Etiology and management of *Neofabraea* lenticel decay (bull's eye rot) of apples in the Western Cape of South Africa

by

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DECLARATION

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SUMMARY

Postharvest lenticel decay of apple and pear fruit caused by *Neofabraea malicorticis*, *N. perennans*. *N. vagabunda* and *N. kienholzii* is a disease more commonly known as Bull's eye rot. In South Africa, only *N. vagabunda* has been identified to cause this disease on apple fruit in Western Cape apple orchards, especially on the late harvested cultivar 'Cripps Pink'. The pathogen infects the lenticels of fruit in the orchard and disease symptoms only become visible months after harvest. Symptoms include decay spreading outward from an infected lenticel as concentric dark and light brown discoloured rings. This disease does not spread in postharvest storage and preharvest infections thus ultimately determine disease incidence. Preharvest management strategies reduce infection levels by the pathogen, but the postharvest application of fungicides can reduce the decay incidence of already infected fruit. There are, however, no fungicides registered against bull's eye rot in South Africa.

To confirm the current causal pathogen of bull's eye rot in South Africa, *Neofabraea* spp. were isolated from symptomatic fruit received from packhouses in the Western Cape. *Neofabraea* species were identified using a multiplex-PCR. A total of 91 isolates were all identified as *N. vagabunda*. Subsequently, *N. vagabunda* isolates from the Western Cape were tested on key apple cultivars Fuji, Cripps Pink and Golden Delicious to evaluate cultivar susceptibility. The isolates were equally pathogenic on tested cultivars with low variation between the isolates. 'Fuji' and 'Cripps Pink' were found highly susceptible to disease development, averaging lesion diameters of 8.36 mm and 8.15 mm respectively 14 days after inoculation. 'Golden Delicious' was significantly less susceptible averaging only 6.28 mm in lesion diameter after 14 days.

Two fungicides registered for use on pome fruit in South Africa, that have reportedly been found to effectively control bull's eye rot in other studies, are the phenyl pyrrole fludioxonil, and the anilinopyrimidine pyrimethanil. The curative ability of these fungicides was tested on *N. vagabunda* inoculated 'Fuji' and 'Cripps Pink' apple fruit. The fungicide efficacy was compared as a dip, drench and thermo-fog application. Dip application with fludioxonil effectively controlled bull's eye rot incidence on 'Fuji' by 83% and 'Cripps Pink' by 84% compared to the untreated control fruit. Pyrimethanil did not control bull's eye rot incidence as a dip application. As a drench however, pyrimethanil could control incidence on 'Fuji' by 27%. Fludioxonil was less effective as a drench and controlled disease incidence on 'Fuji' by 73%, and on 'Cripps Pink' by 41%. Pyrimethanil was the most effective as a thermo-fog application, controlling incidence of bull's eye rot on 'Fuji' by 59%. On 'Cripps Pink' however, pyrimethanil thermo-fogging only controlled bull's eye rot incidence by 18%. As a thermo-fog treatment, fludioxonil had moderate efficacy, controlling bull's eye rot on 'Fuji' by 47% and 'Cripps Pink' by 28%.

To investigate pyrimethanil inefficacy in controlling bull's eye rot, the sensitivity of different *N. vagabunda* isolates on inoculated fruit were evaluated towards pyrimethanil, as well as the effect of incubation time before curative fungicide application. *Neofabraea vagabunda* isolates did not differ in their sensitivity towards pyrimethanil and reacted equally to a 500 mg/L and 1000 mg/L concentration fungicide treatment. Fludioxonil was effective regardless of the incubation time. Pyrimethanil was significantly more effective when incubation time was shortened to 6 hours before treating fruit with the fungicide.

In conclusion, *Neofabraea vagabunda* is the causal organism of bull's eye rot in the Western Cape province of South Africa, and the late harvest apple cultivars 'Fuji' and 'Cripps Pink' are highly susceptible to this pathogen. Fludioxonil can effectively reduce *N. vagabunda* bull's eye rot disease incidence when applied postharvest. Pyrimethanil had variable efficacy towards the pathogen but should not be discarded as a postharvest treatment for bull's eye rot in South Africa, as the inoculation method used in the trials did not truly simulate natural infection of fruit by the pathogen.

OPSOMMING

Na-oes lentisel verrotting van appel en peer vrugte wat veroorsaak word deur *Neofbraea malicorticis*, *N. perennans*, *N. vagabunda* en *N. kienholzii* is a siektekompleks wat meer algemeen as “Bull’s eye” vrot bekend staan. In Suid-Afrika is nog slegs *N. vagabunda* geïdentifiseer as die oorsaak van hierdie siekte op appel vrugte in appel boorde in die Wes-Kaap, veral op die laat seisoen kultivar ‘Cripps Pink’. Hierdie patogeen infekteer die lentiselle van vrugte in die boord en siekte simptome kom eers maande na oes te voorskyn. Simptome behels verrotting wat as konsentriese donker en lig bruin verkleurde ringe uitwaarts versprei vanaf die geïnfekteerde lentisel. Die siekte versprei nie in opberging na oes nie en infeksies in die boord bepaal maksimum moontlike siekte voorkoms. Voor-oes bestuurs strategieë verlaag die infeksie vlakke van die patogeen, maar die toediening van fungisiedes na-oes kan siekte ontwikkeling verlaag in reeds geïnfekteerde vrugte. Daar is egter geen fungisiedes geregistreer teen “Bull’s eye” vrot in Suid-Afrika nie.

Ten einde te bevestig watter patogeen huidiglik bull’s eye vrot in Suid-Afrika veroorsaak, was *Neofabraea* spp. geïsoleer vanaf simptomatiese vrugte wat ontvang was van pakhuise in die Wes-Kaap. *Neofabraea* spesies was geïdentifiseer met ‘n veelvuldige-PCR. ’n Totaal van 91 isolate was almal geïdentifiseer as *N. vagabunda*. Gevolglik was sleutel appel kultivars Fuji, Cripps Pink en Golden Delicious getoets teen *N. vagabunda* isolate vanuit die Wes-Kaap om kultivar vatbaarheid te evalueer. Die isolate was ewe patogenies op die getoetse kultivars met lae vlakke van variasie tussen die isolate. ‘Fuji’ en ‘Cripps Pink’ was hoogs vatbaar vir siekte ontwikkeling met ‘n gemiddelde letsel deursnee van onderskeidelik 8.36 mm en 8.15 mm 14 dae na inokulasie. ‘Golden Delicious’ was aansienlik minder vatbaar met ‘n gemiddelde letsel diameter van 6.28 mm na 14 dae.

Twee fungisiedes wat geregistreer is op kern-vrugte in Suid-Afrika, en na bewering in ander studies gevind was om effektief te wees in die beheer van bull’s eye vrot, is die feniel-pirrol fludioxonil, en die anilinopirimidien pyrimethanil. Die kuratiewe vermoë van hierdie fungisiedes was getoets op *N. vagabunda* geïnokuleerde ‘Fuji’ en ‘Cripps Pink’ appel vrugte. Die fungisied effektiwiteit was vergelyk as ‘n dompel, drenk en termoberoking. Dompel toediening met fludioxonil het bull’s eye vrot voorkoms effektief beheer op ‘Fuji’ met 83% en ‘Cripps Pink’ met 84% in vergelyking met die onbehandelde kontrole vrugte. Pyrimethanil het nie bull’s eye vrot beheer as a dompel toediening nie. As ‘n drenking het pyrimethanil egter voorkoms op ‘Fuji’ beheer met 27%. Fludioxonil was minder effektief as ‘n drenking en het siekte voorkoms op ‘Fuji’ beheer met 73%, en op ‘Cripps Pink’ met 41%. Pyrimethanil was die mees effektiewe as ‘n termoberoking toediening en het bull’s eye vrot voorkoms op ‘Fuji’ beheer met 59%. Op ‘Cripps Pink’ het pyrimethanil termoberoking egter voorkoms met slegs

18% beheer. As ‘n thermoberoking toediening het fludioxonil matige effektiwiteit gehad met bull’s eye vrot beheer van 47% op ‘Fuji’ en 28% op ‘Cripps Pink’.

Ten einde die rede vir die oneffektiwiteit van pyrimethanil om bull’s eye vrot te beheer te ondersoek, was die sensitiwiteit van *N. vagabunda* isolate op geïnokuleerde vrugte teenoor pyrimethanil geëvalueer, sowel as die effek van inkubasie tyd voor kuratiewe toediening van funigisiedes. *Neofbraea vagabunda* isolate het nie verskil in hul sensitiwiteit teenoor pyrimethanil nie en het dieselfde gereageer op die 500 mg/L en 1000 mg/L konsentrasies fungisied behandelings. Pyrimethanil was beduidend meer effektief wanneer inkubasie tyd verkort was tot 6 ure voordat vrugte behandel was met die fungisied.

