFLP group 4 most closely resembled that of *P. glabrum*, but morphological identification was difficult. The PCR-RFLP group 7 isolate morphologically resembled *P. chermisinum*, although it produced brown exudates and

a light red brown soluble pigment that are not known characteristics of this species. Although there was sequence variation within the *P. ramulosum* prov. nom. PCR-RFLP groups (2, 5 and 8), the isolates were morphologically indistinguishable and they were all considered to belong to this species. Furthermore, phylogenetic analysis did not support a separate species status (unpublished data).

All the sequenced and morphologically identified isolates were submitted to the Stellenbosch University culture collection (STEU) at the Department of Plant Pathology. The GenBank (<http://www.ncbi.nlm.nih.gov/Genbank/>) accession numbers of the ITS and  $\beta$ -tubulin sequences of the isolates as well as their culture collection numbers are presented in Table 2.

After the identification of all the isolates to the species level, the association of specific *Penicillium* species with asymptomatic, WCR, DCR and mouldy core apples in the different isolation positions was investigated. The association of the different *Penicillium* species with each of the different symptoms and asymptomatic apples is presented in Table 3. In DCR apples, *P. expansum* and *P. ramulosum* prov. nom. were the predominant species isolated from lesions (positions 1 and 2) in both growing seasons. These species were also isolated from the tissue surrounding the calyx tube (positions 3 and 4). In asymptomatic apples, which only consisted of 8 isolates, the predominant species was *P. ramulosum* prov. nom. These isolates were mainly isolated from the calyx tube region (positions 3 and 6) from five orchards in Ceres. In the MC apples only *P. ramulosum* prov. nom. was isolated, mainly from the calyx tube region (positions 3 and 4). In both seasons the main *Penicillium* species that was isolated from the WCR lesion area (positions 1 and 2), and from almost all the other isolation positions, (except position 7) was *P. ramulosum* prov. nom. In WCR apples, *P. expansum* was only isolated in the 2005/06 season, mainly from the lesion area (Table 3).

Aside from *P. expansum* and *P. ramulosum* prov. nom., other *Penicillium* species that were isolated occasionally from DCR, WCR and asymptomatic apples included *P. glabrum* (WCR, asymptomatic, DCR) and *P. chermisinum* (WCR) and *P. chloroloma* (Table 3).

**Evaluation of the pathogenicity of 84 *Penicillium* isolates using a toothpick surface inoculation method.** The inoculation of apples with the surface toothpick inoculation method showed that the isolates varied in the size of lesions that they caused, as well as the appearance (colour and texture) of the lesions (Table 3; Figs. 7 and 8). The appearance of lesions was quite a stable character, since it was reproducible for most isolates between experiments, and three distinct lesion types (L1 to L3) could be distinguished (Fig. 7). However, for 11 of the *P. ramulosum* prov. nom. isolates the lesion types were not consistent, with some apples showing an L2 and other apples showing an L3 lesion type within the same experiment (Table 3). The control, inoculated with uncolonised toothpicks, never showed any lesion (Fig. 7 D), nor was *Penicillium* isolated from the inoculation site. In *Penicillium* inoculated apples, this genus was re-isolated from lesions, fulfilling Koch's postulates.

The three lesion types (L1 to L3) each had a distinct colour and texture. Lesion type L1 had a light brown and very wet appearance, L2 had a medium brown and spongy lesion that was sometimes slightly wet, and L3 had a medium brown colour and was a dry lesion that was very small. There were significant differences between the lesion sizes associated with specific lesion types (data not shown). Lesion sizes associated with a specific lesion type also varied between the tested isolates (Table 3). The lesion size of the L1 type ranged from 41 mm to 85.8 mm (average 58 mm), that of the L2 lesion type ranged from 2.3 mm to 16.3 mm (average 9.5 mm) and that of the L3 lesion type ranged from 0.00 to 6.3 mm (average 1.6 mm). Lesions caused by *P. expansum* isolates were all of the L1 lesion type (light brown and very wet appearance), whereas the *P. ramulosum* prov. nom. isolates caused either a L2 or L3 type (Table 3). The L2 lesion type most closely resembled the WCR lesions that were originally found pre-harvest in orchards.

Analyses were conducted to determine whether specific *Penicillium* species were associated with specific lesion sizes. The data of the two repeats of the trials could not be pooled since external lesion diameter differed significantly between the two repeats of the trial. The analysis showed that for some species isolates within the same species differed significantly with regard to the size of lesion they caused (Fig. 8). Isolates within *P. expansum*, *P. glabrum* and *P. species* (aff. *dendriticum*) did not differ significantly from each other in the lesion sizes they caused. However, isolates



within the *P. ramulosum* prov. nom. group did differ significantly from each other in the lesion sizes caused (data not shown). The lesion sizes caused by *P. expansum* ranged from 41 to 85.7mm (average 59mm), *P. ramulosum* prov. nom. lesions ranged from 0 to 18 mm (average 6.95 mm), *P. species* (aff. *dendriticum*) lesions ranged from 14 to 20.5 mm (average 17.2 mm), *P. glabrum* lesions ranged from 0 to 2.8 mm (average 0.83) and *P. chloroloma* lesions ranged from 1.8 to 2.3 mm (average 2 mm). Analyses of lesion sizes according to species groups showed that *P. expansum* caused significantly larger lesions than any of the other species. *Penicillium chloroloma*, *P. chermisinum* and *P. glabrum* did not differ significantly from each other with regards to lesion size (Fig. 8). *Penicillium species* (aff. *dendriticum*) caused significantly larger lesions than *P. ramulosum* prov. nom, *P. chloroloma*, *P. glabrum* and *P. chermisinum* in both experiments. *Penicillium ramulosum* prov. nom. caused significantly larger lesions than *P. chloroloma*, *P. glabrum* and *P. chermisinum* in both experiments (Fig. 8). Only one isolate of *Penicillium chermisinum* was found, which was non-pathogenic (Table 3).

A comparison of the pathogenicity of *Penicillium* isolates obtained from different isolation positions in WCR apples showed that all the isolates obtained from isolation position 1 and 2 (21 isolates) were pathogenic, i.e. causing a lesion diameter and/or either lesion type L2 or L3. In isolation position 3, 100% (8/8) of the isolates were pathogenic, and in position 4, 89% (8/9) of the isolates were pathogenic. Most of the *Penicillium* isolates (80%) from asymptomatic apples were also pathogenic.

**Evaluation of the pathogenicity of 10 *Penicillium* isolates using two calyx end toothpick inoculation methods.** In the calyx-end inoculation studies, two different methods were used for inoculating the *Penicillium* isolates, resulting in wounding or non-wounding of the apple tissue.

In the wounding experiment, where the toothpick was inserted off-centre from the calyx-tube through to the core region, most of the isolates caused lesions after a 4 week incubation period. The exception was the *P. expansum* isolate that already caused extensive rotting 2 weeks after inoculation, causing the L1 lesion type (Table 3; Fig. 9A). Most of the *P. ramulosum* prov. nom. isolates (D103, D101, W10, W159, W202, W224) that were tested caused the same lesion type (L2) in this

experiment (Fig. 9B) as in the surface toothpick inoculation trial. However, there were a few exceptions. For example *P. ramulosum* prov. nom. isolate W177 and *P. glabrum* isolate W234 caused the L3 lesion type in surface inoculation studies, but in the calyx-end wounding experiment the lesion type was not distinctive and varied between the different inoculated apples. *Penicillium glabrum* isolate W234, produced a L1 or L2 lesion type in different apples in the wounding experiment, whereas isolate W177 produced all three lesion types in the different apples (Table 3). The control of the wounding experiment did not cause a lesion, only a slight browning where wound healing occurred (Fig. 9D).

In the non-wounding method where toothpicks were inserted into the calyx tube, only two of the isolates, representing *P. expansum* (D97) and *P. species* (aff. *dendriticum*) (W44), caused distinct lesions. In these experiments the *P. expansum* isolate (D97) caused all 5 apples to completely rot in both experiments only two weeks after inoculation, causing a typical L1 lesion type (light brown and very wet lesion) (Table 3; Fig. 10A). This isolate also caused a L1 lesion type in the shallow toothpick inoculation experiment. The *P. species* (aff. *dendriticum*) isolate (W44) also caused a lesion (L2) in both experiments, but only after four weeks of incubation (Table 3; Fig. 10B). These were similar lesions to the lesion type (L2) caused by this isolate in the toothpick surface inoculation trial. The remainder of the isolates caused no lesion in the non-wounding experiment after 4 weeks of incubation (Table 3; Fig. 10C). No lesion type L3 was caused by this inoculation method. No lesions were observed in the control apples (Fig. 10C).

## DISCUSSION

Pre-harvest wet core rot (WCR) of apples was detected in all three production regions and in 10 of 11 orchards. Although the Ceres orchards contained somewhat higher incidences of WCR than the Grabouw orchards, definite conclusions regarding the incidence of WCR in the two regions can not be made since too few orchards were sampled. Disease incidence was also very low within orchards, and different cultivars were sampled within each region. In orchards with WCR the incidence ranged from

0.3% to 1.7%. In general, the incidence of WCR (average 0.8%) was lower than that of DCR (average 3.5%) and MC (7.6%) in most of the orchards.

Wet core rot is mostly known as a post-harvest rot where *Penicillium* infects the core region during fruit dipping practices in pack houses (Spotts, 1990). However, careful scrutiny of core rot literature in South Africa showed that WCR also has been reported previously as a pre-harvest rot in South Africa by De Kock *et al.* (1991). Although not quite clear, the publications of Combrink *et al.* (1985) and Serdani *et al.* (1998) may have identified pre-harvest WCR, since the apples were harvested from trees and stored directly for three to eight months before evaluation, without treatment in pack houses. The report of pre-harvest WCR by De Kock *et al.* (1991) did not indicate the incidence of WCR separate from DCR, but only reported the incidence of total core rot which ranged from 3.3% to 12% in two seasons at harvest (De Kock *et al.*, 1991). Although Combrink and Ginsburg (1973) reported incidences of WCR as high as 4% to 20%, this was a post-harvest WCR specifically associated with the use of diphenylamine-emulsions in dip tanks.

*Penicillium* was found to be the main fungal genus isolated from WCR apples. Identification of the isolated *Penicillium* species showed that the most common *Penicillium* species obtained from WCR apples was *P. ramulosum* prov. nom. Combrink *et al.* (1985) and De Kock *et al.* (1991) reported *P. funiculosum* as the main species associated with WCR, which were most likely all pre-harvest infections. Serdani *et al.* (1998) only mentioned that they isolated *P. funiculosum* and *P. expansum* from apples with WCR that were picked from trees and stored directly, but they did not elaborate on the contribution of each species to WCR. The isolates that were identified in all three of the aforementioned studies as *P. funiculosum*, may have been similar to the currently identified *P. ramulosum* prov. nom. as well as *P.* species (aff. *dendriticum*) isolates, since the latter two species have culture characteristics similar to that of *P. funiculosum* (personal communication Dr. K. Jacobs). In the current study *P. expansum* was only isolated from WCR apples in one season. Previously, *P. expansum* has been associated with post-harvest WCR in South Africa (Combrink and Ginsburg, 1973) as well as possibly pre-harvest infections (Serdani *et al.*, 1998).

In pre-harvest WCR apples, *Penicillium* was isolated from several internal positions including the calyx tube (positions 3 and 6) and flower parts (position 8). This suggests that, should *Penicillium* be able to sporulate in these regions of apples, WCR apples from infected orchards may be an important source contributing inoculum to the dip tank water. The relative high frequency of isolation of *Penicillium* in WCR apples in asymptomatic fleshy tissue, i.e. 1 cm away from the calyx tube (position 4) as well as the lesion area (position 2) is interesting. Isolations from position 2 were specifically made to increase the probability of finding the causative agent at the asymptomatic edge of the rotted tissue. Pathogenicity testing using the surface inoculation method showed that all WCR isolates obtained from positions 1 and 2 were pathogenic, as were most isolates (>89%) from positions 3 and 4. Thus, as suggested in earlier literature studies most core rot pathogens (Brien, 1937; Carpenter, 1942), including *Penicillium* isolates identified in this study, are most likely facultative pathogens that grow saprophytically and only cause disease when environmental and host conditions become favourable.

*Penicillium* was isolated from DCR, MC and asymptomatic apples, although the incidence was in general much lower than in WCR apples from all isolation positions. Similar to WCR apples, the most common *Penicillium* species isolated from DCR, MC and asymptomatic apples was *P. ramulosum* prov. nom. *Penicillium expansum* was only isolated from DCR lesions, which has not been reported previously. *Penicillium* is not a fungal genus that is generally associated with the internal regions of apple fruits, and reports on the presence of this genus are not specific concerning the incidence or species of *Penicillium* found. Serdani *et al.* (1998) did not report isolating this genus from healthy fruit, whereas Teixido *et al.* (1999) reported that *Penicillium* was seldom isolated from internal fruit regions. Ellis and Barrat (1983) only mentioned that *Penicillium* was one of the minor genera isolated from the core region of apples, but that *Alternaria* was the main genus isolated from this region.

The surface toothpick inoculation method was useful for investigating the pathogenicity and virulence of *Penicillium* isolates. Pathogenicity is defined here as the ability to cause disease, whereas virulence refers to the amount of disease caused. This inoculation method has previously only been used for evaluating the

pathogenicity of *Alternaria* isolates (Serdani *et al.*, 1998). The surface inoculation method revealed the presence of three lesion types (L1 to L3) that were relatively reproducible. Lesion type 1 was only caused by *P. expansum* isolates, whereas the remainder of the species caused lesion type L2 or L3. The L2 lesion type produced in the surface inoculation studies most closely resembled the initial WCR symptoms seen in orchards. The lesion types caused by the surface inoculation method were reproduced in the calyx-end wounding inoculation studies in most cases.

Based on the surface inoculation method, *Penicillium expansum* followed by *P. species* (aff. *dendriticum*) were the most virulent species since they caused significantly larger surface lesions than the other species. Isolates from these two species were also the only species that caused lesions in the non-wounding calyx end inoculation study, although lesion development was much faster for *P. expansum* (2 weeks) than *P. species* (aff. *dendriticum*) (4 weeks). In contrast, the *P. ramulosum* prov. nom. isolates are all considered to have low virulence, since they only caused relatively small lesion sizes (L2 and L3 type) in the surface inoculation method, and they also did not cause lesions in the non-wounding calyx end inoculation method. Based on these results, the surface inoculation method seems useful for the initial screening of isolates for pathogenicity. However, in order to determine whether isolates can cause disease without wounding and determine their virulence, the non-wounding calyx-end inoculation method must be used and may be able to identify more virulent isolates.

Several methods have been published previously for evaluating the pathogenicity of *Penicillium* on apples. These include the inoculation of spore suspensions to surface wounds (Spotts *et al.*, 1999), wound inoculation by placing conidia and hyphae into 3mm deep surface wounds (Combrink *et al.* 1985; Spotts *et al.*, 1988), and injecting spores into the core region using a syringe (Combrink and Ginsburg, 1973; Michailides *et al.*, 1994; Spotts *et al.* 1988). In preliminary trials, the usefulness of spore inoculation of surface wounds was investigated, but this was found to be unreliable for evaluating isolates with low virulence, since the results varied greatly within and between experiments if for example *P. ramulosum* prov. nom. isolates were used (unpublished data). The use of spore suspensions injected into the core region is associated with problems related to low infection frequencies,

as well as possibly resident spores from the calyx region being washed into the core region. This will thus result in infections not always being caused by the fungal inoculum.

The study was not able to conclusively show that *Penicillium*, or a specific *Penicillium* species is the sole causative agent of pre-harvest WCR. However, there was some evidence that supported the fact that *P. ramulosum* prov. nom. may be involved since (1) it was isolated at higher incidence in WCR apples than in other apples, (2) it was isolated from WCR lesions and (3) it produced a lesion similar to the original lesions observed in orchards in the surface inoculation studies. It is however important to note that isolates within the species seemed to vary in their virulence. *Penicillium expansum* and *P. species* (aff. *dendriticum*) most likely also play a role in pre-harvest wet core rot due to their high virulence.

Wet core rot may be caused by a complex of *Penicillium* species since not one species was isolated consistently from the lesions, and other fungal genera may also be involved. The species that initially start infection may be obscured in the isolation studies, since lesions had already progressed substantially when isolations were made. At this stage of disease development, saprophytic or minor pathogens may have started to colonise the lesion. The fact that in inoculation studies (surface inoculation and non-wounded calyx end inoculations) *P. expansum* and the *P. species* (aff. *dendriticum*) isolates caused the largest lesions, and that both species were isolated from WCR lesions especially in the 2005/06 season, may suggest that these species can start the infection, but subsequent symptom development is modified by other facultative and minor pathogens including some *P. ramulosum* prov. nom. isolates.

Much still needs to be learned about pre-harvest WCR. Future studies should investigate the contribution of WCR to pre-harvest fruit drop by determining the number of symptomatic fruit on the orchard floor during the growing season. In the 2005/06 season in one orchard where fruit on the orchard floor was investigated at the end of the season 2.3% fruit had WCR symptoms (unpublished data). Yield losses due to fruit drop in orchards could be substantial, especially if virulent species such as *P. expansum* and *P. species* (aff. *dendriticum*) are involved, since they cause rapidly expanding lesions that are most likely to cause fruit drop. It should also be

investigated whether orchards with a high incidence in pre-harvest WCR have (1) high *Penicillium* inoculum concentrations and (2) an increased incidence of post-harvest WCR after apples have been stored. Currently, it is not known if pre-harvest WCR contributes to post-harvest WCR losses. Future studies should also investigate the usefulness of the calyx end non-wounding method by investigating a larger set of isolates and comparing the lesions formed to those in the surface inoculation method.

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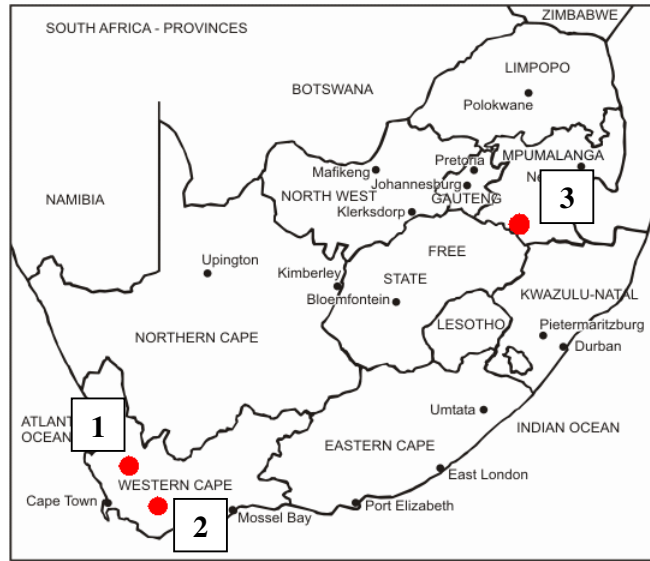
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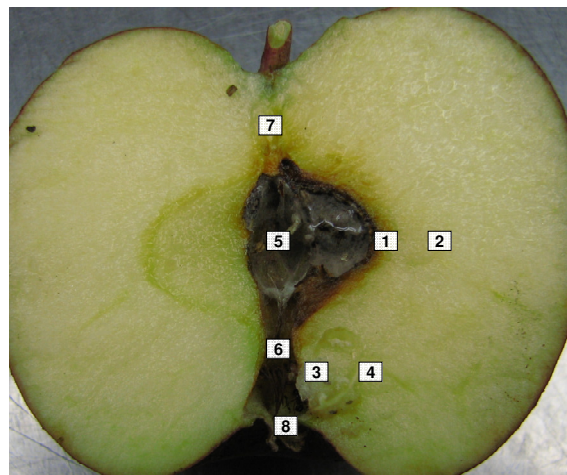
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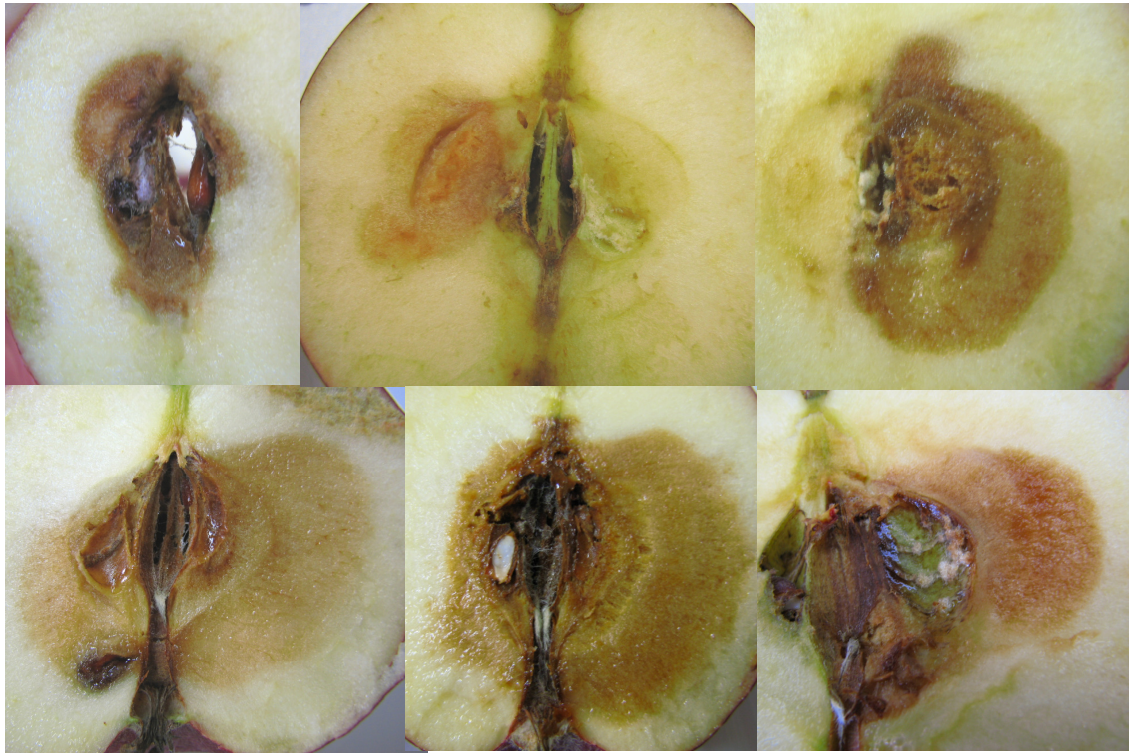
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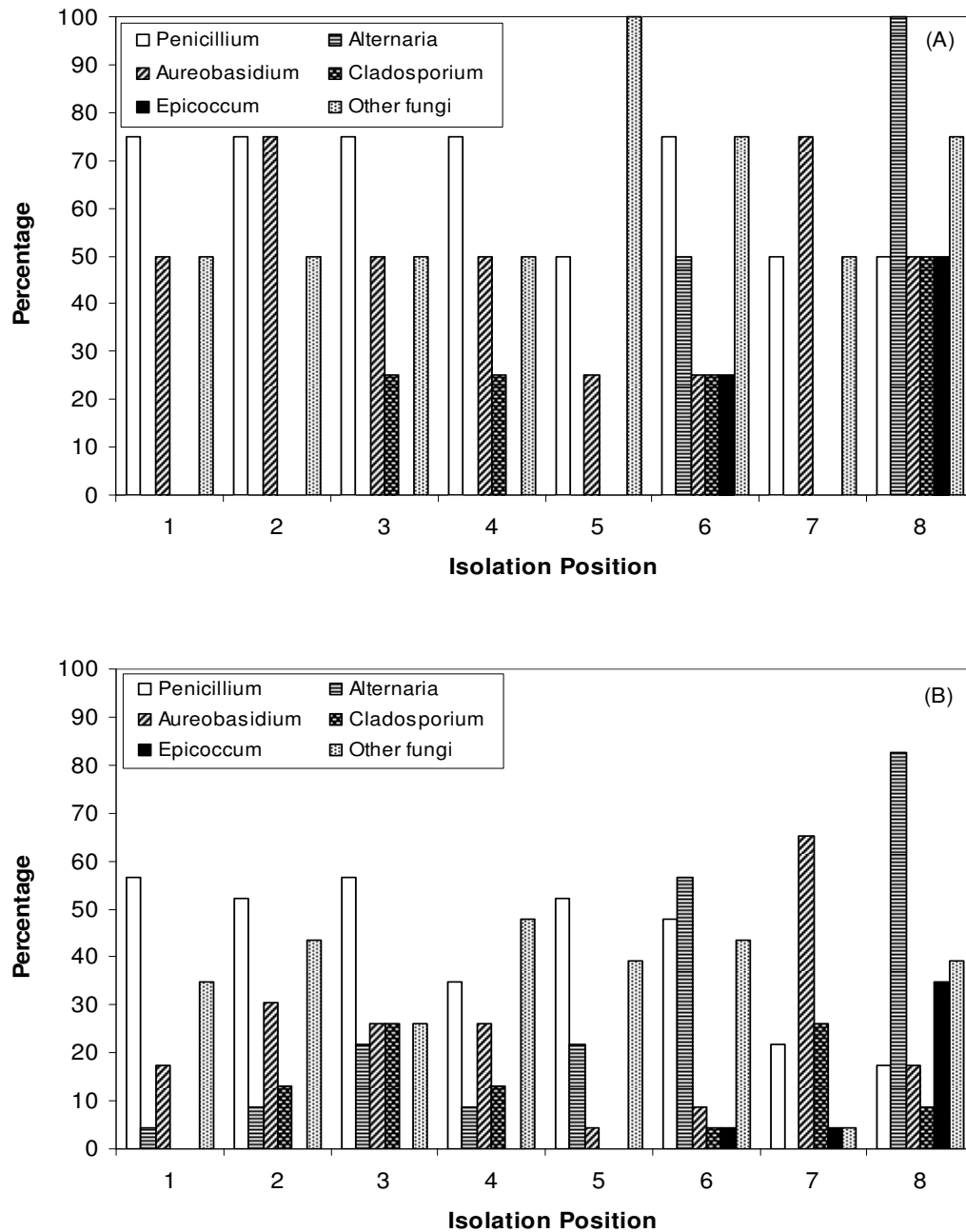
**Fig. 1.** Map of South Africa showing the two regions in the Western Cape (Ceres (1) and Grabouw (2)), and one region in Mpumalanga (Ermelo (3)) that were surveyed for the presence of wet- and dry core rot.



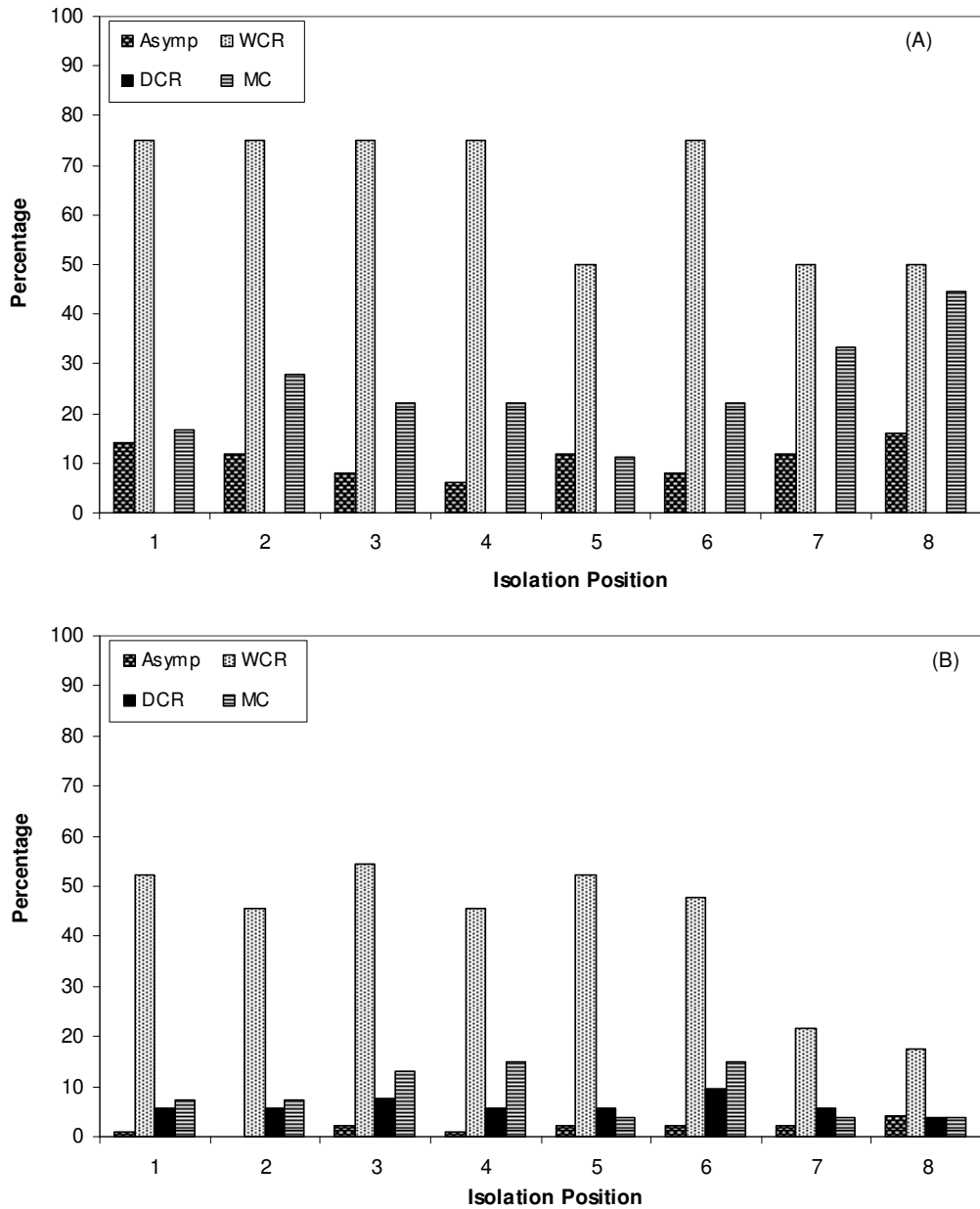
**Fig. 2.** Eight internal positions within an apple from which fungal isolations were made.