Ter afsluiting, *Neofabraea vagabunda* is die oorsaaklike organisme van bull’s eye vrot in die Wes-Kaap provinsie van Suid-Afrika en die laat-oes appel kultivars, ‘Fuji’ en ‘Cripps Pink’, is baie vatbaar vir die patogeen. Fludioxonil kan effektief *N. vagabunda* bull’s eye vrot siekte voorkoms verlaag wanneer dit toegedien word as ‘n na-oes behandeling. Pyrimethanil het wisselvallige effektiwiteit getoon teenoor die patogeen, maar kan nie uitgeskakel word as ‘n na-oes behandeling vir bull’s eye vrot in Suid-Afrika nie, omdat die inokulasie metode wat gebruik was in proewe, nie werklike natuurlike infeksie van vrugte deur die patogeen naboots nie.

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CONTENTS

CHAPTER 1

A review of *Neofabraea* lenticel decay (bull's eye rot) of pome fruit

INTRODUCTION

Apple (*Malus domestica* L.) is considered one of the most important of all the deciduous fruits grown for the South African economy. In the 2016/2017 growing season, apple production in South Africa totalled to a gross value of R5.5 billion (DAFF, 2018). This means that apple production alone amounts to 35.5% of South Africa's total deciduous fruit industry (DAFF, 2018). Apple production in South Africa has seen a considerable rise from 2006 to 2016 with a production of 633 000 tonnes increasing to 918 000 tonnes (FAOUN, 2018). The Western Cape region is the main producer of apples in the country due to its favourable climate resembling that of the Mediterranean area, which is well suited for apple production (Den Breeyen and Lennox, 2012). Postharvest decay is of great importance to the value of the deciduous fruit industry. Postharvest diseases caused by fungal and bacterial pathogens lead to major losses of fruit in storage and can lead to the disruption of various aspects of the fruit industry (Spotts *et al*., 1999; Mari *et al*., 2014). Harvesting, packing, storage, transportation and export of fruit are all factors adapted to reduce postharvest decay. Detection of postharvest decay in a packhouse or consignment can lead to fruit having to be repacked, consignment rejected and export to be suspended. These factors lead to a loss of income for the apple industry.

Apple fruit has relatively high susceptibility to fungal decay due to its low levels of pH, high moisture content and favourable nutrient composition (Tahir *et al*., 2014). A postharvest disease that has sporadically occurred on apple throughout the Western Cape is bull's eye rot (Den Breeyen and Lennox, 2012). Bull's eye rot is a disease-complex associated with four different *Neofabraea* species namely, *N. malicorticis* Jacks, *N. perennans* Kienholz, *N. vagabunda* Guthrie (syn. *Phlyctena vagabunda* Desm.) and *N. kienholzii* Seifert, Spotts and Levesque (Verkley, 1999; Spotts *et al*., 2009). However, in the Western Cape province of South Africa only *N. vagabunda* has been identified as the causal agent of bull's eye rot (Den Breeyen and Lennox, 2012; Den Breeyen *et al*., 2019). The disease infects the lenticels of apple fruit in the orchard and symptoms only arise 3 to 5 months into storage (Bompeix, 1978). Lesions on fruit are concentric circles light to dark brown in colour surrounding infected lenticels (Grove, 1990). Fruit are particularly susceptible when wet conditions occur just before harvest, as mature fruit are highly susceptible to the pathogen and water removes lenticel protecting chemicals (Edney *et al.,* 1977). Major sources of inoculum for the pathogens include dead bark and leaf litter, twigs, mummies, fruit spurs, pruning material as well as cankers (Verkley, 1999; Henriquez *et al.*, 2006; Köhl *et al.*, 2018).

Management strategies for bull's eye rot pathogens in South Africa include practices mostly aimed at controlling apple scab (*Venturia inaequalis* Cooke) and other fungal pathogens (Rochefort, 2015). The most important strategies are the elimination of inoculum sources, and more importantly the application of fungicides. Removing cankers, fruit mummies, leaf-litter, pruning material and twigs reduces inoculum levels not only in the next season, but the current season as well, because fruit can become infected anytime during the growing season (Grove *et al.*, 1992; Henriquez *et al.*, 2006; Spotts *et al.*, 2009; Wenneker and Köhl, 2014; Köhl *et al.*, 2018).

The application of fungicides has proven to be very effective in controlling bull's eye rot pathogens (Henriquez *et al.*, 2006; Spotts *et al.*, 2009; Aguilar *et al.*, 2018). However, there are currently no fungicides registered for use specifically for bull's eye rot pathogens in South Africa as well as no set management strategies aimed at controlling said pathogens. Although several studies have tested the efficacy of fungicides against *Neofabraea spp.*, it is apparent that fungicides effective against one species may not be effective against the other (Henriquez *et al.*, 2004; Spotts *et al.*, 2009; Lolas *et al.*, 2016; Wood and Fisher, 2017).

The following chapter will review the bull's eye rot pathogens, their epidemiology and management strategies, including fungicide applications, in order to identify the components of a potential integrated management strategy for bull's eye rot.

PATHOGENS THAT CAUSE BULL'S EYE ROT OF POME FRUIT

Lenticel decay of pome fruit caused by *Neofabraea* spp cause disease symptoms such as cankers on apple tree trunks or branches as well as postharvest decay of fruit. The disease is more commonly known as 'Bull's eye rot' (Fisher, 1925). Other names it has gone by include black spot or dead spot (Cordley, 1900), apple anthracnose or bitter rot (Clinton, 1902), delicious spot (Wilkinson, 1945) and *Gloeosporium* rot (Lockhart, 1967). Four species of the genus *Neofabraea* have been recorded to cause this disease on pome fruit, they are *N. malicorticis*, *N. perennans*, *N. vagabunda* and *N. kienholzii*.

The first of these to be described was *N. malicorticis*, identified as the teleomorph stage of the described *Gloeosporium malicorticis* Cordley in 1913 by H. S. Jacks (Verkley, 1999). *Neofabraea malicorticis* is known to cause cankers on apple trees and is commonly referred to as apple anthracnose (Kienholz, 1939). It has been described as the most aggressive of the four pathogens because of its ability to penetrate the healthy bark of apple trees directly (Kienholz, 1939; Henriquez *et al.*, 2004). The presence of *N. malicorticis* has been reported in Canada, the Pacific North West and eastern areas of North America, Chile and China (Kienholz, 1939; Abeln *et al.,* 2000; Henriquez *et al.,* 2004; Spotts *et al.,* 2009; Soto-Alvear *et al*., 2013; Michalecka *et al*., 2016). In Europe apple anthracnose infections were identified in

various countries including Denmark, Germany, the Netherlands, Poland, Portugal, Sweden, the United Kingdom and recently Italy (Abeln *et al.*, 2000; de Jong *et al.*, 2001; Tahir *et al.*, 2014; Cameldi *et al*., 2016).

The second bull's eye rot pathogen was described in 1925 by Zeller and Childs as *Neofabraea perennans* (Childs, 1929; Verkley, 1999). The species name refers to the word perennial, not because the cankers caused by *N. perennans* are perennial in occurrence, but because of the infections increasing annually around already present cankers (Childs, 1929). *N. perennans* has been reported in the United States as well as Canada (Kienholz, 1939; de Jong *et al.*, 2001; Spotts *et al.*, 2009). Countries in Europe where it has been reported includes the United Kingdom, Sweden, Portugal, Germany, Denmark, the Netherlands, Czech Republic, Lithuania and Norway (Maxin *et al*., 2005; Børve *et al.*, 2013; Hortova *et al*., 2014; Kingsnorth *et al*., 2017; Pešicová *et al*., 2017). Brazil also reported *N. perennans* on their 'Cripps Pink' apple cultivars (Blum *et al.*, 2005). *Neofabraea perennans* was identified for the first time in Australia in 2004, not only on apple fruit but also causing branch cankers (Cunnington, 2004).

Neofabraea vagabunda was first described in 1952 when the perfect state of the fungus *G. album* was observed in England but can be traced back to as early as 1847 (Guthrie, 1959). The key characteristic of this species is that it lives saprophytically on dead plant material (Tan and Burchill, 1972). Until recently, the species *N*. *vagabunda* was called *N. alba*, but Johnston *et al.* (2014) made recommendations for changes of generic names in the order *Letiomycetes* (Ascomycota) in hopes of better understanding and interpreting confusing taxonomy.