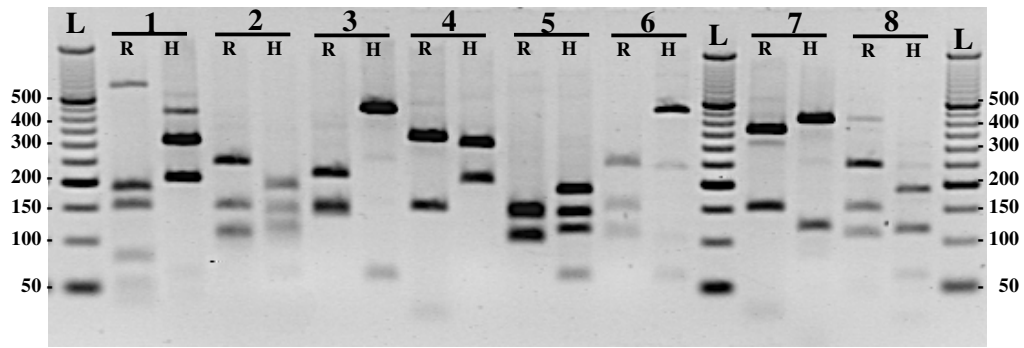
**Fig. 3.** Range of wet core rot symptoms in apples harvested from orchards.



**Fig. 4.** Fungal genera isolated from apples with wet core rot (WCR). Fungi that were only represented by three or four isolates were grouped under “Other fungi”. The percentage WCR apples containing each of the fungal genera within eight different isolation positions (1 to 8; see Fig. 2) are shown for apples that were collected (A) in the 2005/06 season in one orchard (n = 4) and (B) in the 2006/07 season in 11 orchards (n = 24).

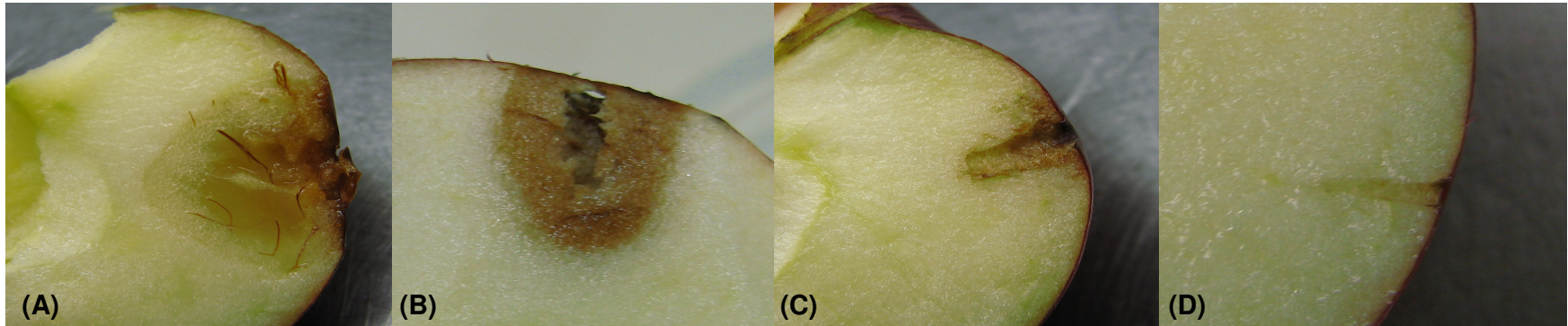


**Fig. 5.** Percentage apples with wet core rot (WCR), dry core rot (DCR), mouldy core (MC) and asymptomatic apples (Asymp) containing *Penicillium* within eight different isolations positions (1 to 8; see Fig. 2) in the (A) 2005/6 season in one orchard and (B) 2006/07 season in 11 orchards.

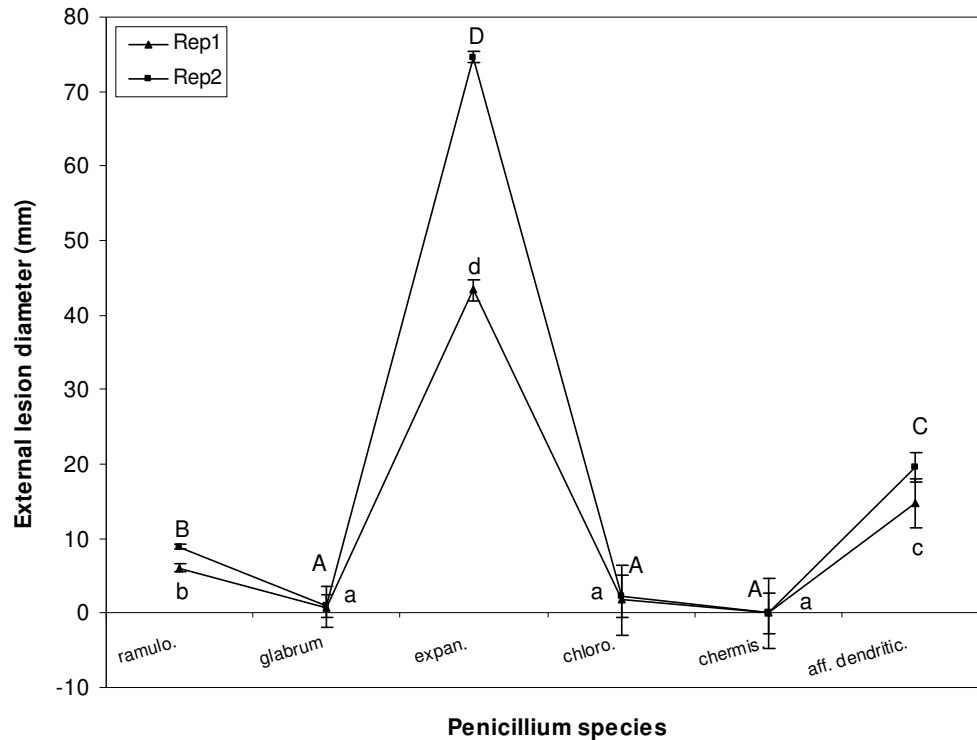


**Fig. 6.** Eight PCR-RFLP groups (1 to 8) that were identified among *Penicillium* isolates obtained from apples. The PCR-RFLP groups were identified by conducting *RsaI* (R) and *HaeIII* (H) restriction digests on PCR products of the partial  $\beta$ -tubulin gene. A 50-bp DNA ladder (L) was run along with the digest products.

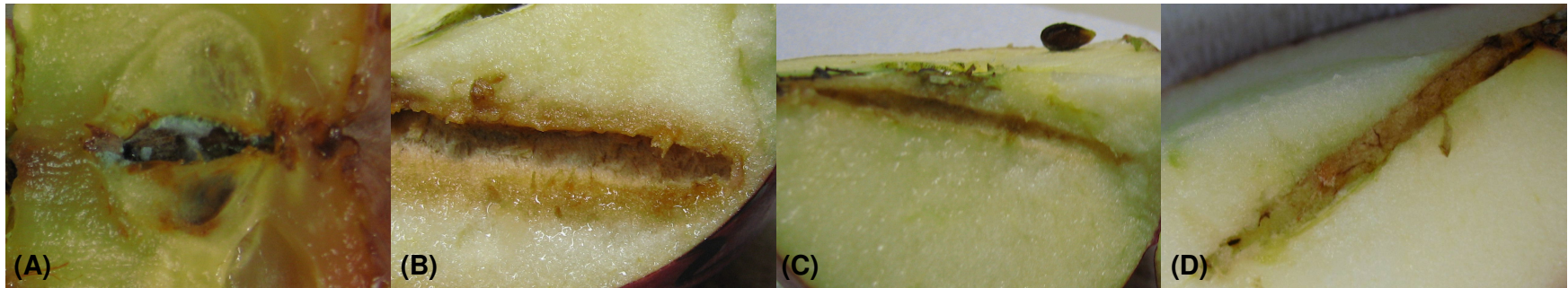




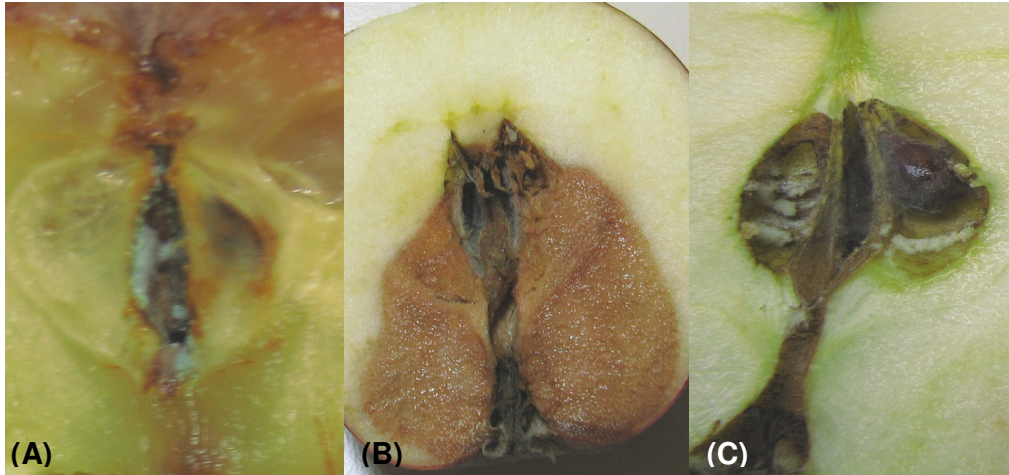
**Fig. 7.** Three main lesion types caused by *Pencillium* isolates in a surface toothpick inoculation trial. Lesion type 1 was light brown and very wet (A), lesions type 2 was medium brown and spongy (B), and lesion type 3 was a medium brown, dry and small lesion (C). Control toothpicks consisting of toothpicks that were placed on uninoculated PDA-plates (D) was also included in the trial.



**Fig. 8.** External lesion diameter on the surface of apple fruits inoculated with toothpicks colonised by *Penicillium* isolates representing six species, including *P. ramulosum* prov. nom. (*ramulo.*), *P. glabrum* (*glabrum.*), *P. expansum* (*expan.*), *P. chloroloma* (*chloro.*), *P. chermisinum* (*chermis.*) and a putative new species with closest affinity to *P. species* (*aff. dendriticum*) (*aff. dendritic.*). The data was analysed using repeated measures analysis of variance for external lesion diameter vs *Penicillium* species in two independent trials (Rep1 and Rep 2). A, B, C and D are significantly different at the 5% significance level. Significant differences are also present between a, b, c and d at the 5% significance level.



**Fig. 9.** Three main lesion types caused by *Penicillium* isolates that were inoculated with a *Penicillium* colonised toothpick that was inserted off-centre from the calyx end, through to the core region causing wounding of the apple tissue. Lesion type 1 was light brown and very wet (A), lesions type 2 was medium brown and spongy (B), and lesion type 3 was a medium brown, dry and small lesion (C). Control toothpicks consisting of toothpicks that were placed on uninoculated PDA-plates (D) was also included in the trial.



**Fig. 10.** Two main lesion types caused by *Penicillium* isolates that were inoculated with a *Penicillium* colonised toothpick that was inserted through the calyx-tube into the core region, not causing wounding. Lesion type 1 was light brown and very wet (A) and lesions type 2 was medium brown and spongy (B). Control toothpicks consisting of toothpicks that were placed on uninoculated PDA-plates (C) were also included in the trial.

**Table 1.** Incidence of wet core rot, dry core rot and mouldy core in apples harvested from trees.

Orchard number	Production region	Cultivar	Season	% DCR <sup>1</sup>	% WCR <sup>1</sup>	% MC <sup>1</sup>	Total number analysed
CSC1	Ceres	Oregon Spur	2005/06	0.4	1.7	13.9	238
CSC1	Ceres	Oregon Spur	2006/07	6.0	1.3	12.0	300
CSC2	Ceres	Oregon Spur	2006/07	5.7	0.7	8.0	300
CSC3	Ceres	Oregon Spur	2006/07	2.7	1.0	13.0	300
CSC4	Ceres	Oregon Spur	2006/07	3.3	0.7	6.3	300
CSC5	Ceres	Oregon Spur	2006/07	3.3	1.0	6.3	300
CSC6	Ceres	Oregon Spur	2006/07	2.7	1.7	16.0	300
CSE	Ermelo	Top Red	2006/07	5.7	0.3	8.7	300
CSG1	Grabouw	Top Red	2006/07	2.8	0.3	4.1	290
CSG2	Grabouw	Top Red	2006/07	5.7	0.0	2.3	300
CSG3	Grabouw	Top Red	2006/07	2.5	0.7	0.0	282
CSG4	Grabouw	Top Red	2006/07	1.7	0.3	1.0	290
Average				3.5	0.8	7.6	

<sup>1</sup>DCR = Dry Core Rot, WCR = Wet Core Rot, MC = Mouldy Core

**Table 2.** *Penicillium* isolates used in this study, their culture collection number,  $\beta$ -tubulin-PCR-RFLP group as well as GenBank accession numbers of their  $\beta$ -tubulin and internal transcribed spacer region (ITS) sequences.

Isolate code	STEU culture number <sup>1</sup>	PCR-RFLP group <sup>2</sup>	Species ID based on sequence and morphological identification	ITS accession number	B-tubulin accession number
W5	6561	1	<i>P. expansum</i>	FJ491783	FJ491757
W91	6562	1	<i>P. expansum</i>	FJ491784	FJ491758
D104	6563	1	<i>P. expansum</i>	FJ491785	FJ491759
D6	6564	1	<i>P. expansum</i>	FJ491786	FJ491760
W110	6565	2	<i>P. ramulosum</i> prov. nom.	FJ491796	FJ491771
W10	6566	2	<i>P. ramulosum</i> prov. nom.	FJ491792	FJ491763
W202	6567	2	<i>P. ramulosum</i> prov. nom.	FJ491790	FJ491762
W224	6568	2	<i>P. ramulosum</i> prov. nom.	FJ491787	FJ491761
W170	6569	2	<i>P. ramulosum</i> prov. nom.	FJ491789	FJ491772
W235	6570	2	<i>P. ramulosum</i> prov. nom.	FJ491791	FJ491773
W159	6571	2	<i>P. ramulosum</i> prov. nom.	FJ491794	FJ491774
W198	6572	2	<i>P. ramulosum</i> prov. nom.	FJ491797	FJ491766
W123	6573	2	<i>P. ramulosum</i> prov. nom.	FJ491798	FJ491765
D3	6574	2	<i>P. ramulosum</i> prov. nom.	FJ491793	FJ491767
W44	6575	3	<i>P. species</i> aff. <i>dendriticum</i>	FJ491806	FJ491781
W45	6576	3	<i>P. species</i> aff. <i>dendriticum</i>	FJ491807	FJ491780
A92	6577	4	<i>P. glabrum</i>	FJ491804	FJ491776
D99	6578	4	<i>P. glabrum</i>	FJ491805	FJ491777
W177	6579	5	<i>P. ramulosum</i> prov. nom.	FJ491800	FJ491768
A94	6580	5	<i>P. ramulosum</i> prov. nom.	FJ491799	FJ491770
W181	6581	5	<i>P. ramulosum</i> prov. nom.	FJ491795	FJ491764
D98	6582	6	<i>P. chloroloma</i>	FJ491803	FJ491779
W118	6583	7	<i>P. chermisinum</i>	FJ491802	FJ491778
W53	6584	8	<i>P. ramulosum</i> prov. nom.	FJ491788	FJ491769
D103	6588	2	<i>P. ramulosum</i> prov. nom.	FJ491801	FJ491775

<sup>1</sup>All *Penicillium* cultures were submitted to the Stellenbosch University culture collection (STEU).

<sup>2</sup> The PCR-RFLP groups were identified by conducting *RsaI* (R) and *HaeIII* (H) restriction digests on PCR products of the partial  $\beta$ -tubulin gene. Isolates that had the same restriction digest pattern for both enzymes were classified into the same PCR-RFLP group.

**Table 3.** Characteristics of *Penicillium* isolates obtained from asymptomatic apples, as well as apples showing mouldy core, dry core- and wet core rot symptoms.

<i>Penicillium</i> species	Year of isolation	Production region	Isolation position from apple <sup>1</sup>	Lesion characteristics caused in surface toothpick inoculation method <sup>2</sup>		Lesion type caused in calyx-end toothpick inoculation study <sup>3</sup>	
				Lesion type <sup>4</sup>	Average lesion diam. (mm) <sup>5</sup>	Lesion type non-wounding <sup>4</sup>	Lesion type wounding <sup>4</sup>
<b><i>Penicillium</i> isolates obtained from apples with DCR symptoms</b>							
<i>P. expansum</i>	2005/06	Ceres	1, 4	L1 (3)	58.13	-	-
<i>P. expansum</i>	2006/07	Ceres and Ermelo	1	L1 (2)	55.52	L1 (D97)	L1 (D97)
<i>P. ramulosum</i> prov. nom.	2006/07	Grabouw	2, 4, 5	L2 (3)	14.75	NL (D103, D101)	L2 (D103, D101)
<i>P. ramulosum</i> prov. nom.	2005/06	Ceres	1	L2 (1)	8.96	-	-
<i>P. ramulosum</i> prov. nom.	2005/06	Ceres	3	L2 or L3 <sup>6</sup> (1)	4.88	-	-
<i>P. glabrum</i>	2005/06, 2006/07	Ceres	2, 4	L3 (2)	2.50	-	-
<i>P. chloroloma</i>	2006/07	Grabouw	1	L3 (1)	2.00	-	-
<b><i>Penicillium</i> isolates obtained from asymptomatic apples</b>							
<i>P. ramulosum</i> prov. nom.	2006/07	Ceres	6	L3 (1)	0.75	-	-
<i>P. ramulosum</i> prov. nom.	2006/07	Ceres	3, 6	L2 (2)	7.19	-	-
<i>P. glabrum</i>	2006/07	Ceres	6	L3 (1)	0.00	-	-
<i>P. ramulosum</i> prov. nom.	2006/07	Ceres	5	L2 or L3 (1)	3.25	-	-
<b><i>Penicillium</i> isolates obtained from apples with mouldy core</b>							
<i>P. ramulosum</i> prov. nom.	2006/07	Ceres	3	L2 or L3 (1)	6.63	-	-
<i>P. ramulosum</i> prov. nom.	2006/07	Ceres	2, 3, 4	L3 (4)	1.12	-	-
<b><i>Penicillium</i> isolates obtained from apples with wet core rot symptoms</b>							
<i>P. expansum</i>	2005/06	Ceres	1, 2, 5	L1 (4)	59.31	-	-
<i>P. expansum</i>	2005/06	Ceres	2, 4	L1 (3)	59.96	-	-
<i>P. ramulosum</i> prov. nom.	2005/06	Ceres	1, 3, 4, 5, 6, 8	L2 (18)	9.70	NL (W10, W159, W202, W224)	L2 (W10, W159, W202, W224)
<i>P. ramulosum</i> prov. nom.	2006/07	Ceres	1, 2, 3, 4, 5, 6, 8	L2 (14)	7.65	-	-
<i>P. ramulosum</i> prov. nom.	2006/07	Grabouw	5	L2 (1)	7.50	-	-
<i>P. ramulosum</i> prov. nom.	2005/06	Ceres	2, 3	L2 or L3 (3)	4.04	-	-

<i>P. ramulosum</i> prov. nom.	2006/07	Ceres	1, 5	L2 or L3 (6)	4.92	-	-
<i>P. ramulosum</i> prov. nom.	2005/06	Ceres	3, 5	L3 (3)	2.25	-	-
<i>P. ramulosum</i> prov. nom.	2006/07	Ceres	5	L3 (2)	0.63	NL (W177)	L1, L2, L3 (W177)
<i>P. sp. (aff. dendriticum)</i>	2005/06	Ceres	1, 4	L2 (2)	17.19	L2 (W44)	L2 (W44)
<i>P. glabrum</i>	2006/07	Ceres	8	L3 (1)	0.00	NL (W234)	L2 or L1 (W234)
<i>P. chermisinum</i>	2006/07	Grabouw	4	L3 (1)	0.00	-	-

<sup>1</sup>Isolates were obtained from different isolation positions within apples, as shown in Fig. 2.

<sup>2</sup>The ability of the isolates to cause lesions in apples was determined using a toothpick inoculation method, where *Penicillium* colonised toothpicks were inserted approximately 8mm into the surface of apples.

<sup>3</sup>The ability of the isolates to cause lesions was also determined by inserting a *Penicillium* colonised toothpick through the calyx tube (non-wounding) or off-centre from the calyx tube through to the core region (wounding).

<sup>4</sup>The lesion type produced by *Penicillium* isolates was determined by characterising the colour and texture (type) of lesions. The three lesion types that were distinguished included L1 = light brown and very wet, L2 = medium brown and spongy, L3 = medium and dry, very small lesion and NL = no lesion. Numbers in brackets in the surface lesion type column indicate number of isolates tested. Specific isolate identity numbers are in brackets in the calyx end wound and non-wound lesion type column.

<sup>5</sup>The lesion size is the average of two independent repeat trials, of isolates tested within in each line.

<sup>6</sup>For isolates that produced two different lesion types in the four (surface toothpick inoculation) inoculated apples within an experiments, both lesion types observed in the different apples are shown and indicated by “or”



### **3. THE ECOLOGY OF *TARSONEMUS* MITES IN APPLE ORCHARDS, AND THEIR ASSOCIATION WITH CORE ROT DISEASES AND FUNGI**

#### **ABSTRACT**

Information on the role of *Tarsonemus* mites in the epidemiology of apple core rot diseases is limited. The incidence of apple core rot diseases differ for different apple cultivars. Most Red Delicious varieties are prone to developing the disease, whereas the occurrence of core rots are extremely rare in other cultivars such as Granny Smith. The aims of the study were to (1) investigate the ecology of *Tarsonemus* mites in Red Delicious and Granny Smith orchards, (2) determine if there is a significant association of *Tarsonemus* mites with diseased (wet- and dry core rot) fruits and (3) determine if potential core rot pathogenic fungi are associated with mites. The ecology of the mites was investigated by assessing different apple developmental stages (buds, blossoms, 4cm diameter fruit, mature fruit and mummies) for the presence of mites in two Red Delicious (Oregon Spur) and two Granny Smith orchards. In all the assessed developmental stages, *Tarsonemus* mites were identified as the dominant mite genus, having the highest incidence in mummies and mature fruits from Red Delicious and Granny Smith orchards. The high incidence of mites in mummies might suggest that this may be their overwintering site. Investigations into the presence of *Tarsonemus* mites in diseased and healthy mature fruits from 11 Red Delicious (Oregon Spur and Top Red) and four Granny Smith orchards showed that the *Tarsonemus* mites are found within the core and/or calyx region of Red Delicious fruits. In contrast, mites were restricted to the calyx tube in Granny Smith fruits, and did not enter the core region. In Red Delicious fruits there was a significant association between dry core rot as well as total core rot (wet- and dry-core rot) with the presence of mites in the core, as well as total mites (mites in core and calyx tubes). Interestingly, during the analyses of Granny Smith fruits for mites, a new calyx tube decay was detected. In these fruits, there were no significant association of mites with the calyx tube decay. Fungal isolation studies from the *Tarsonemus* mites showed that they carried potential core rot fungal pathogens within

the genera *Penicillium* and *Alternaria*. The mites were not able to reproduce on *Alternaria* or *Penicillium* cultures although they ingested the culture, whereas the mites were able to consistently reproduce and complete their life cycle on *Cladosporium* cultures.

## INTRODUCTION

*Tarsonemus* mites are common in nature and have been collected from many plant species, fungi, leaf litter, soil, as well as stored food products (Lindquist, 1986; Zhang, 2003). The genus *Tarsonemus* belongs to the family Tarsonemidae, subfamily Tarsoneminae and contains more than 200 described species (Lindquist, 1986). The majority of species within the genus are considered to be fungivorous (McDaniel, 1979; Lindquist, 1986). Species such as *Tarsonemus floricolus* Canestrini and Fanzago and *T. myceliophagous* Hussey are known pests in mushroom houses (Hussey and Gerney, 1967). *Tarsonemus confusus* Ewing occasionally occurs as a minor pest on plants in greenhouses (Zhang, 2003).

The fungivorous nature of *Tarsonemus* mites inevitably also creates the potential for them to vector the fungi with which they come in contact with. They thus play a role in fungal dispersal and ecology. Studies on the role of *Tarsonemus* mites have been focused on the vectoring of Ophiostomatoid fungi in the Northern Hemisphere (Lombardero *et al.*, 2003; Hofstetter *et al.*, 2006). The best example is vectoring of the plant pathogenic blue-stain fungus, *Ophiostoma minus*, by *Tarsonemus ips* Lindquist, *Tarsonemus krantzii* Smiley and Moser, and *Tarsonemus fusarii* Cooreman (Moser, 1976). These three mite species are phoretic on the Southern pine beetle, *Dendroctonus frontalis* (Coleoptera: Scolytidae), which attacks and kills pine trees in the United States (Thatcher *et al.*, 1980). *Tarsonemus* mites are thought to play an important role in the dispersal of *Ophiostoma* spores between various coniferous trees (Moser *et al.*, 1995), thus spreading the disease (Dowding, 1969; Klepzig, 2001). In South Africa, *Tarsonemus* mites that are carried on beetles (*Genuchus hottentottus* (F), *Trichostetha fascicularis* L. and *T. capensis* L.) are also thought to be the primary vector that disperses non-pathogenic *Ophiostoma* species present in *Protea* infructescences between *Protea* spp. (Roets *et al.*, 2007; 2008).

An important plant disease in which *Tarsonemus* mites have also been postulated to play a role, is core rot diseases of apples (Michailides *et al.*, 1994). Core rot diseases are important in many countries, including the United States of America, South Africa, Australia, New Zealand, Canada, the United Kingdom and the Netherlands. The disease is problematic in cultivars such as the Red Delicious varieties, Golden Delicious, Gravenstein and Idared, which all have a fruit with a high incidence of open calyx tubes (Spotts, 1990). In South Africa, core rot is one of the most important post-harvest diseases of apples (Combrink and Ginsburg, 1973), causing post-harvest losses between 5 and 8%. The disease is mainly associated with susceptible Red Delicious apple varieties (Serdani *et al.*, 1998). In other cultivars such as Granny Smith, core rots are extremely rare, and have only been reported by Combrink (1983) at a very low incidence (0.04%) after examining a large number (46 800) of apples. Core rot also causes apples to drop prematurely in the orchard, thus also reducing yields prior to harvest (Combrink and Ginsburg, 1973; Michailides *et al.*, 1994). Based on the symptoms and causative agents, apple core rots can be divided into dry- and wet core rot. Both wet- (WCR) and dry core rot (DCR) consists of a rot that spreads from the core region into the fleshy tissue that surrounds the seed cavity (Spotts *et al.*, 1988).

The specific fungal pathogens that cause WCR and DCR diseases are not well understood, but may include several fungal genera. The reported causative agents of DCR include several fungal genera such as *Coniothyrium*, *Alternaria*, *Epicoccum* and *Pleospora* (Combrink *et al.*, 1985a). However, the fungi that have most frequently been indicated as causing DCR are small-spored *Alternaria* species (Serdani *et al.*, 2002). Earlier studies have indicated that *Alternaria alternata* (Combrink *et al.*, 1985b) and the *Alternaria tenuissima* species group (Combrink and Ginsburg, 1973; Serdani *et al.*, 1998) are the main causative agents of DCR. However, more recent studies have suggested that a complex of small-spored *Alternaria* species are the causative agents, with pathogenic and non-pathogenic isolates occurring within the same morphological species group (McLeod *et al.*, 2008; Smit, unpublished data). Wet core rot is known to be caused by *Penicillium*, but the specific species involved have been poorly characterised. In South Africa, *Penicillium expansum* and *Penicillium funiculosum* (Combrink and Ginsburg, 1973; Combrink *et al.*, 1985a; De

Kock *et al.*, 1991; Serdani *et al.*, 1998) have been reported as causing WCR, whereas a more recent study indicated that a new *Penicillium* species, *P. ramulosum* prov. nom. and *P.* species (aff. *dendriticum*), which morphologically resembles *P. funiculosum* (Visagie *et al.*, unpublished data) are also associated with the disease (Chapter 2). In Australia, *Penicillium roquefortii* has been found associated with WCR (Spotts *et al.*, 1988).