Previously the asexual morph of *N. alba* was called *Phlyctema vagabunda* until Johnston *et al.* (2014) argued that since Verkley (1999) in his monograph of the genus *Pezicula*, accepts both *Phlyctema* and *Neofabraea* under the same genus *Neofabraea*, that these two taxonomically coincide. It was decided to keep the genus name *Neofabraea* since it was better characterized phylogenetically, and the new name *N. vagabunda* was adopted (Johnston *et al.*, 2014). Although, a more recent study by Chen *et al*. (2016) found *Neofabraea vagabunda* to fall in the separate phylogenetic clade, *Phlyctema*, compared to the other bull's eye rot causing *Neofabraea* species. However, there were discrepancies between the amplified integral transcribed spacer (ITS) and the RNA polymerase II second largest subunit region (*rpb*2) gene areas of the two tested isolates (Chen *et al*., 2016).

As of September 2019, the name *Neofabraea vagabunda* is still accepted on both the MycoBank and the Index Fungorum databases. Thus, it was decided to refer to this species as *N. vagabunda* in the current study as it is more recognized from a bull's eye rot disease perspective. *Neofabraea vagabunda* is established in various countries which include Australia, Canada, Chile, Czech Republic, Denmark, Germany, Italy, the Netherlands, New Zealand, Poland, South Africa, Spain, the United Kingdom and the United States of America

(USA) (Grove *et al.*, 1992; de Jong *et al.*, 2001; Cunnington, 2004; Henriquez *et al.*, 2004; Johnston *et al.,* 2005; Den Breeyen and Lennox, 2012; Maxin *et al.,* 2012; Børve *et al.*, 2013; Soto-Alvear *et al.*, 2013; Hortova *et al.*, 2014; Wenneker and Köhl, 2014; Michalecka *et al*., 2015; Romero *et al*., 2016; Pešicová *et al.*, 2017; Köhl *et al.*, 2018).

The latest bull's eye rot species to be identified is *N. kienholzii* (Spotts *et al.*, 2009). Although it was first described in 2009, it was discovered in the United States (USA) five years prior to its description (Henriquez *et al.*, 2004). Not much information is available on this species due to its recent discovery. As earlier mentioned *N. kienholzii* is present in the USA but has been found in other countries such as Australia, Czech Republic, the Netherlands, Poland and Portugal (de Jong *et al*., 2001; Cunnington, 2004; Henriquez *et al*., 2004; Spotts *et al*., 2009; Michalecka *et al*., 2016; Pešicová *et al*., 2017).

DISEASE CYCLE

Inoculum sources

The epidemiology of species in the bull's eye rot complex although similar varies in certain aspects. *Neofabraea vagabunda* is classified as a saprophyte and was first found to live and sporulate on pruning snags, fruit mummies and dead buds (Sharples, 1959; Tan and Burchill, 1972). The pathogen is prevalent on dead branches and twigs and has been found on whole diseased trees (Verkley, 1999). Senescent tree leaves have also been shown to be a critical source of inoculum, causing significant levels of disease even when other inoculum sources have been removed (Tan and Burchill, 1972). The fungus can grow on senescent leaves but will only sporulate once the leaves are dead or critically damaged (Tan and Burchill, 1972). The natural infection of apple tree branches or bark by *N. vagabunda* is an uncommon sight and is generally not regarded as problematic. Despite *N. vagabunda* being a known saprophyte, low levels of pathogenicity and formation of small cankers have been reported on apple tree branches. However, sporulation from these cankers was initially not observed (Corke, 1956). White and Wilkinson (1962) successfully induced *N. vagabunda* lesions artificially on tree shoots, which subsequently led to an increase in natural disease incidence. Nevertheless, despite increased incidence, the ability of these lesions to produce spores was not proven.

Henriquez *et al.* (2006) tested *N. vagabunda* and *N. perennans'* ability to produce cankers on 'Granny Smith' apple and 'd'Anjou' pear trees. *Neofabraea vagabunda* could effectively produce canker symptoms beyond the inoculation point for both tree types, but these cankers were smaller than those produced by *N. perennans*. Furthermore, Hortova *et al*. (2014) also reported canker lesion formation on *N. vagabunda* inoculated apple tree branches. In hindsight, the pathogenicity of this species on living apple tree tissue was up until recently

confirmed only with artificial inoculation. Consequently, sporulation from artificially produced cankers was found with abundant amounts of conidia being produced (Henriquez *et al.*, 2006).

Considering artificially induced cankers, *N. vagabunda* was found to be the lead cause of apple branch cankers in California, but the sporulation ability of these cankers was not reported (Rooney-Latham, 2013). In the Netherlands, *N. vagabunda* caused high levels of cankers on the twigs of apple trees and moderate levels on pear trees (Köhl *et al.*, 2018). Interestingly, sporulation of coin cankers produced by *N. vagabunda* on ash trees in Michigan, United States, has been reported (Putnam and Adams, 2005). Conidia were sampled from acervuli sprouting from the centre of canker lesions on the ash trees, meaning these cankers serve as a source of inoculum for the pathogen (Rossman *et al.* 2002). *Neofabraea vagabunda* has been identified to cause cankers on branches and twigs of olive trees in Spain (Romero *et al.*, 2016).

Canker formation on pome trees is more commonly associated with the species *N. malicorticis* and *N. perennans*. *Neofabraea malicorticis* produces cankers that present a 'fiddle-string' like appearance, due to the pathogens inability to attack the bast (phloem) fibres surrounding the infected area (Kienholz, 1939). Cankers start as irregular brown discolouration and slightly depressed (Cordley, 1900). Later development causes small spots that are reddish-brown in colour and internally discoloured bark that extends inward to the cambium (Powell *et al.*, 1965). Canker development ceases after one season, but sporulation has been reported up to three years after canker formation (Dugan *et al.*, 1993). Development of the canker is slow during autumn and winter and rapidly starts to spread in the warmer temperatures that comes with spring (Childs, 1929; Kienholz, 1939). The older the canker gets the darker in colour it becomes. As the dead tissue separates from the living tissue, irregular cracks form at the border of the canker (Cordley, 1900). Matured cankers crack or fall off revealing the stringy bast fibres that give apple anthracnose cankers their characteristic fiddle-string like appearance (Dugan *et al.*, 1993; Aguilar *et al.*, 2017).

Perennial cankers are caused by *N. perennans*. These canker symptoms are similar to that of *N. malicorticis* previously mentioned, except canker development does not cease after one season. New infections of old cankers occur each season when conditions are favourable and the cankers subsequently enlarge (Childs, 1929). After one year of infection canker lesions are a few centimetres in diameter, clearly sunken as well as dark brown in colour with a cracked margin that is separated from the living surrounding tissue (Kienholz, 1939, Henriquez *et al.*, 2006). Cankers produced by both *N. malicorticis* and *N. perennans* only start sporulating once the canker is substantially developed (Kienholz, 1939). Canker production for both species is highest during the colder temperatures at the end of autumn and winter months when precipitation occurs (Grove *et al.*, 1992). Higher canker incidence can be expected in cold seasons with high rainfall if not properly managed. When sporulation

occurs from the cankers, the surface becomes uniformly covered with acervuli that, under relative moisture conditions, erupt with creamy masses of conidia (Kienholz, 1939). Sporulation in cankers is observed in the first summer months where the cream coloured spore masses erupt from avervuli (Cordley, 1900). In cases where the infection is severe and young tree branches are involved, these pathogens can cause the girdling of branches (Kienholz, 1939). Trees of all ages are susceptible to infection and canker formation by *N. malicorticis* and *N. perennans* (Kienholz, 1939; Henriquez *et al.*, 2006).

Infection

According to Edney (1964) there are two stages to the bull's eye rot disease, the first is the pathogen's manifestation and infection in the orchard, and secondly, the latent period where the pathogen remains dormant until symptom expression. Although the various *Neofabraea* species that cause bull's eye rot produce almost indistinguishable disease symptoms, the conditions around their infection processes differ slightly.

The infection of tree bark or branches and the formation of cankers that serve as inoculum source are associated with *N. malicorticis* and *N. perennans.* The main difference between the two species is that *N. malicorticis* infects the apple trees through healthy bark, whereas *N. perennans* requires wounds to infect (Kienholz, 1939). Even though trees of all ages are susceptible, *N. malicorticis* favours the infection of young apple trees (Kienholz, 1939). Kienholz (1939) reported the species to typically infect smaller branches rather than older larger branches. This could be the reason why the species can infect tree branches without wounds, as it targets the younger branches with softer bark. Infection occurs with mycelium penetrating the bark cuticle (Powell *et al.*, 1965).