The epidemiology of core rot diseases, including the mode of entry of core rot fungal pathogens into apple core regions, is poorly understood. It is generally accepted that DCR pathogens, which cause infection during the growing season, enter the core region by growing through the open calyx tube of susceptible cultivars, since they colonise blossom parts early in the season (Combrink and Ginsburg, 1973; Ellis and Barrat, 1983; Miller 1959; Spotts *et al.*, 1999). Infection of apples with WCR pathogens is known to be dependent on an open calyx tube since infections mainly occur during post-harvest dipping of fruits in DPA emulsion (Combrink and Ginsburg, 1973; Combrink *et al.*, 1987), although infections can also take place during the growing season (Chapter 2). Although it is mainly thought that an open calyx tube contributes to the susceptibility of cultivars to core rot diseases (Miller, 1959; Combrink and Ginsburg, 1973.), Serdani *et al.* (1998) and Teixido *et al.* (1999) suggested that core rot fungal pathogens are endophytic in apple tissues. Furthermore, Niem *et al.* (2007) recently suggested that the susceptibility of cultivars to core rots is due to their seed locules differing in susceptibility to colonisation, as well as subsequent infection into the mesoderm by *A. alternata*. They also suggested that the higher mesoderm pH of Red Delicious varieties results in enhanced pathogen virulence due to increased gene expression of endo- and exo-glucanases by *Alternaria* spp. in susceptible versus resistant varieties. Some support for this can be found in the work of Combrink (1983) who suggested that the susceptibility of Starking apples was due to their lower malic and fumaric acid content.

The role of *Tarsonemus* mites in the epidemiology of core rot diseases, specifically DCR caused by *Coniothyrium*, has only been investigated by Michailides *et al.* (1994), although their work has not been published in a peer-reviewed journal. They hypothesised that *Tarsonemus* mites carry core rot pathogen spores into the apple core region through the open calyx tube. The mites may also cause small

wounds in the core region that facilitate pathogen entry and disease development (Michailides *et al.*, 1994). Michailides *et al.* (1994) found a high incidence of *Tarsonemus confusus* within the core region of apples with DCR caused by *Coniothyrium*, but not in healthy apples. They furthermore showed in preliminary experiments that inoculation of apples with *Coniothyrium* and mites resulted in a higher incidence of DCR than when apples were only inoculated with *Coniothyrium* (Michailides *et al.*, 1994).

The three main aims of this study were to (1) investigate the ecology of *Tarsonemus* mites within two Red Delicious and two Granny Smith orchards, (2) determine if there is a significant association of *Tarsonemus* mites with diseased (WCR and DCR) and healthy fruits and (3) determine if potential core rot pathogenic fungi are associated with the mites and whether the mites can complete their life cycle on *Alternaria*, *Penicillium* and *Cladosporium* cultures.

## MATERIALS AND METHODS

**The ecology of *Tarsonemus* mites within Oregon Spur and Granny Smith apple orchards.** Four apple orchards were selected on one farm in the Ceres area. Two of the orchards (CSC1 and CSC2) contained the Red Delicious cultivar Oregon Spur, and had a history of core rot development. The other two orchards (CRC1 and CRC2) contained the cultivar Granny Smith in which core rot symptoms has not been reported. In each of the four orchards twenty-five trees were randomly selected and marked, from which different apple developmental stages were sampled for mite analyses. The developmental stages that were sampled in all four orchards in the 2006/07 season included blossoms at full bloom (October 2006), fruit with a 4cm diameter (November 2006), mature fruit just before harvest (March 2007) and mummies from trees (undeveloped fruit from the previous season that mummified). In addition to these samplings, in orchard CSC1 buds were also sampled (September 2006), as well as mature fruits (April 2006) and mummies. In all orchards and seasons, mummies were collected from trees at the start of the growing season, when sampling was done for buds, as well as later in the growing season. In each orchard five buds, blossoms, 4cm dia. fruits and mature fruits were collected from each tree.

Thus in total, in each orchard 125 samples of each developmental stage (blossoms, buds, 4cm. diam. fruits and mature fruits) were collected for mite analyses.

The different developmental stages were inspected for the presence of mites using the 40× magnification of a stereomicroscope. Buds (only orchard CSC1) and mummies were dissected in order to investigate the presence of mites. The 4cm diameter fruits, as well as mature fruits and mummies, were cut longitudinally through the core region, and inspected for mites within the core cavity as well as the calyx tube. The presence of mites was noted as either being present within the core (this also included fruits that contained mites in their calyx tube and core), or just associated with the calyx tube. All mites were identified to family level. Mites within the family Tarsonemidae were also identified to genus level.

**The association of *Tarsonemus* mites with the core and calyx region of healthy and diseased mature Red Delicious (Oregon Spur and Top Red) and Granny Smith fruits.**

*Sampling strategy.* The association of *Tarsonemus* mites with healthy and core rot diseased mature fruits was studied in 11 Red Delicious and 4 Granny Smith orchards from the 2005/06 to 2007/08 seasons (Tables 1 and 2). The orchards with Red Delicious fruits were situated in three main apple production regions of South Africa including Ceres (orchards with CSC prefix), Ermelo (orchards with CSE prefix) and Grabouw (orchards with CSG prefix) (Table 1). The orchards with Granny Smith fruits were all situated in Ceres (orchards with CRC prefix) (Table 2). In each orchard 40 to 304 fruits were sampled by randomly harvesting apples from trees throughout the orchard, not harvesting more than four apples per tree. The total number, or subsets of fruits, were analysed for the presence of mites and core rot diseases (Tables 1 and 2).

*Analyses of mature fruits for the presence of core rot diseases (WCR and DCR) and mites within the core and calyx region.* For mite and core rot disease analyses the apples were first cut open longitudinally through the core. The presence of mites was investigated by inspecting the core and calyx tube under a stereomicroscope at 25 to 40× magnification. The identification of core rot diseases

was based on the specific symptom present in the tissue surrounding the core, with WCR being identified as a wet rot extending into the fleshy tissue, and DCR as a dry rot that extended from the core into the fleshy tissue of the apple.

*Isolation and characterisation of fungi associated with a calyx tube decay in Granny Smith fruits.* The analyses of Granny Smith mature fruits sampled in orchards CRC1 and CRC2 in the 2006/07 season revealed the presence of a new calyx tube decay, which was often observed as occurring at a small crack within the calyx tube (Fig. 1). The symptom consisted of a dry decay, which has not previously been reported in South Africa. Therefore, the occurrence and causative agent of this dry decay was further investigated in fruits collected in the 2007/08 season (Table 2) through isolation and characterisation studies.

Isolations for fungi were made from fruits collected in the 2007/08 season only. In total, isolations were made from 22 asymptomatic and 21 calyx tube decay fruits, representative of the four orchards. Isolations were made from the margin between healthy and diseased tissue of the lesion and at a position 1cm away from the lesion in symptomatic apples. Isolations were also made in the same number of asymptomatic apples from the same isolation positions. The isolations 1 cm away from the lesion was made in an attempt to increase the probability of finding the pathogen at the “leading edge” of the decay, since it was expected that tissue at the margin may also contain secondary colonisers. The isolated tissue sections were plated on PDA plates containing 0.04g/L Streptomycin (PDA+ plates) and incubated on the laboratory bench at 25°C ± 2°C. Fungal growth was sub-cultured onto 65mm diameter PDA+ plates, single spored, stored and identified to genus level as previously described (Chapter 2).

The pathogenicity of 10 *Alternaria* isolates obtained from the calyx tube decay as well as 11 isolates from asymptomatic apples was investigated using a toothpick inoculation method (Serdani, 1999; Serdani *et al.*, 2002). The isolates were representative of isolations made from fruits collected from the four orchards, with one to six isolates being tested from asymptomatic fruits in each of the orchards, as well as one to five isolates from calyx tube decay fruits from each of the orchards. Four colonised toothpicks of each isolate were randomly inoculated into surface

sterilised apples (30 seconds in 70% ethanol, 2 minutes in 1% NaOCl and 15 seconds in 70% ethanol). A total of 19 apples were inoculated, each apple containing 5 toothpicks each with a different isolate, as well as a control toothpick. The inoculated apples were incubated 10 to 14 days at  $25^{\circ}\text{C} \pm 4^{\circ}\text{C}$  in Perspex moisture chambers until lesions could be distinguished. At the end of the incubation period, the toothpicks were removed from apples and the apples were split longitudinally across the inoculation points with a sterile knife. Lesions were characterised with regard to diameter (mm), colour and texture (dry, wet or spongy). The experiment was repeated twice.

*Statistical analyses to determine whether the presence of disease is dependent on the presence of mites.* Results of the presence and absence of disease symptoms and mites were reported in 2x2 contingency tables. These were used in association analyses (Clewer and Scarisbrick, 2006). A contingency table provides a technique for investigating suspected relationships. The null hypothesis that was tested was whether two characteristics occur independent of one another, i.e. the probability that an individual falls in a certain class is not affected by another class the individual happens to belong to. When the variables are independent, it means that knowledge of one provides no information about the other variable. When they are dependent, the knowledge of one variable is helpful in predicting the value of the other variable (Clewer and Scarisbrick, 2006).

In this investigation the detection of core rot may be considered a rare event, thus Fisher's exact test is appropriate for testing the hypotheses (Clewer and Scarisbrick, 2006). Fisher's exact test does not depend on any large-sample distribution assumptions, and it is therefore applicable even for small sample sizes and for sparse tables. Fisher's exact test is a test of association between the row and column variables. This test assumes that the row and column totals are fixed, and then uses the hypergeometric distribution to compute probabilities of possible tables with these observed row and column totals (Clewer and Scarisbrick, 2006).

For susceptible Red Delicious fruits it was important to investigate whether the development of DCR, WCR and total Core Rot (WCR + DCR) were dependent on the presence of mites in the calyx, core or both the calyx and core (total mites). The



null hypothesis is that the occurrence of rot (WCR, DCR and Total CR) was independent of the presence of mites (in calyx, core or both).

No core rot was detected for Granny Smith fruits. However, calyx tube decay occurred and mites were detected in the calyx tube. Thus, it was also investigated whether the development of calyx tube decay was dependent on the presence of mites in the calyx tube. The null hypothesis is that the occurrence of calyx tube decay was independent of the presence of mites in the calyx tube.

#### **The association of potential core rot fungal pathogens with *Tarsonemus* mites.**

*Isolation of fungi from mites.* Mites obtained from the four orchards (CSC1, CSC2, CRC1 and CRC2) that were studied in the mite ecological study were examined for the presence of fungi. This was conducted by transferring 62 mites from the mummies, 21 from blossoms, 29 from the calyx and 24 from the core cavity of fruits using a very fine needle, onto PDA plates. In the 2005/06 season isolations were only made from mites obtained from mature fruits in orchard CSC1, whereas in the 2006/07 season isolations were made from mites collected from the different developmental stages in all four orchards. Fungal hyphae emerging from the plated mites were sub-cultured onto 65-mm PDA-plates, and subsequently single spored. Pure cultures were stored at 4°C on PDA slants as well as in 20 mL bottles containing sterile water. The single spored isolates (74 in total) were identified to the genus level, except for two isolates that could not be identified and were grouped under “other fungi”.

*Characterisation of the mite-isolated potential core rot pathogens within the genus *Penicillium* and *Alternaria*.* *Penicillium* and *Alternaria* isolates that were isolated from mites were further characterised in order to determine their potential for being core rot pathogens. Three randomly selected *Penicillium* isolates were identified to species level using PCR-RFLP as previously described (Chapter 2). Six randomly selected *Alternaria* isolates were also characterised for their pathogenic potential using a toothpick inoculation method as previously described (Serdani, 1999; Serdani *et al.*, 2002). The inoculation and evaluation of inoculated apples were done as described above for *Alternaria* isolates obtained from the calyx tube decay

symptomatic fruits. The identification of small spored *Alternaria* species is very controversial and were therefore not pursued further.

*The ability of Tarsonemus mites to complete their life cycle on Alternaria, Penicillium and Cladosporium cultures.* The ability of the *Tarsonemus* mites to use *Alternaria*, *Penicillium* and *Cladosporium* as a food source was investigated by determining whether the mites could reproduce on one isolate of each of these genera. In addition to the aforementioned isolates, a red fluorescing *Alternaria* isolate was also included in the study to determine if the mites ingested the fungus. The red fluorescent *Alternaria* isolate, obtained from a DCR lesion, was labeled with the red fluorescent protein DsRed-Express, as previously described for *Phaemoniella chlamydospora* (Mclean *et al.*, 2009). Ten *Tarsonemus* mites collected from the core region of Oregon Spur mummies were transferred to two 7-day old PDA<sup>+</sup> cultures (90-mm plates) of the red fluorescent *Alternaria* isolate as well as two 7-day old culture plates of an untransformed *Alternaria* isolate. Ten mites were also transferred to two 7-day old cultures plates (90-mm PDA<sup>+</sup> plates) of a *Penicillium ramulosum* prov. nom. isolate and two 5-day old culture plates (90-mm PDA<sup>+</sup> plates) of a *Cladosporium* isolate. Controls consisted of two PDA<sup>+</sup> plates (90mm) that were not inoculated with any fungus. For each of the inoculated isolates and control, one plate was incubated at 25°C, and one plate was incubated at 30°C. The plates were inspected for eggs and nymphal stages of the mites after 7 and 14 days. The experiment in which the enumeration of mites on *Alternaria* and *Penicillium* cultures was investigated was conducted twice at both temperatures, whereas the *Cladosporium* experiments were only conducted once at 30°C, and twice at 25°C.

The association of *Alternaria* spores and mycelial content with the mites was inspected using an epifluorescent Zeiss Axioscope (West Germany) microscope, equipped with a HQ:TRITC filter with excitation filter of 545 nm, emission filter of 620 nm and beam splitter Q570lp (Chroma Technology Corp.). The mites feeding on the fluorescent culture were also viewed using a stereo microscope. Images were captured with a Nikon digital camera DXM1200 and Automatic Camera Tamer (ACT-1) computer software.

## RESULTS

**The ecology of *Tarsonemus* mites within Oregon Spur and Granny Smith orchards.** In all samples where mites were found, the predominant mite genus consisted of a small *Tarsonemus* species (Fig. 2) (Table 3). Although mites from other families including Phytoseiidae, Tetranychidae, Tydeidae, Bdellidae, Ascidae, Oribatidae, Dolichocybidae, Pyemotidae, Iolionidae, Stigmaeidae, Cunaxidae and Acaridae were identified, these were present at very low frequencies (less than 10%) compared to the *Tarsonemus* species (Table 3).