Perennial canker development resumes each season due to favourable colder winter conditions for the pathogen being present once a year where it proceeds to infect the healthy surrounding tissue (Childs, 1929). *Neofabraea perennans* requires wounds to infect bark and pruning wounds have been identified as the primary infection site (Childs, 1929; Kienholz, 1939; Grove *et al.*, 1992). Apple trees form callus tissue around already present cankers to serve as a physical defence mechanism to impede the spreading of the canker (Grove *et al.*, 1992). However, in the winter season, freezing temperatures cause the callus tissue to crack, and this serves as new infection portals (Dugan *et al.*, 1993). Woolly apple aphids (*Eriosoma lanigerum* Hausmann*)* assist and are vital for the revival of perennial canker infection and development (Grove *et al.*, 1992). The aphids do not serve as vectors for the fungus, but rather as a propagator of infection sites (Grove *et al.*, 1992). They feed on the callus tissue surrounding the cankers, creating openings that are susceptible to infection (Grove *et al.*, 1992). With the feeding on callus tissue comes the formation of galls which then crack under severe environmental conditions that subsequently creates more infection sites (Childs, 1929;

Grove *et al.*, 1992). When pruning wounds and cankers are infested by woolly apple aphids, infection rates by *N. perennans* can be extremely high when optimal environmental and host conditions are present (Childs, 1929). Woolly apple aphid infested pruning wounds and cankers can have infection rates as high as 90% for *N.* perennans in optimal conditions (Zeller and Childs, 1925).

Neofabraea vagabunda survives as a saprophyte on dead plant material (Tan and Burchill, 1972). Pathogen propagules are splash-dispersed from inoculum sources during rain and are carried to susceptible plant materials by the wind (Tan and Burchill, 1972; Edney 1974). Spores are produced throughout most of the year but peak at the end of summer and during autumn (Henriquez *et al.*, 2006). Although *N. vagabunda* is a saprophyte, it grows epiphytically on apple leaves during the summer but only sporulates once leaves are damaged or become moribund during cold, wet conditions (Tan and Burchill, 1972).

Infection of apple fruit can occur as early as one month after bloom resulting in long infection periods, especially in the case of late harvested cultivars (Grove *et al.*, 1992). Conidia are washed on to fruit surfaces through water splash where they adhere and remain until favourable germination conditions occur. Conidia require very high levels of relative humidity (RH) to germinate. Optimal conditions for a high germination success rate require extended wet periods and a temperature margin between 15 to 20°C (Edney, 1974). More than 95% RH for 72 hours at 10°C or higher can lead to at least 95% spore germination (Edney, 1974). Moreover, low humidity levels close the lenticels and helps impede the pathogen's ability to infect fruit (Bompeix, 1978). Closed lenticels are completely resistant to *Neofabraea* infection (Bompeix, 1978). Importantly, Edney (1974) found the germination ability of conidia to completely deteriorate when unsuitable conditions were present for three weeks or longer. Conidia can only germinate when suitable conditions are present, and when lenticels are susceptible, otherwise they will remain dormant (Edney, 1974).

When germination occurs germ-tubes are produced, which swell to form thick-walled appressoria leading to infection of fruit lenticels (Edney, 1956). Appressorium formation is essential for infection and under ideal conditions infection hyphae from the appressorium invade the lenticel cavity (Edney, 1956). The appressorium firmly attaches to the fruit surface where it cannot be washed off (Edney, 1958). Cases when no appressorium is immediately formed, the fungus hyphae have been observed to grow on the surface of fruit only ceasing once an appressorium is formed (Edney, 1958). This is because the direct penetration of the suberized layer of cells covering the lenticels does not occur without an appressorium (Edney, 1958). Infection threads only develop and penetrate epidermal cells when tissues are not suberized or if the layer is damaged, otherwise the threads will only penetrate a very short distance into the underlying tissues of the fruit skin (Edney, 1958; Neri *et al*., 2019).

Latency and symptom expression

An explanation for the diseases' latency period cannot be attributed to a single host or pathogen factor, but rather a network of factors and the effect they have on each other (Bompeix, 1978). The reason for the latency of infections until symptom expression in fruit is not fully understood. When it comes to fruit maturity, young fruit could be resistant to the pectolytic enzymes produced by the pathogen (Edney, 1964). Edney (1964) found that leuco-anthanin phenolic compounds have an inhibiting effect on the pathogens pectolytic enzymes and that the phenolics decreases as the fruit matures.

The duration of the latency period is due to changing levels in fruit physiological resistance and natural biochemicals during maturation in long term storage (Creemers, 1989). Lattanzio *et al.* (2001) proposed that the host's biochemical reaction to infection to impede on fungal development. The phenolics phloridzin and chlorogenic acid have a germination inhibiting effect on *N. vagabunda* (Lattanzio *et al.*, 2001). These phenolics are present at the infection site and gets catalysed by the enzyme polyphenol oxidase to produce fungitoxic quinones which makes conditions unfavourable for the fungus (Lattanzio *et al.*, 2001). However, phloridzin and chlorogenic acid levels decreases significantly in long term storage and this subsequently leads to reduced fungitoxic ability over time (Lattanzio *et al.*, 2001).

Eventually, a break in latency or host resistance will occur after three to five months in storage, when fruit start to senesce and phenolic production is significantly reduced. The fungal mycelium then spreads and produces pectolytic enzymes which disintegrate the host tissue (Edney, 1964). High levels of nitrogen have been found to increase the pectolytic activity of enzymes (Edney, 1964). Disease incidence increases the longer the apple fruit stays in cold storage, not because infections spread, but because more infections overcome host resistance (Lolas *et al.*, 2016). The pathogen does not spread during storage, infection prior to storage ultimately determines maximum disease incidence (Dugan *et al.*, 1993).

Fruit lesions develop as small brown spots which start at the infected lenticels. Lesions enlarge circularly and become sunken, spreading outward from the lenticel. Older lesions have distinctive light brown concentric rings appear surrounded by dark brown zones (Wilkinson, 1945). Lesions are not soft to the touch and advanced lesions will develop a white mycelial mat on the surface (Spotts *et al.*, 2009). On mature lesions, irregularly spaced acervuli will erupt from the lesion, having a grey-black colour (Wilkinson, 1945). Under moist humid conditions, light-yellow conidial masses can be produced (Spotts *et al.*, 2009).

HOST RANGE

The bull's eye rot species-complex is mostly known for infecting and causing disease on apple and pear (*Pyrus*) but have also been found on other crops (Verkley, 1999). *Neofabraea*

vagabunda has been reported on several berry trees (*Rubus* spp. and *Sambucus* spp.), some flowering bane species (*Aconitum* spp. and *Erigeron* spp.) as well as spindle tree species (*Euonymus* spp.) (Verkley, 1999; Rossman *et al.*, 2002). Other crops include olives (*Olea europea* L.), where it causes leaf anthracnose, leaf spot as well as leprosy, and ash trees (*Fraxinus*), as previously mentioned, where it was reported to cause coin cankers (Putnam and Adams, 2005; Rooney-Latham *et al.,* 2013; Romero *et al.,* 2016).

Identified hosts of *N. malicorticis* and *N. perennans* include *Rosaceae* species such as quince (*Cydonia oblonga* L.), hawthorn species (*Crataegus* spp.), Japanese flowering quince (*Chaenomeles japonica* (L.) Thunberg) and wild mountain ash species (*Sorbus* spp.) (Kienholz, 1939). More important hosts are stone fruit like peach, apricot, plum and cherry (*Prunus* spp.) on which the fungi successfully produce cankers (Kienholz, 1939). de Jong *et al.* (2001) isolated *N. malicorticis* from a rose stem canker.

MANAGEMENT OF BULL'S EYE ROT

Control measures for bull's eye rot differ between the causal species. The different *Neofabraea* species and isolates respond differently to fungicides. For effective management of bull's eye rot, control measures must focus on the particular characteristics of the causal *Neofabraea* species (Henriquez *et al.*, 2004; Spotts *et al.*, 2009; Wood and Fisher, 2017).

Cultural practices in the orchard

Bull's eye rot pathogens such as *N. malicorticis* and *N. perennans* that form cankers, which serves as their primary source of inoculum, can be managed with an informed pruning programme which removes these cankers in the orchard to prohibit new infections from taking place in the next season (Powell *et al.*, 1965). Removal of cankers significantly reduces infection pressure for the next season (Creemers, 1989). In the case of *N. perennans* cankers, orchards would benefit from a management programme for woolly apple aphids (Grove, 1990). As mentioned earlier, the aphids contribute to infection portals for *N. perennans* which leads to more cankers being produced and more inoculum present in the orchard (Dugan *et al*., 1993).

Increasing the fruit's natural resistance duration by harvesting at optimal maturity before fruit respiration increases will, in turn, reduce total lenticel size at harvest and susceptibility to *Neofabraea* infection (Creemers, 1989; Spotts, 1985). Fruit internal resistance decreases with the ripening process (Creemers, 1989). In a study by Henriquez *et al*. (2008), there was a significant increase in bull's eye rot disease of pears, caused by *N. perennans,* when the fruit was harvested later in the season than those harvested earlier in the same season. A recent study showed that fruit harvested one month earlier had an average decrease of 11% in

Neofabraea lenticel decay over three years with one season having a decrease as high as 77% (Børve *et al.*, 2013).

The higher incidence of bull's eye rot in late-harvested fruit is not only because of increasing fruit susceptibility but possibly a higher spore count and dispersal due to the cold-wet conditions that come with the winter season. Avoiding overhead irrigation can also reduce incidence. Overhead irrigation leads to increased release and dispersion of bull's eye rot spores due to splash-dispersal (Henriquez *et al.*, 2008). Increased periods of wetness can also lead to higher disease incidence in storage with the incidence increasing by 10% for every hour of wetness (Henriquez *et al.*, 2008).

Standard practices like minimising orchard density and planting orchard rows to maximise airflow to reduce wetness periods can minimise favourable conditions for the pathogen. Use of fungicides in controlling cankers seems dependent of the causal *Neofabraea* species. Henriquez *et al*. (2006) found copper sulphate to be effective against *N. vagabunda* cankers on pear trees, but Garton *et al*. (2019) reported low efficacy of available fungicides on limiting canker expansion and preventing new infections of *N. malicorticis*. This could be due to *N. vagabunda* being a weak canker pathogen and *N. malicorticis* an aggressive canker pathogen (Dugan *et al*., 1993; Henriquez *et al*., 2006; Aguilar *et al*., 2017). Proper orchard sanitation is also important especially for *N. vagabunda* infested orchards. Removal of pruning litter, fruit mummies and leaf litter are vital in reducing disease pressure of *N. vagabunda*. Weeds and pollinator trees such as crabapple, have been found as a source of inoculum for the *Neofabraea* pathogens and should be managed accordingly (Tan and Burchill, 1972; Grove, 1990; Rochefort, 2015; Köhl *et al*., 2018).

Fungicide control

Preharvest

Fungicide application is very important for bull's eye rot management. If the pathogen is present in an orchard, fungicides can protect infection sites from inoculum already present and which cultural practices could not eradicate. Early application of fungicides during spring and early summer from fruit set through fruit development can greatly reduce the early onset of bull's eye rot infection. Ziram, mancozeb and thiram are multi-site inhibiting dithiocarbamate contact fungicides that impede on the biochemical processes within the cell cytoplasm and mitochondria (Gullino *et al.,* 2010). Captan is a phthalimide fungicide that is a multi-site contact fungicide which inhibits fungal nitrogen respiration (Yang *et al*., 2011). In South Africa, mancozeb, thiram and captan are registered on apple for managing apple scab in a preharvest application, captan is also registered as a disinfectant in apple and pear packhouses against postharvest decay. Ziram is not registered for use on any crop (www.agri-intel.com). There is

a low risk of resistance development against dithiocarbonates and phthalimides due to their multi-site mode of action (Hahn, 2014).

Application of ziram as an orchard spray can reduce initial inoculum present in the season when applied at petal-fall and up to two weeks thereafter (Kienholz, 1956; Henriquez *et al.*, 2006). Moreover, applying ziram before or after high disease pressure events such as high rainfall or relative humidity reduces the number of dispersed spores and successful infection of the pathogen (Henriquez *et al.,* 2008). However, ziram's *in vitro* and *in vivo* efficacy proved moderately effective against *N. vagabunda* and *N. perennans* whilst poor efficacy was observed on *N. malicorticis* and *N. kienholzii* (Spotts *et al*., 2009). Recently, a study found ziram to be ineffective in reducing *N. perennans* or *N. kienholzii* disease incidence when, respectively, applied either 2 or 14 days before harvest (Aguilar *et al.,* 2018).

The fungicide, mancozeb, proved more effective as a preharvest spray than a postharvest curative dip application (Spotts *et al.,* 2009). There is a discrepancy in this fungicide's efficacy as mancozeb is highly effective against isolates *in vitro* but could not achieve a significant reduction in bull's eye rot disease incidence *in vivo* (Spotts *et al.*, 2009; Grantina-Levina, 2016). Application of thiram and captan on weekly intervals early in the season gives control of *N. vagabunda* and *N. malicorticis* lenticel decay (Powell *et al.*, 1965: Burchill and Edney, 1972). The fungicide captan has been recommended for application in the orchard for control of bull's eye rot in New Zealand due to its 14-day interval and recurring application throughout the season (Wood and Fisher, 2017). Applying two extra cover sprays with captan before harvest 12 days apart can effectively reduce the postharvest incidence of bull's eye rot (Ross and Lockhart, 1960).

Benzimidazole is a systemic fungicide group that inhibits the production of the β-tubulin-protein which in turn prevents microtubule formation during germ-tube elongation and hyphal growth (Davidse, 1995). The use of the benzimidazoles has been recommended due to the fungicide group's systemic action, which penetrates the fruit and enables it to reach the latent infections inside the lenticels (Creemers, 1989). Benzimidazole fungicides were first applied as a preharvest spray against bull's eye rot in the 1970's when the disease was responsible for 90% of fruit rot in Belgium. With the use of these fungicides, disease incidence was drastically reduced (Creemers, 1989). The application of thiabendazole and benomyl during the early season months proved effective in reducing *N. vagabunda* lenticel decay incidence in the United Kingdom (Burchill and Edney, 1972).

A recent study on the use of benomyl showed a decrease in efficacy against bull's eye rot fungi (Weber and Palm, 2010). Benomyl has effectively controlled *N. vagabunda* and *N. perennans* in Germany since 1970 as an orchard spray, but both pathogens developed resistance to the fungicide due to prolonged use (Weber and Palm, 2010). The fungicide thiabendazole, which has been used routinely in the United States against bull's eye rot,

experienced some resistance development in *N. vagabunda* in France in 1997 (Bompeix and Cholodowski-Faivre, 1997). Contrary to these findings, thiabendazole gives good control of all *Neofabraea* species *in vitro* and *in vivo* (Spotts *et al.*, 2009). However, Aguilar *et al*. (2018) found the benzimidazole thiophanate-methyl to significantly reduce *N. perennans* and *N. kienholzii* disease incidence when it was applied two days before harvest.

Thiophanate-methyl has been used in Germany against bull's eye rot, however resistance development against the fungicide has been reported in *N. vagabunda* and *N. perennans* (Weber and Palm, 2010). Cameldi *et al*. (2016) found thiophanate-methyl to be highly effective against *N. vagabunda* in Italy when it was applied 14 or 7 days before harvest, proving it can still be effective against *Neofabraea* populations with low resistance to benzimidazoles.

Thiabendazole, benomyl and thiophanate-methyl are all benzimidazoles and should be used with caution due to the high risk of resistance development in pathogens (Weber and Palm, 2010). Wood and Fisher (2017) treated the fruit with the fungicide carbendazim before inoculating it with *N. vagabunda*, which significantly reduced the incidence of bull's eye rot compared to untreated inoculated fruit. Benzimidazoles are single-site inhibiting fungicides and a single point mutation in the fungal β-tubulin gene can lead to complete resistance against the fungicide (Hahn, 2014). Managing of resistance development on fruit crop can be achieved by mixing or alternating fungicides with a high-risk for resistance development with low-risk fungicides (Russell, 1995). Benomyl is registered for use preharvest in South Africa against various diseases of apple. Thiabendazole is registered against postharvest decay only as a preharvest application, but not recommended for use on fruit going to the export market. Carbendazim is not registered for use on pome fruit in South Africa (www.agri-intel.com).

Strobilurin fungicides are an effective group of fungicides against bull's eye rot and many other crop diseases. Strobilurins are quinone outside inhibitors (QoI), which impede on mitochondrial respiration (Fernández-Ortuño *et al*., 2008). QoIs are also high-risk fungicides for resistance development due to their single-site action (Ding *et al*., 2019). Therefore, a combination of pyridine-carboxamide fungicide consisting of high-risk pyraclostrobin and low-risk boscalid proved to control all four *Neofabraea* species *in vitro* and was proposed for use in spring orchard sprays (Spotts *et al.*, 2009).