In the Oregon Spur orchards CSC1 and CSC2, the *Tarsonemus* mites were present in almost all of the developmental stages. In orchard CSC1 where sampling was already initiated in the 2005/06 season the *Tarsonemus* mites were found very early in the season in buds (20%) as well as blossoms, although the incidence on blossoms was low (less than 2%). Later in the season the mites were also found in 4cm diameter fruit as well as in the core and calyx of mature fruits (Table 3). In orchard CSC2, where blossoms were the first developmental stage sampled, the mites were present in less than 1% of blossoms. In this orchard the mites were also found in the core and calyx region of 4cm diameter fruits as well as in mature fruits. In both of the Oregon Spur orchards all the mummies contained the *Tarsonemus* mites (Table 3).

In the Granny Smith orchards CRC1 and CRC2, the *Tarsonemus* mites had a more restricted occurrence and were not found in the core region of mature fruits or in blossoms. The mites were only found in the calyx region of 4 cm diameter fruits, as well as in the calyx tube of mature fruits. Similar to the Oregon Spur orchards, the mites were found in all the sampled mummies from these orchards (Table 3).

**The association of *Tarsonemus* mites with the core and calyx region of healthy and diseased Red Delicious (Oregon Spur and Top Red) and Granny Smith mature fruits.**

*Analyses of mature fruits for the presence of core rot diseases (WCR and DCR) and mites within the core and calyx region.* *Tarsonemus* mites were found in both Granny Smith and Red Delicious fruits. However, in Red Delicious fruits, the

mites were present in the core and calyx regions (Table 1), whereas in Granny Smith fruits the mites were restricted to the calyx tube (Table 2).

DCR and WCR were found in fruits of almost all of the Red Delicious orchards. The incidence of DCR in these orchards ranged from 0.4% to 10%, and that of WCR from 0.3% to 2% (Table 1). In Granny Smith orchards, no DCR and WCR were found. However, a different kind of symptom that consisted of a dry decay within the calyx tube (hereafter referred to as calyx tube decay) was observed for the first time. Calyx tube decay was found in apples collected from all orchards with the incidence varying from 0.3% to 9.7%.

*Tarsonemus* mites were found in the core region of Red Delicious fruits, and/or calyx tube of healthy Red Delicious and Granny Smith fruits, as well as diseased fruits. In Red Delicious fruits the incidence of mites in the core region of diseased fruits varied from 40% to 94.7%, whereas in healthy fruits it varied from 28% to 66.7% (Table 1). In Granny Smith fruits the incidence of the mites in the calyx tube varied from 16.7% to 100% in fruits with calyx tube decay, whereas in healthy fruits their incidence varied from 0% to 58.4% (Table 2).

*Isolation and characterisation of fungi associated with calyx tube decay in Granny Smith fruits.* The calyx tube decay lesions resembled DCR lesions in that they consisted of dark brown, dry and hard lesions that sometimes penetrated the soft tissue surrounding the calyx tube to a depth of 1 to 2 mm (Fig. 1). *Alternaria* was isolated from 100% of the fruits with calyx tube decay lesions, as well as at a position 1cm away from the lesions. Similarly, in 95% of the asymptomatic fruits, isolations from the calyx tube also yielded *Alternaria*. Although *Alternaria* was the main genus isolated from asymptomatic and diseased fruits, *Penicillium*, *Epicoccum*, *Cladosporium* and *Ulocladium* were also sometimes isolated.

Pathogenicity testing of a subset of the *Alternaria* isolates from healthy (11 isolates) and calyx tube decay fruits (10 isolates) showed that most of the isolates were able to cause a dry lesion. Seventy-three percent of the *Alternaria* isolates from asymptomatic fruits, as well as 91% of *Alternaria* isolates from calyx tube decay

fruits were able to cause a dry lesion upon *in vitro* inoculation with colonised toothpicks.

*Statistical analyses to determine whether the presence of disease is dependent on the presence of mites.* The results of the tests for independence are presented in Table 4. In general, considering all core rots and calyx tube decay, the presence of mites in the calyx tube did not seem important since, in both Red Delicious and Granny Smith fruits, the presence of core rot was either independent of the presence of mites in the calyx, or when significant, there was an inverse relationship, i.e. in diseased apples a very low percentage contained mites in the calyx tube in comparison to the larger percentage of healthy apples containing mites in the calyx tube (Table 4). In susceptible Red Delicious fruits the development of WCR was independent of the presence of mites in the core ( $P = 0.44$ ), as well as the total mites ( $P = 0.66$ ). This might be due to the low number of WCR apples (28) found in the survey, compared to the higher number of DCR apples (177). The development of DCR was dependent on the presence of mites in the core ( $P < 0.05$ ) as well as the total mites ( $P < 0.05$ ). Similarly, considering the presence of both core rots (DCR and WCR) in Red Delicious fruits there was a significant association with mites in the core ( $P < 0.05$ ) as well as total mites ( $P = 0.01$ ).

#### **The association of potential core rot fungal pathogens with *Tarsonemus* mites.**

*Isolations of fungi from mites.* Isolations from mites yielded different fungal genera (Fig. 3), which have also been isolated most frequently from apple core regions (chapter 2). In the 2005/06 season the main isolated fungal genera were *Cladosporium* and *Aureobasidium*, when only mites obtained from mature fruits from one orchard were investigated. In the 2006/07 season, when mites were investigated that were collected from all the different developmental stages in all four orchards, the incidence of *Cladosporium* and *Alternaria* were highest, with *Penicillium* also being isolated (Fig. 3). Fungal genera grouped under “other” were genera that only consisted of two or less isolates.

*Characterisation of the mite-isolated potential core rot pathogens within the genus *Penicillium* and *Alternaria*.* Three *Penicillium* isolates and six *Alternaria*

isolates obtained from the mites were characterised. Identification of the *Penicillium* isolates to the species level using PCR-RFLP showed that the isolates belonged to three different species, namely *P. expansum*, *P. ramulosum* prov. nom. and a putative new species currently known as *P. species* (aff. *dendricitum*) (Chapter 2). All three *Penicillium* spp. caused lesion type 2 (Chapter 2). Characterisation of the *Alternaria* isolates using pathogenicity studies showed that all isolates were able to cause a dark brown, dry lesion typical of DCR.

*The ability of Tarsonemus mites to complete their life cycle on Alternaria, Penicillium and Cladosporium cultures.* The *Alternaria* isolate obtained from a DCR lesion was stably transformed with the red fluorescent protein gene DsRed-Express. The transformant exhibited constitutive fluorescence in spores when viewed using epi-fluorescent microscopy (Fig. 4). The level of expression of the protein was high, frequently also resulting in spores having a pink colour when viewed with a light microscope.

The survival of the mites that were placed on the *Penicillium* and *Alternaria* cultures were influenced by temperature. After 7 days, the 10 mites that were transferred to the plates and incubated at 30°C were still alive, whereas more than 80% of the mites that were incubated at 25°C were no longer detected, or were dead. After 14 days of incubation only one or two mites were detected on plates incubated at 30°C, whereas no mites were detected on plates incubated at 25°C. The mites that were transferred to the control PDA plates that were not inoculated with any fungus were no longer visible.

Although the *Tarsonemus* mites were not able to reproduce or complete their life cycle on the *Alternaria* cultures, observations on mites placed on the red fluorescent *Alternaria* cultures suggested that they did ingest the fungus. These mites contained a light red to pink colour when viewed under the stereomicroscope (Fig. 4). Visualisation of these mites using fluorescent microscopy also revealed the presence of bright fluorescence within the intestines of the mites with no discernable fungal structures being present within the mite bodies (Fig. 4). Mites that were placed on untransformed *Alternaria* cultures did not contain a light red colour, nor showed any fluorescence when viewed using epi-fluorescence microscopy (data not shown).

The survival and enumeration of mites on *Cladosporium* cultures were also influenced by temperature. This genus seemed to be a much better food source for the mites than *Alternaria* and *Penicillium*. After 7 days of incubation, mites placed on *Cladosporium* plates that were incubated at 30°C multiplied extensively and could not be counted accurately, but at least 150 mites were present on the plates. In contrast, plates that were incubated at 25°C only showed a two to three fold (20 to 30) increase in mite numbers. After 14 days of incubation the mites incubated at 30°C increased to 250 to 300, whereas at 25°C fewer mites ( $\pm 50$ ) were observed.

## DISCUSSION

This study has increased our understanding of the ecology of *Tarsonemus* mites within apple orchards, as well as their association with core rot diseases (WCR and DCR). It was shown that *Tarsonemus* mites differentially colonise several important apple developmental stages (buds, blossoms, 4 cm. dia. fruit, mature fruits and mummies) during the growing season. The mites may be potential vectors of core rot pathogenic fungi within the genus *Alternaria* and *Penicillium*, since these fungi were isolated from the mites that were collected from some developmental stages. The *Tarsonemus* mites were associated with the core and calyx tube region of Red Delicious fruits known to develop core rot, whereas in Granny Smith fruits that in general do not show core rot symptoms, the mites were only associated with the calyx tube. When Red Delicious fruits were analysed for the presence of mites and core rot diseases, a significant association of the mites within the core region could be found with core rot symptomatic (DCR and WCR) fruits.

Sampling of several apple developmental stages in four different orchards gave some clues as to the ecology of *Tarsonemus* mites within Oregon Spur, as well as Granny Smith orchards. At the start of the season the high incidence of mites in mummies, mostly undeveloped fruit mummies, from the previous season suggests that the mites can overwinter in the mummies. From here, they could then emerge in very small numbers in spring, from where they can colonise buds and blossoms. The mites would preferentially leave the mummies as a niche in spring, since overcrowding can

occur when the mites become active and start multiplying, which would also result in exhaustion of their food source. Tarsonemid mites can also overwinter in the soil in organic debris, moving upwards with insects emerging from the soil, thus enabling them to migrate to the new growth in spring and summer (Lindquist, 1986). The *Tarsonemus* mites showed a moderate (~ 20%) colonisation rate of buds, although this would need more investigation since buds were only analysed in one orchard. The blossoms did not seem to be a good niche for the mites since they were only detected at very low levels (0.8 to 1.6%) in the Oregon Spur orchards. This is most likely due to the fact that blossoms are an exposed and temporary niche that does not last long enough for mites to settle in. Mites present on the blossoms, as well as possibly leaves, twigs and branches, may then colonise 4 cm. diam. fruits, mainly their calyx tube, at relatively low levels (5.04% to 11.2%). The occurrence of mites on leaves, twigs and branches were not investigated in this study, and needs further investigation in order to determine the importance of these areas as niches.

The *Tarsonemus* mites most likely begin vectoring the fungi at the start of the season when they emerge from mummies, since *Alternaria* and *Penicillium* were isolated from mites obtained from the mummies. Furthermore, when mummies were inspected for mites under the microscope, fungal spores, including *Alternaria* spores was frequently observed in undeveloped fruit mummies. The mites may also pick up *Alternaria* and *Penicillium* spores when they move into mature Oregon Spur fruits and pass along the dried flower parts at the calyx end. The dried flower parts are colonised by different fungi (chapter 2), and *Alternaria* spores were frequently observed on and isolated from the dried floral parts. Other fungal genera that are commonly isolated from core regions such as *Aureobasidium* and *Cladosporium*, were also isolated from the mites. This further supports the hypothesis that *Tarsonemus* mites contribute to the composition of microbial populations within apple core and calyx regions. The reason for the differences in the fungal genera isolated between seasons is most likely due to only one orchard being investigated in the 05/06 season and four orchards in the 06/07 season.

The calyx tube and core region of mature apple fruits are ideal sites for mites since colonisation of these were high (34% to 85%) within all four orchards. These



areas are ideal niches for the *Tarsonemus* mites since they can find food here, and are protected from predators and acaricides. The small size of Tarsonemid mites may also be to their advantage in entering the core region through the calyx tube, since Tarsonemid mites are 100 to 300µm in size, whereas the Tetranychidae average at about 400µm, Acarid mites are 320 to 420µm and Phytoseiids 250 to 400µm (Zhang, 2003). However, since the calyx tube diameter of Red Delicious fruits in this study was 2 to 3 mm, which is similar to the range (1.8 to 2.4 mm) reported by Combrink (1983) for this cultivar, the size of most mites would in theory not limit their entry into the core. It is thus possible that *Tarsonemus* mites are more attracted to the fungi on which they feed that grow in the core region, and consequently have a higher incidence in core regions than other mite families. The increased diversity in mite families found within core and calyx regions also supports the fact that these are ideal niches for mites, including the *Tarsonemus* mites. Mature fruits, as well as undeveloped aborted fruit left on the trees at the end of the harvesting period will continue to serve as a niche for mites to multiply in, until they become dormant when temperatures and food availability decrease during autumn and winter. It is also possible that at the end of the growing season when food and shelter becomes scarce that mites remaining on leaves and twigs can migrate to fruit (developed and undeveloped) that remain on the trees.

*Tarsonemus* mites are not the only mites that can potentially vector fungal spores to the core region of mature fruits, since mites in the families Phytoseiidae, Tetranychidae, Tydeidae and Dolichocybidae were also found in mature fruits. However, since these mites were found in very low frequencies (0 % to 3.40 %) they most likely play a minor role in dispersal of core rot pathogens. The Phytoseiidae was most prevalent within the core region followed by the Tydeidae. Phytoseiids are used for biological control of spider mites (Tetranychidae) which are phytophagous and have the ability to cause damage to a wide range of crops (Gerson *et al.*, 2003). Mites in the family Tydeidae are potential fungal vectors, since they are omnivorous and feed on fungi as well as plant litter, and could even cause injury to some crops (Jeppson *et al.*, 1975). The Tydeidae are known as cleaners and are always on the move, with fungal spores being able to stick to setae in their bodies as larvae feed on fungi eg. *Penicillium* and *Colletotrichum* (McCoy *et al.*, 1969).

The *Tarsonemus* species found in California by Michailides *et al* (1994) was identified as *Tarsonemus confusus*. In South Africa, the specific *Tarsonemus* mite species present in apple orchards could not be identified, and it could also not be established with certainty that only one specific species was present. There are more than 200 described *Tarsonemus* species, with the taxonomy of some still being controversial, and there are only a few specialised taxonomists working in this field. Investigations are ongoing in order to determine the specific *Tarsonemus* species present in South African apple orchards. Thus far, it has been possible to establish that the *Tarsonemus* mites in South Africa are not *T. confuses* and that the species to which they have closest similarity is *T. bilobatus* (personal communication R. Ochoa, Systematic Entomology Laboratory, U.S. Department of Agriculture). An interesting characteristic of the South African *Tarsonemus* species is their ability to survive and reproduce better at 30°C than at 25°C. According to Lindquist (1986) the optimal temperature for tarsonemid mites to reproduce is 30°C, with the reproduction rate slowing down at temperatures below 20°C. *Alternaria* and *Penicillium* did not seem to be a good food source for the *Tarsonemus* mites in South Africa, since they were unable to reproduce on these cultures. In contrast, *Cladosporium*, which was also isolated from the mites, seemed to be a good food source for the mites that enabled them to reproduce and complete their life cycle.