Neofabraea malicorticis, in particular, has shown high sensitivity towards a mixture of pyraclostrobin and boscalid *in vitro* (Grantina-Levina, 2016). However, Aguilar *et al*. (2018) found the application of pyraclostrobin and boscalid inadequate in controlling bull's eye rot incidence when it was applied shortly before harvest. A mixture of pyraclostrobin and boscalid is not available in South Africa, only a mixture of pyraclostrobin and dithianon, which is registered as a preharvest spray against scab and powdery mildew. Dithianon is a multi-site anthra-quinone fungicide and has a low risk for resistance development. Trifloxystrobin effectively inhibits germination of *N. perennans* on cankers and protects the fruit from

N. vagabunda infection (Henriquez *et al.*, 2006; Wood and Fisher, 2017). Trifloxystrobin is registered against apple scab and is recommended to be applied at green-tip until 75% flowering or at early fruit set, although it is required that mancozeb be included in the spray programme to avoid resistance development.

Although the use of copper fungicides are under heavy debate due to its high levels of toxicity to humans, animals, beneficial insects and the environment, applying these fungicides at the beginning of autumn can prevent pathogen dispersal and subsequent infection during the tree's dormant phase (Kienholz, 1939). Copper ions react with critical exudates produced by the pathogen breaking them down making the survival of the pathogen impossible (McCallan, 1949). The application of copper sulphate on *N. vagabunda*-produced cankers on pear trees can successfully reduce sporulation and was effective for up to one month after application (Henriquez *et al.*, 2006). Lime sulphur has been tested as a possible organic product and showed to be effective in controlling *N. vagabunda in vitro* (Wood and Fisher, 2017).

Postharvest

The control of postharvest diseases benefits from the application of fungicides postharvest. Advantages of applying fungicides postharvest include no selection pressure from infection sources, better coverage of fruit with fungicide, reduced risk of a fungal population developing resistance and less fungicide used than a preharvest orchard spray (Creemers, 1989). Applying fungicides postharvest against bull's eye rot is strictly a curative action as these pathogens infect fruit in the orchard and do not spread from one fruit to another in storage (Dugan *et al.*, 1993). Very few fungicides are registered and used on pome fruit postharvest due to strict maximum residue levels and active ingredients allowed for the export markets. Postharvest application of fungicides includes dipping, drenching, spraying and thermal fogging of fruit. Postharvest fungicide dipping of fruit is usually applied protectively against postharvest diseases such as *Penicillium* spp. and *Botrytis cinerea* (Leibinger *et al.*, 1997). But with latent infections, fungicide application would be curative or inhibitory.

The anilinopyrimidine fungicide, pyrimethanil, is a reduced-risk broad-spectrum fungicide that inhibits methionine biosynthesis and in turn impedes hyphal growth and germ tube elongation (Milling and Richardson, 1995; Rosslenbroich and Stuebler, 2000). Pyrimethanil was first registered in 2004 in the United States and is effective against a wide range of postharvest diseases. It has been used extensively in several countries on various crops (Sholberg *et al*., 2005). Pyrimethanil has proven effective in controlling all four bull's eye rot species on pear fruit when applied as a dip (Spotts *et al.*, 2009). Moreover, it also showed high efficacy in controlling *N. perennans* and *N. kienholzii* on apple cv. Fuji, where it effectively

controlled bull's eye rot incidence when applied as a dip treatment before storage (Aguilar *et al.,* 2018).

The contact fungicide fludioxonil is a phenylpyrrole and inhibits the transport-associated phosphorylation process of glucose and glycerol synthesis, thus preventing spore germination, germ-tube elongation and mycelial growth (Rosslenbroich and Stuebler, 2000). Fludioxonil effectively controls bull's eye rot caused by *N. vagabunda* when applied as a drench (Lolas *et al.*, 2016). Spotts *et al.* (2009) found a fludioxonil dip to be effective against *N. vagabunda* as well as *N. malicorticis* on pear fruit. Fludioxonil is however, not effective against *N. perennans* and *N. kienholzii* on apple fruit when applied as a dip (Aguilar *et al.,* 2018).

Drench treatments of fruit with fludioxonil shortly after harvest, as well as a mixture of fludioxonil and thiabendazole, have shown to significantly reduce bull's eye rot incidence in fruit stored for a period of two to three months (Lolas *et al.*, 2016). Dip application of the benzimidazoles, thiophanate-methyl and thiabendazole respectively, controls all four bull's eye rot species on inoculated pear fruit (Spotts *et al.*, 2009). Only thiabendazole has been tested on bull's eye rot infected apple fruit, and effectively controlled incidence of *N. vagabunda, N. perennans* and *N. kienholzii* (Bertolini *et al.*, 1995; Aguilar *et al.,* 2018). However, thiabendazole was found to be ineffective in controlling bull's eye rot on 'd'Anjou' pear fruit when applied as a postharvest dip before fruit went into storage (Lennox *et al*., 2004).

Pyrimethanil, fludioxonil and thiabendazole are registered for use postharvest against bull's eye rot (*N. malicorticis*, *N. perennans*, *N. vagabunda* and *N. kienholzii*) on pome fruit in the United States (Aguilar *et al*., 2018). All three of these fungicides are registered for postharvest use on pome fruit in South Africa (www.agri-intel.com).

An alternative method of applying fungicides postharvest is thermo-fogging, also known as fumigation or thermo-nebulisation. A fog is produced by vaporizing or atomizing the fungicide inside a machine and the vapour then rapidly condenses when exiting the machine after mixing with the cooler outside air. With this method, fungicide is directly applied to fruit in cold storage and can be re-applied throughout the storage term (Delele *et al*., 2012). There are however challenges experienced with fogging, inconsistency in treatments due to the non-uniform distribution of fungicide deposition within storage as well as between fruit, and loss of fungicide particles to non-target materials such as the fruit bins have been reported (Brown and Craig, 1989; Delele *et al*., 2014). However, optimising parameters like the air circulation rate, circulation intervals and the stacking pattern of the bins can increase fungicide uniformity significantly (Delele *et al*., 2014). Bertolini *et al.* (1995) found inverting the fruit container halfway through application also improved deposition uniformity.

Pyrimethanil, fludioxonil and thiabendazole have been applied as fog treatments and delivered positive results against bull's eye rot. Pyrimethanil and fludioxonil controlled

N. perennans and *N. kienholzii* on apple cv. Fuji (Aguilar *et al.,* 2018). Bertolini *et al.* (1995) compared thiabendazole efficacy against *N. vagabunda* on apple fruit as a dip and fog treatment and found that the fog treatment achieved better control.

The synthetic cyclic olefin, 1-methylcyclopropene (1-MCP) is used on stored apple to extend fruit firmness in storage and increase fruit marketability (Saftner *et al*., 2003). It is applied as a fumigant and blocks the ethylene-binding receptor on fruit inhibiting ethylene production. More importantly, it has shown the ability to delay the decay of fruit in long term storage (Cao and Zheng, 2010; Zhang *et al*., 2012). 1-MCP effectively reduced bull's eye rot incidence, caused by *N. vagabunda*, on 'Cripps Pink' apples by delaying fruit senescence and thus extending fruit resistance (Cameldi *et al*., 2016). Not only has 1-MCP been effective on apple but it also reduced bull's eye rot on 'd'Anjou' pear fruit in long term cold storage (Spotts *et al*., 2007).

Use of multiple fungicides postharvest is not as necessary as in preharvest application. Fungicides are only applied once or twice postharvest, whereas they are applied multiple times during the preharvest stage. A postharvest application programme would rather rotate fungicides year-to-year to avoid resistance development or loss of efficacy.

Hot water treatment

This method is of special importance in organic apple fruit production as this is the only viable treatment these growers have against postharvest diseases (Maxin *et al.*, 2005; Mbili, 2015). Warm water treatment of fruit for specifically targeting bull's eye rot has been shown to be effective (Maxin *et al.*, 2005).

Treating fruit by dipping in hot water (49-53°C) for 120-180 seconds can reduce disease incidence by 83% (Maxin *et al.*, 2005). Treating fruit by rinsing has had similar success to dipping (Maxin *et al.*, 2012). Rinsing fruit with 55°C water for 25 seconds can effectively reduce bull's eye rot incidence (Maxin *et al.*, 2012). However, treating the fruit with such high-temperature water leads to physiological skin disorders. Hot water dipping fruit at 50°C or higher for 3 minutes or longer leads to heat-damaged fruit (Maxin *et al.*, 2005; Maxin *et al.,* 2012). Hot water rinsing fruit at temperatures higher than 60°C even for just 25 seconds, leads to significant heat damage (Maxin *et al.*, 2012). Although heat damage does not affect the internal qualities of the fruit, such as firmness, starch and sugar content, it does lead to superficial scald and damaged parts that are susceptible to infection by opportune wound fungi such as *Penicillium* (Maxin *et al.*, 2012). Between the two application methods, hot water dipping is more effective in controlling bull's eye rot and other postharvest diseases, but hot water rinsing is better for integration into pack house lines due to shorter application time required for controlling disease (Maxin *et al.*, 2012).

Heat treating fruit without water has also shown promise, especially when combined with controlled atmosphere storage. Heat treating fruit at 40°C for a minimum of 24 hours just before storage can significantly reduce bull's eye rot incidence by up to as much as 80% (Tahir *et al.*, 2009). Increased resistance of fruit against *Neofabraea* sp. can also be induced by heat treatment (Tahir *et al.*, 2009). This is possibly due to a delayed ripening and therefore softening of fruit which impedes on the pathogens required conditions for infection development (Janisiewicz *et al.,* 2003). Another effect of hot water treatment is the melting of the fruit's natural wax that fills surface microcracks, covering germinated spores, hyphae and conidia, and thus preventing inoculation and growth in storage by exposing the latent fungi (Lurie *et al.*, 1995; Tahir *et al.*, 2009).

Storage conditions

Storing fruit at specific environmental conditions can prevent the ripening process by retarding fruit ripening and senescence. These specific environmental conditions for storing fruit are known as controlled atmosphere (CA) storage and allows the fruit to be kept in storage for long periods thus allowing for an increased marketability time.

Ideal storage conditions suggested for most apple cultivars include cooling of fruit to -0.5°C within 48 hours after harvest and keeping fruit at that temperature for the remainder of storage. Furthermore, a high RH (90% - 95%) keeps fruit moisture loss to a minimum and a gas regime of 3.0% O_2 and 1.0% CO_2 delays fruit ripening (Van Bodegom *et al*., 2013).

Fruit respires during ripening and in this process, stored organic materials are metabolised. During metabolisation, O_2 gets taken up by fruit and CO_2 is produced. Maintaining low external gas levels slows down the ripening process. Reducing O_2 levels in the atmosphere means less O_2 uptake and thus slower fruit metabolism. However, a specific gas composition must be maintained to avoid the negative effects that can occur under a low O_2 atmosphere, such as browning of fruit and superficial scald. CA storage conditions can also prevent the incidence of postharvest diseases. *In vitro* conditions with low CO_2 (5-10%) and even lower O_2 (0-5%) levels leads to a significant reduction in growth for *N. vagabunda* (Lockhart, 1967). This is due to the fact that pectolytic enzymes produced by the pathogen are reduced by low CO_2 levels (Edney, 1964).

Low humidity levels have an inhibiting effect on the pathogen as well as closes the lenticels (Bompeix, 1978). Bompeix (1978) tested CA conditions of 3% O_2 and 5% CO_2 at 1°C and found it did not affect the *in vivo* mycelial growth of *N. vagabunda* or *N. malicorticis*. Furthermore, there was no significant difference between the growth rate of *N. vagabunda* on unripened and senescent fruit when stored in CA at 20°C (Bompeix, 1978). Tahir *et al.* (2009) found that combining heat treatment with CA storage at 2.0% O_2 and 2.0% CO_2 can significantly reduce bull's eye rot incidence. Using CA storage for reducing postharvest decay

does not inhibit pathogen development, it merely reduces the tempo of decay development increasing marketability time of stored fruit (Creemers, 1989). However, changing CA conditions for postharvest decay management is not always possible and conditions for decay control could negatively affect fruit quality.

Biological control agents

Overuse of chemical fungicides in the agricultural industry has led to several concerns related to environmental pollution, human health implications and the development of fungicide resistant pathogens (Ippolito and Nigro, 2000). Leibinger *et al.* (1997) studied the application of antagonistic organisms as a preharvest orchard spray and its ability to control latent *Neofabraea* spp. infection. They found that applying a mixture of *Bacillus subtilis* Ehrenberg and *Aureobasidium pullulans* de Bary early in the growing season could reduce *Neofabraea* infections that would occur later in the season, they reported significantly lower bull's eye rot incidence on treated fruit after storage compared to the untreated control (Leibinger *et al.*, 1997).

More recently, Vanwalleghem *et al.* (2016) tested several biological control organisms against *Neofabraea* spp., including a registered preharvest product of *A. pullulans*. The biological agents were applied as a curative action via thermo-fogging on inoculated apple fruit. Vanwalleghem *et al.* (2016) did not mention which biological control agents were used but several agents, including *A. pullulans*, reduced bull's eye rot incidence to less than 25% (Vanwalleghem *et al.,* 2016). The compounds alkylresorcinols, which are found naturally in the outer layer of cereal grains, has been tested against *Neofabraea* infections (Tahir *et al.*, 2014). These compounds showed curative antifungal abilities by reducing bull's eye rot incidence by as much as 77% in artificially inoculated fruit (Tahir *et al.*, 2014).

Essential oils have also shown promise in their antifungal capabilities. The use of garlic extract, when applied as a volatile, inhibits *N. vagabunda* mycelial growth *in vitro* (Daniel *et al.*, 2014). However, the *in vivo* capabilities of the extracts showed no inhibition of bull's eye rot incidence when applied as a curative treatment (Daniel *et al.,* 2015). Mbili (2015) tested lemon, lime and lemongrass oil as well as mixtures of these oils against bull's eye rot, or more specifically *N.* vagabunda, as a volatile application. She found all the oils to significantly reduce *N. vagabunda* incidence by at least 90% when the fruit was kept in CA storage. The lime essential oil was the most effective, inhibiting incidence by at least 96% (Mbili, 2015). Using essential oils as treatments for postharvest fungal decay costlier than chemical fungicides. But, applying mixtures of oils instead of each oil individually would reduce the cost and furthermore, their benefits in terms of human health and environmental sustainability, support their use in postharvest apple treatment against decay (Mbili, 2015).

CONCLUSION

Neofabraea infection on apples (c.o. Bull's eye rot of apples) can result in significant levels of fruit decay in storage. In the 2010/2011 season, incidence levels varied from 0-73% throughout the Western Cape province of South Africa (Den Breeyen and Lennox, 2012). The sporadic occurrence of this disease and the absence of routine management strategies make this disease a phytosanitary risk to export markets. In 2013 and 2018 China temporarily closed off its apple import market from Chile and New Zealand due to bull's eye rot infected apples. South African apple production is largely aimed at the export market with 440 000 tonnes exported in the 2015/2016 season amounting to a net worth of R4.6 billion (DAFF, 2018). Abiding by the phytosanitary requirements of other countries and reducing the risk of embargo's due to fruit decay is of the utmost importance to the apple industry. The most important export markets for South African apples include Africa, Asia and Europe. The aim of this study aimed at developing an integrated management strategy for *N. vagabunda* of apple in South Africa.

Bull's eye rot causal species require different methods of management as the species differ in epidemiology as well as susceptibility to fungistatic compounds. The objectives of this study included the identification of the *Neofabraea* spp. causing bull's eye rot in the Western Cape of South Africa, and to examine which key cultivars in the growing region are most susceptible and require management attention (Chapter 2).

Fungicide control is an important management strategy for the bull's eye rot disease. Preharvest application prevents infection of fruit and postharvest application reduces or inhibits disease development in already infected fruit. A reliable orchard spray programme with readily available fungicides will help manage the disease and prevent further infection of apples and apple trees. Postharvest application of fungicides is more complicated due to bull's eye rot causing *Neofabraea* spp. that react differently to fungicides and there are currently no fungicides registered against the disease in South Africa. The objective was to evaluate postharvest fungicides, that are registered on pome fruit, against *N. vagabunda* (isolated in South African orchards) and their ability to inhibit bull's eye rot disease incidence (Chapter 3).

REFERENCES

Abeln, E.C.A., De Pagter, M.A. and Verkley, G.J.M. 2000. Phylogeny of *Pezicula*, *Dermea* and *Neofabraea* inferred from partial sequences of the nuclear ribosomal RNA gene cluster. Mycologia 92: 685–693.

Aguilar, C.G., Mazzola, M. and Xiao, C.L. 2017. Timing of apple fruit infection by *Neofabraea perennans* and *Neofabraea kienholzii* in relation to bull's-eye rot development in stored apple fruit. Plant Disease 101: 800-806.