The *Tarsonemus* species present in South African apple orchards could potentially play a role in vectoring core rot pathogens. Since most *Tarsonemus* mites are fungivorous it is most likely that the species that was found in the orchards is also fungivorous. This was supported by the fact that the mites could complete their life cycle on cultures of *Cladosporium* as a sole food source. Furthermore, several fungal genera including species known to be associated with WCR such as *P. expansum* (Combrink and Ginsburg, 1973) and *P.* species (aff. *dendriticum*) (Chapter 2) were also isolated from the mites. Due to controversy on the *Alternaria* species involved in DCR development, as well as the validity of the published *in vitro* pathogenicity test of Serdani *et al.* (2002), it can not be said with certainty whether the isolates of *Alternaria* that were isolated from the mites are core rot pathogens. The *Alternaria* isolates from mites caused a dry lesion in inoculation studies, which according to Serdani *et al.* (2002) was not the lesion type caused by DCR pathogens. However, a

recent study suggested that at this stage it can not be said with certainty that a specific lesion type is caused by DCR pathogens (McLeod *et al.*, 2008). Future studies should use alternative pathogenicity testing of *Alternaria* isolates in order to determine if they can cause DCR. Although the incidence of fungi, including the core rot pathogen *Alternaria*, isolated from the *Tarsonemus* mites seem low (13 to 17.5%), this is in agreement with findings of Roets *et al.* (2007), who also found that the fungi vectored by mites had an incidence of 4 to 15.8% on the mites.

Apart from the vectoring of fungi, Michailides *et al.* (1994) also hypothesised that the *Tarsonemus* mites may cause small wounds that allow infection of core rot fungi. This could indeed be true since some *Tarsonemus* mites are known to feed on plant material as well as on fungi (Lindquist, 1986), thus being able to produce small wounds that predispose the fruit to fungal infections. Michailides *et al.* (1994) found some support for this hypothesis, since they were able to show in preliminary experiments that a higher disease incidence was present when fruits were inoculated with *Coniothyrium* and mites, than when fruits were only inoculated with *Coniothyrium* (Michailides *et al.*, 1994). The role of wounds in the development of core rot diseases has also been suggested previously by Miller (1959) but may be underestimated in disease development. Miller (1959) hypothesised that as apples mature on the trees, environmental and other factors cause apples to grow too fast and this causes wounds in the tissue lining in the core that serves as entry for core rot pathogens that are most likely weak pathogens, requiring a wound for entry. In the current study some support for the importance of wounds was found when the calyx tube decay was observed in Granny Smith fruits, since the decay was often associated with a small crack in the calyx tube.

Analyses of core rot susceptible apples for the presence of mites within diseased and healthy fruits showed a significant association of *Tarsonemus* mites within the core region of fruits with total core rots (DCR and WCR) as well as DCR. This is in accordance with findings reported by Michailides *et al.* (1994) who also found a high incidence (50% to 86%) of *T. confuses* in diseased fruits. However, unlike Michailides *et al.* (1994), a high incidence of *Tarsonemus* mites was also found within the core region of healthy fruits in the current study. Michailides *et al.* (1994) did not find the mites in the core region of healthy fruits, but most (83%) of these

apples had the mites along the floral parts or the calyx tube. Although in the current study a significant association was found with mites within the core region and diseased Red Delicious fruits, the high incidence of mites within the core region of healthy fruits suggests that if the mites do play a role in disease development, several other factors may also be important in ultimately determining disease development.

Two important observations were made during the analyses of Granny Smith fruits for mites, which included the detection of a new calyx tube decay symptom, as well as the absence of macroscopic fungal hyphae within the seed locule of these fruits. The Granny Smith calyx tube decay symptom was observed at frequencies (0.33% to 9.68%) similar to that found for DCR (0.43% to 10%) in Red Delicious fruits. It was interesting to note that none of the Granny Smith fruits contained fungal hyphae in their core region when viewed using a stereomicroscope, whereas more than 90% of the Red Delicious fruits contained fungal hyphae in their core region, regardless of the presence or absence of mites (data not shown). This may be due to environmental conditions not being favourable for fungal growth in the closed-off core region of Granny Smith fruits, or due to the fact that fungal spores can not enter the core region. Alternatively, the lack of hyphae in the core region may be due to the core region of Granny Smith fruits being less susceptible to *Alternaria* colonisation when compared to Red Delicious fruits, as recently suggested by Niem *et al.* (2007). These authors also suggested that cultivars that do not develop core rot, have a lower pH in mesoderm tissue just outside of the core region than susceptible cultivars, which results in reduced pathogen virulence. This could also explain the small and restricted calyx tube decay lesion that was observed in Granny Smith fruits. In the current study Granny Smith fruits were only sampled from one production region (Ceres), which precludes definite conclusions from being made about the presence of mites, and the occurrence and etiology of the calyx tube decay associated with this cultivar.

Although *Alternaria* was isolated from the calyx tube decay symptom observed in Granny Smith fruits, results from the isolation and inoculation studies are inconclusive, as this genus was also isolated from the calyx tube of asymptomatic Granny Smith fruits. This ubiquitous nature of *Alternaria* in apple fruits is in agreement with findings of Serdani *et al.* (1998) and Teixido *et al.* (1999) that showed

that *Alternaria* is a ubiquitous endophytic genus within apple cultivars that develop core rots as well as those that do not develop core rots. Furthermore, Ellis and Barrat (1983) also found that in Red Delicious fruits *Alternaria* could be isolated from the core region and calyx tube of 100% of asymptomatic fruits at the end of the season, even though core rot rarely developed in any of the fruits. Thus, considering all of the above, we currently still do not know whether some of the *Alternaria* isolates obtained from fruits are perhaps non-pathogenic thus not contributing to disease development, or whether all isolates are pathogenic, with symptom expression only occurs under certain environmental conditions (including wounding and high humidity) or changes in host responses. These will be important aspects to investigate in future studies. Although the pathogenicity of *Alternaria* isolates obtained from asymptomatic and calyx tube decay lesions were investigated using a toothpick assay, the results are inconclusive as discussed above for testing of *Alternaria* isolates from mites.

This study suggests that *Tarsonemus* mites may play a role in the etiology and epidemiology of apple core rot diseases, due to 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. However, much still remains to be elucidated before any definite conclusions can be made on their role in the etiology and epidemiology of core rot diseases. Therefore, future studies should include a more detailed investigation into the movement and seasonal patterns of *Tarsonemus* mites within South African apple orchards, as well as their population sizes within Red Delicious orchards having a history of low and high core rot disease incidences. Dispersal of mites over long distances should also be investigated, as this will give an indication whether other insects (phoretic associations) could also be involved in the epidemiology of the disease. It will also be important to conduct inoculation studies that will show conclusively that co-inoculation of core rot pathogenic fungi and *Tarsonemus* mites results in higher disease incidence than when only core rot pathogens are inoculated. In the current work these studies could not be conducted, since the reproduction of the mites was inconsistent and never yielded enough mites for inoculation experiments. Thus, conditions for reproduction of *Tarsonemus* mites in South Africa need to be optimised by further investigating the effect of different temperatures as well as different fungal species and genera on reproduction of the

mites. The feeding habits of the mites also need further investigation to determine whether they can feed on apple tissue, thus creating wounds, or whether they are strictly fungivorous.

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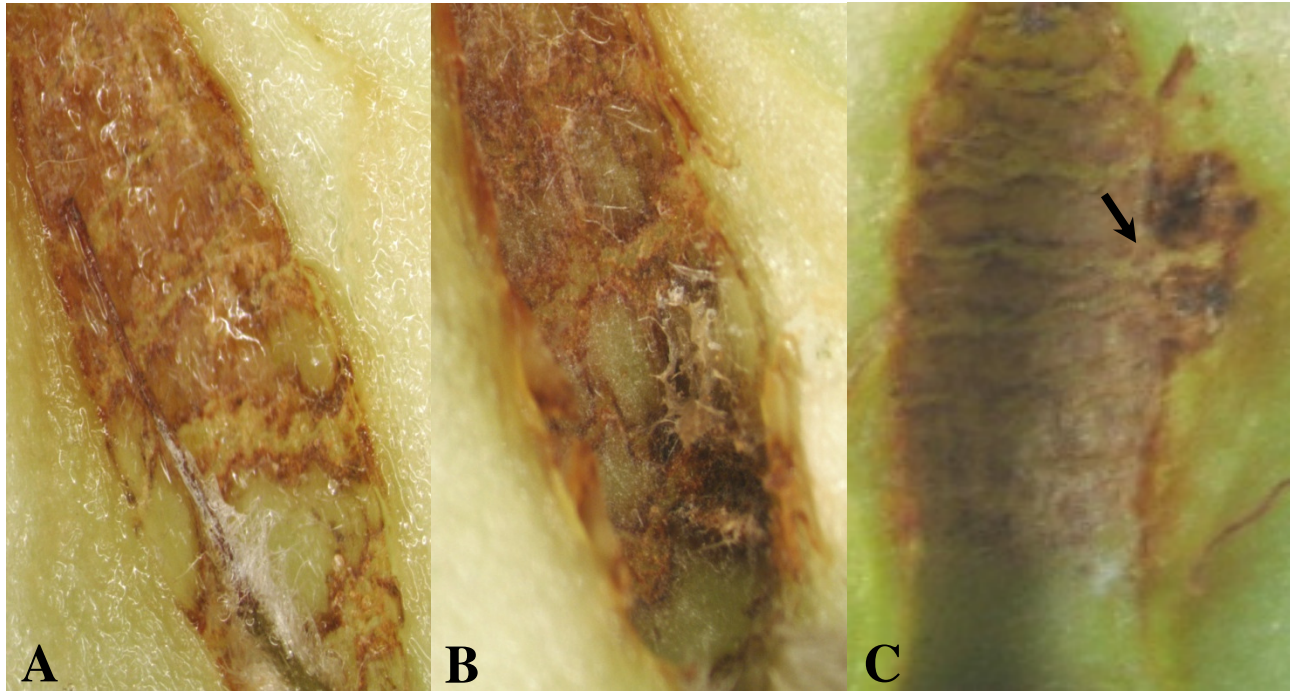
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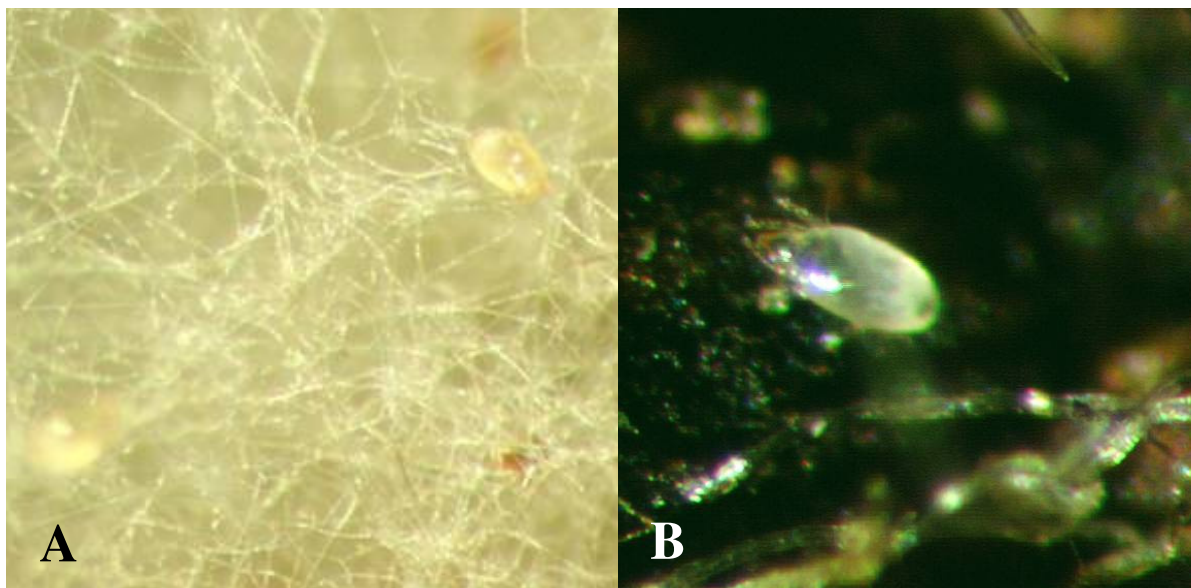
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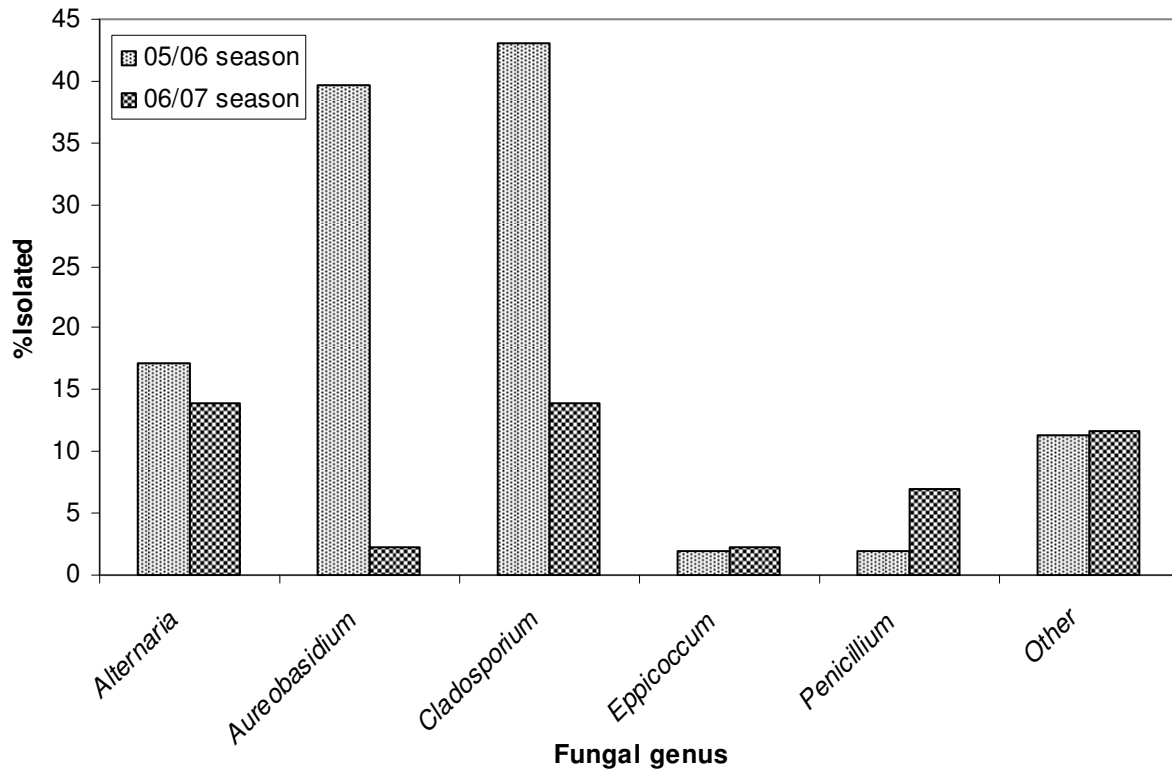
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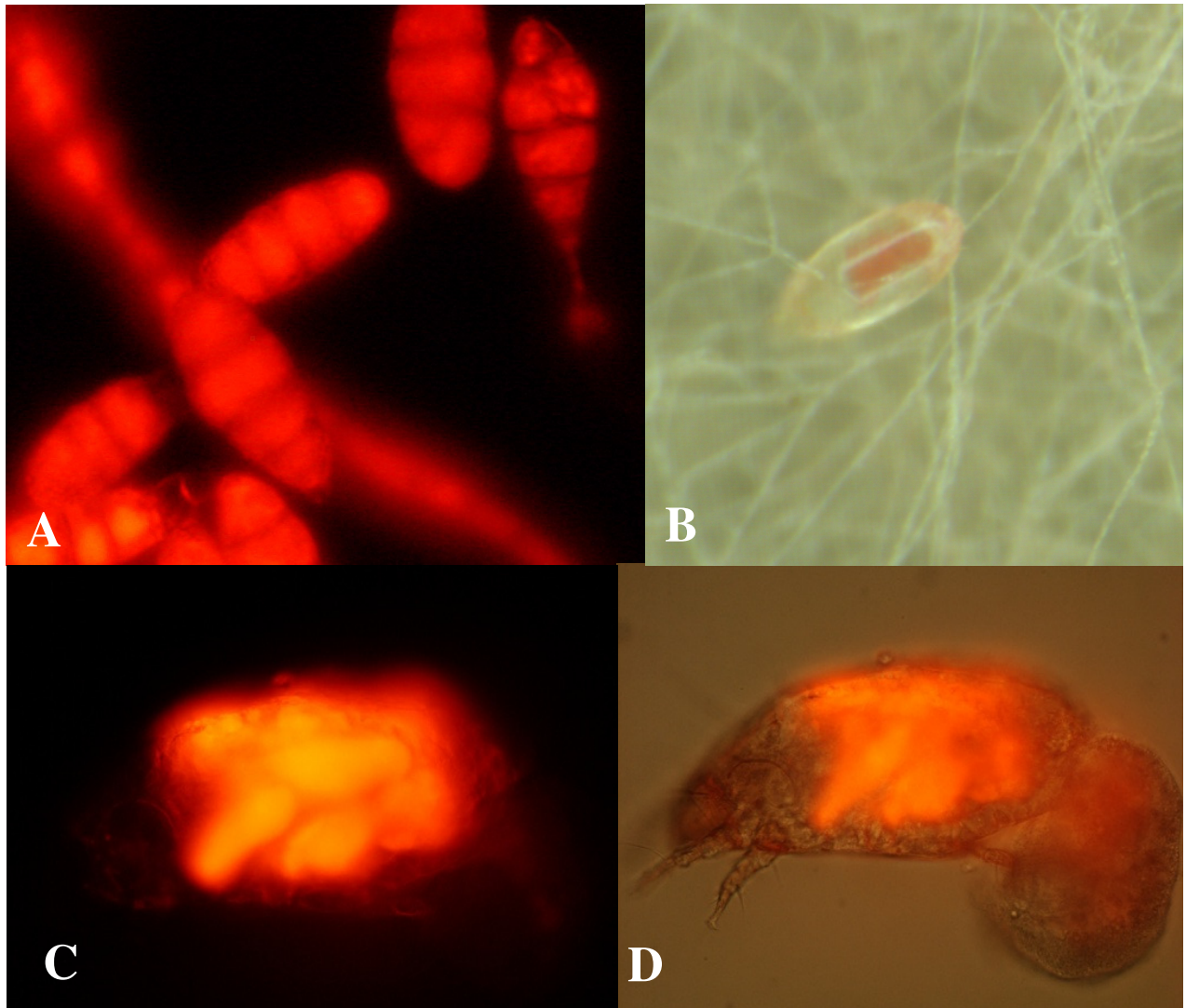
**Fig 1.** Calyx tube region of Granny Smith mature fruits showing (A) no symptoms (asymptomatic), (B) calyx tube decay symptom and (C) calyx tube decay symptom associated with cracks (arrow) within the calyx tube.



**Fig. 2.** *Tarsonemus* mite species present within an (A) apple core region among fungal hyphae and (B) apple mummy.



**Fig. 3.** Incidence of different fungal genera isolated from *Tarsonemus* mites. In the 2005/06 season, isolations were made from mites that were obtained from mature fruits harvested from only one orchard (CSC1), whereas in the 2006/07 season isolations were made from mites obtained from apple mummies, blossoms and mature fruits in two Red Delicious (CSC1 and CSC2) and two Granny Smith orchards (CRC1 and CRC2).



**Fig. 4.** Culture of a *Alternaria* transformant expressing the DsRed-Express gene within conidia as viewed using epifluorescent microscopy (A). *Tarsonemus* mites that were fed on the DsRed-Express *Alternaria* transformant as viewed using (B) a light stereo microscope and (C & D) epifluorescent microscopy.

**Table 1.** Incidence of dry- and wet core rot diseases, as well as *Tarsonemus* mites within Red Delicious (Oregon Spur and Top Red) mature fruits collected from orchards in different apple production regions of South Africa

Orchard	Cultivar	Season	Production region	Number DCR fruits <sup>1</sup>	Number WCR fruits <sup>1</sup>	Total number fruits analysed	Number healthy fruits with mites in calyx tube <sup>1</sup>	Number WCR + DCR fruits with mites in calyx tube <sup>1</sup>	Number healthy fruits with mites in core region <sup>1</sup>	Number WCR + DCR fruits with mites in core region <sup>1</sup>	Total number fruits analysed
CSC1	Oregon Spur	2005/06	Ceres	1 (0.4)	4 (1.7)	238	48 (20.6)	1 (20.0)	106 (45.5)	2 (40.0)	238
CSC1	Oregon Spur	2006/07	Ceres	18 (6.0)	4 (1.3)	300	15 (14.7)	0 (0.0)	67 (65.7)	19 (86.4)	124
CSC2	Oregon Spur	2006/07	Ceres	17 (5.7)	2 (0.7)	300	18 (17.8)	0 (0.0)	67 (66.3)	18 (94.7)	120
CSC3	Oregon Spur	2006/07	Ceres	8 (2.7)	3 (1.0)	300	7 (18.0)	2 (18.2)	25 (64.1)	6 (54.6)	50
CSC4	Oregon Spur	2006/07	Ceres	10 (3.3)	2 (0.7)	300	6 (16.7)	0 (0.0)	21 (58.3)	10 (83.3)	48
CSC5	Oregon Spur	2006/07	Ceres	10 (3.3)	3 (1.0)	300	5 (13.9)	0 (0.0)	20 (55.6)	11 (84.6)	49
CSC6	Oregon Spur	2006/07	Ceres	8 (2.7)	5 (1.7)	300	10 (25.0)	0 (0.0)	23 (57.5)	12 (92.3)	53
CSE	Top Red	2006/07	Ermelo	17 (5.7)	1 (0.3)	300	9 (13.6)	0 (0.0)	44 (66.7)	17 (94.4)	84
CSG1	Top Red	2006/07	Grabouw	8 (2.8)	1 (0.3)	290	1 (2.9)	0 (0.0)	19 (55.9)	6 (66.7)	43
CSG2	Top Red	2006/07	Grabouw	17 (5.7)	0 (0.0)	300	2 (5.7)	0 (0.0)	21 (60.0)	15 (88.2)	52
CSG3	Top Red	2006/07	Grabouw	7 (2.8)	2 (0.7)	282	3 (37.5)	0 (0.0)	0 (0.0)	4 (57.1)	15
CSG4	Top Red	2006/07	Grabouw	5 (1.7)	1 (0.3)	290	5 (35.7)	0 (0.0)	4 (28.6)	3 (50.0)	20

<sup>1</sup> The number of apples with the specific disease symptom, dry core rot (DCR) and wet core rot (WCR), or mites present is followed by the percentage of apples with this condition in brackets.

**Table 2.** Incidence of calyx tube decay and *Tarsonemus* mites within the calyx tube of mature Granny Smith fruits collected from apple orchards in the Ceres production region of South Africa

Orchard	Cultivar	Season	Number fruits with calyx tube decay <sup>1</sup>	Total number fruits analysed	Number healthy fruits with mites <sup>1</sup>	Number calyx tube decay fruits with mites <sup>1</sup>	Total number fruits analysed
CRC1	Granny Smith	2006/07	12 (9.6)	125	66 (58.4)	7 (58.3)	125
CRC1	Granny Smith	2007/08	5 (1.7)	294	0 (0.0)	2 (40.0)	10
CRC2	Granny Smith	2006/07	3 (7.5)	40	19 (51.4)	2 (66.7)	40
CRC2	Granny Smith	2007/08	9 (3.5)	254	3 (37.5)	6 (66.7)	17
CRC3	Granny Smith	2007/08	6 (2.6)	227	1 (12.5)	1 (16.7)	14
CRC4	Granny Smith	2007/08	1 (0.3)	304	3 (17.7)	1 (100)	18

<sup>1</sup> The number of apples with the specific condition (calyx tube decay and healthy) or mites present is followed by the percentage of apples with this condition in brackets.



**Table 3.** Percentage apple developmental stages containing different mite families within two Oregon Spur and two Granny Smith apple orchards

Orchard <sup>1</sup>	Developmental stage and season sampled <sup>2</sup>	% Incidence of the different mite families								
		Tarsonemidae <sup>3</sup>	Phytoseiidae	Tetranychidae	Tydeidae	Bdellidae	Ascidae	Oribatidae	Dolichocybidae	Acaridae
CSC1	Buds 2006/07	20.00	0.00	37.60	0.00	0.00	0.00	0.00	0.00	0.00
	Blossom 2006/07	1.60	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	4cm. diam. fruit 2006/07	6.40	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	Mature fruit calyx 2005/06	20.85	10.21	8.94	1.28	0.00	0.00	0.43	0.00	0.43
	Mature fruit core 2005/06	45.96	3.40	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	Mature fruit calyx 2006/07	12.00	0.80	0.8	0.00	0.00	0.00	0.00	0.00	0.00
	Mature fruit core 2006/07	68.80	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	Mummies 2005/06	100.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	Mummies 2006/07	100.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
CSC2	Blossom 2006/07	0.80	0.00	0.80	0.00	0.00	0.00	0.00	0.00	0.00
	4cm. diam. fruit 2006/07	5.04	0.00	0.00	0.84	0.00	0.00	0.00	0.00	0.00
	Mature fruit calyx 2006/07	15.00	3.33	3.33	0.83	0.00	0.00	0.00	0.00	0.00
	Mature fruit core 2006/07	70.83	0.83	0.83	2.50	0.00	0.00	0.00	0.83	0.00
	Mummies 2006/07	100.00	6.25	0.00	0.00	0.00	0.00	0.00	0.00	0.00
CRC1	Blossom 2006/07	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	4cm. diam. fruit 2005/06	11.2	0.80	0.00	0.80	0.00	0.00	0.00	0.00	0.00
	Mature fruit calyx 2005/06	52.72	0.00	0.00	2.94	0.00	0.00	0.00	0.00	0.00
	Mature fruit core 2005/06	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	Mature fruit calyx 2006/07	58.87	5.65	4.03	0.81	0.00	0.00	0.00	0.00	0.00

Orchard <sup>1</sup>	Developmental stage and season sampled <sup>2</sup>	% Incidence of the different mite families								
		Tarsonemidae <sup>3</sup>	Phytoseiidae	Tetranychidae	Tydeidae	Bdellidae	Ascidae	Oribatidae	Dolichocybidae	Acaridae
	Mature fruit core 2006/07	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	Mummies 2006/07	100.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
CRC2	Blossom 2006/07	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	4cm. diam. fruit 2006/07	7.76	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	Mature fruit calyx 2006/07	34.44	2.83	2.02	0.41	0.00	0.00	0.00	0.00	0.00
	Mature fruit core 2006/07	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	Mummies	100.00	0.00	0.00	0.00	5.55	0.00	0.00	0.00	0.00

<sup>1</sup>The CSC1 and CSC2 orchards were Oregon Spur orchards, whereas the CRC1 and CRC2 orchards were Granny Smith orchards.

<sup>2</sup>Developmental stages that were sampled included buds, blossoms, 4cm. diam. fruit, mature fruits and mummies. Each mature fruit was inspected for the presence of mites within the calyx tube as well as in the core region. Apples that contained mites within their core as well as calyx tube were classified under “Mature fruit core”, whereas apples classified under “Mature fruit calyx” only contained the mites within the calyx tube region.

<sup>3</sup>All mites that were found within the family Tarsonemidae belonged to the genus *Tarsonemus*.

**Table 4.** Results of association analyses to determine if the presence of rot (WCR, DCR, total rot or calyx tube decay) was dependent on the presence of mites in the calyx, core or both.

Association	Significance (p-value) for Fisher's exact test	Percentage of healthy and diseased apples containing mites		
		No Mites	Mites	
<b>Red Delicious fruits</b>				
DCR with Mites in core	P < 0.0001	DCR	16	84
		Healthy	44	56
DCR with Mites in calyx	P < 0.0001	DCR	98	2
		Healthy	83	17
DCR with Mites total	P = 0.0010	DCR	14	86
		Healthy	27	73
WCR with Mites in core	P = 0.4401	WCR	32	68
		Healthy	40	60
WCR with Mites in calyx	P = 0.1061	WCR	96	4
		Healthy	85	15
WCR with Mites total	P = 0.6594	WCR	29	71
		Healthy	25	75
Core rot total with Mites in core	P < 0.0001	Core rot	19	81
		Healthy	44	56
Core rot total with Mites in calyx	P < 0.0001	Core rot	98	2
		Healthy	83	17
Core rot total with Mites total	P < 0.0001	Core rot	17	83
		Healthy	27	73
<b>Granny Smith fruits</b>				
Calyx tube decay with Mites in calyx	P = 0.7184	Calyx tube decay	47	53
		Healthy	51	49