Aguilar, C.G., Mazzola, M. and Xiao, C.L. 2018. Control of bull's eye rot of apple caused by *Neofabraea perennans* and *Neofabraea kienholzii* uisng pre- and postharvest fungicides. Plant Disease 102: 905-910.

Bertolini, P., Guarnieri, A. and Venturi, P. 1995. Postharvest fog treatment of apples: deposition patterns and control of *Phlyctema vagabunda* and superficial scald. Crop Protection 14: 345-348.

Blum, L.E.B., Amarante, C.V.T. and Valdebenito-Sanhueza, R.M. 2005. Postharvest application of the yeast *Cryptococcus laurentii* reduces apple rots. Acta Horticulturae 682: 2109-2114.

Bompeix, G. 1978. The comparative development of *Pezicula alba* and *P. malicorticis* on apples and in vitro (air and controlled atmosphere). Journal of Phytopathology 91: 97-109.

Bompeix, G. and Cholodowski-Faivre, D. 1997. The use of natural plant products against scald, *Botrytis* sp., *Gloeosporium* sp., *Penicillium* sp. and *Rhizopus* sp. Pages 99-104 in: Non-Conventional Methods for the Control of Postharvest Disease and Microbiological Spoilage. Bologna, Italy.

Børve, J., Røen, D. and Stensvand, A. 2013. Harvest time influences incidence of storage diseases and fruit quality in organically grown "Aroma" apples. European Journal of Horticultural Science 78: 232-238.

Brown, G.E. and Craig, J.O. 1989. Effectiveness of aerosol fungicide applications in the degreening room for control of citrus fruit decay. Proceedings of the Florida State Horticultural Society 102: 181-185.

Burchill, R.T. and Edney, K.L. 1972. An assessment of some new treatments for the control of rotting of stored apples. Annals of Applied Biology 72: 249-255.

Cameldi, I., Neri, F., Ventrucci, D., Ceredi, G., Muzzi, E. and Mari, M. 2016. Influence of harvest date on bull's eye rot of 'Cripps Pink' apple and control chemical strategies. Plant Disease 100: 2287-2293.

Cao, S. and Zheng, Y. 2010. Effect of 1-methylcyclopropene on anthracnose rot caused by *Colletotrichum acutatum* and disease resistance in loquat fruit. Journal of the Science of Food and Agriculture 90: 2289-2294.

Chen, C., Verkley, G.J.M., Sun, G., Groenewald, J.Z. and Crous, P.W. 2016. Redefining common endophytes and plant pathogens in *Neofabraea*, *Pezicula*, and related genera. Fungal Biology 120: 1291-1322.

Childs, L. 1929. The relation of woolly apple aphids to perennial canker infection with other notes on the disease. Oregon State Agricultural College. Station Bulletin 243: 5-31.

Clinton, G.P. 1902. Apple rots in Illinois. University of Illinois (Urbana-Champaign campus). Agricultural Experiment Station: 69.

Cordley, A.B. 1900. Some observations on apple tree cankers. Botanical Gazette 30: 48-58.

Corke, A.T.K. 1956. Bitter rot of apples: II. Seasonal variations in the development and sporulation of cankers of *Gloeosporium* spp. inoculated into apple branches. Journal of Horticultural Science 31: 272-283.

Creemers, P. 1989. Chemical control of parasitic storage diseases on apple and pear. Acta Horticulturae 258: 645-653.

Cunnington, J.H. 2004. Three *Neofabraea* species on pome fruit in Australia. Australasian Plant Pathology 33: 453-454.

Department of Agriculture Forestry and Fisheries (DAFF) of South Africa: Abstract of Agricultural Statistics. 2018. www.daff.gov.za.

Daniel, C.K., Lennox, C.L. and Vries, F.A. 2014. *In vivo* application of garlic extracts in combination with clove oil to prevent postharvest decay caused by *Botrytis cinerea*, *Penicillium expansum* and *Neofabraea alba* on apples. Postharvest Biology and Technology 99: 88-92.

Daniel, C.K., Lennox, C.L. and Vries, F.A. 2015. *In vitro* effects of garlic extracts on pathogenic fungi *Botrytis cinerea*, *Penicillium expansum* and *Neofabraea alba*. South African Journal of Science 111: 1-8.

Davidse, L.C. 1995. Biochemical and molecular aspects of the mechanisms of action of benzimidazoles, N-phenylcarbamates and N-phenylformamidoximes and the mechanisms of resistance to these compounds in fungi. Modern selective fungicides.

de Jong, S.N., Lévesque, C.A., Verkley, G.J.M., Abeln, E.C.A., Rahe, J.E. and Braun, P.G. 2001. Phylogenetic relationships among Neofabraea species causing tree cankers and bull's-eye rot of apple based on DNA sequencing of ITS nuclear rDNA, mitochondrial rDNA, and the β-tubulin gene. Mycological Research 105: 658-669.

Den Breeyen, A. and Lennox, C.L. 2012. Towards the development of a rapid detection method for bull ' s eye rot in apples. Proceedings of the 9th International IOBC-WPRS Workshop on Pome Fruit Diseases 84: 5-8.

Den Breeyen, A., Rochefort, J., Meitz-Hopkins, J.C., Russouw, A. and Lennox, C.L. 2019. Preharvest detection and postharvest incidence of *Neofabraea vagabunda* on 'Cripps Pink' apples in South Africa. Plant Disease [In press].

Delele, M.A., Vorstermans, B., Creemers, P., Tsige, A.A., Tijskens, E., Schenk, A., Opara, U.L., Nicolaï, B.M. and Verboven, P. 2012. Investigating the performance of thermonebulisation fungicide fogging system for loaded fruit storage room using CFD model. Journal of Food Engineering 109: 87-97.

Ding, S., Halterman, D.A., Meinholz, K. and Gevens, A.J. 2019. Distribution and stability of quinone outside inhibitor fungicide resistance in populations of potato pathogenic *Alternaria* spp. in Wisconsin. Plant Disease 103: 2033-2040.

Dugan, F.M., Grove, G.G. and Rogers, J.D. 1993. Comparative studies of *Cryptosporiopsis curvispora* and *C. perennans*. I. Morphology and pathogenic behaviour. Mycologia 85: 551-564.

Edney, K.L. 1956. The rotting of apples by *Gloeosporium perennans* Zeller and Childs. Annals of Applied Biology. 44: 113-128.

Edney, K.L. 1958. Observations on the infection of Cox's Orange Pippin apples by *Gloeosporium perennans* Zeller and Childs. Annals of Applied Biology 46: 622-629.

Edney, K.L. 1964. A comparison of the production of extracellular enzymes and rotting of apples by *Pezicula alba* and *P. malicorticis*. Transactions of the British Mycological Society 47: 215-225.

Edney, K.L. 1974. *Pezicula malicorticis*: Factors affecting spore germination and invasion of apple fruit. Transactions of the British Mycological Society 62: 25-34.

Edney, K.L., Tan, A.M., and Burchill, R.T. 1977. Susceptibility of apples to infection by *Gloeosporium album*. Annals of Applied Biology 86: 129-132.

Fernández-Ortuño, D., Torés, J.A., De Vicente, A. and Pérez-García, A. 2008. Mechanisms of resistance to QoI fungicides in phytopathogenic fungi. International Microbiology 11: 1-9.

Fisher, D.F. 1925. A new apple rot disease “Bull’s eye rot” - caused by *Gloeosporium* Zeller and Childs. Proceedings Washington State Horticultural Association 21: 44-48.

Food and Agricultural Organization of the United Nations (FAOUN) – Production statistics, 2018.

Garton, W.J., Mazzola, M., Alexander, T.R. and Miles, C.A. 2019. Efficacy of fungicide treatments for control of anthracnose canker in young cider apple trees in western Washington. HortTechnology 29: 35-40.

Grantina-Levina, L. 2016. Fungi causing storage rot of apple fruit in integrated pest management system and their sensitivity to fungicides. Rural Sustainability Research 34: 2-11.

Grove, G.G. 1990. Anthracnose and perennial canker. Pages 36-38 in: Compendium of Apple and Pear Diseases. (A. L. Jones and H. S. Aldwinckle, eds.) St. Paul, Minnesota: The American Phytopathological Society.

Grove, G.G., Dugan, F. M. and Boal, R. J. 1992. Perennial canker of apple: seasonal host susceptibility, spore production, and perennation of *Cryptosporiopsis perennans* in infected fruit in eastern Washington. Plant Disease 76: 1109-1114.

Gullino, M.L., Tinivella, F., Garibaldi, A., Kemmitt, G. M., Bacci, L. and Sheppard, B. 2010. Mancozeb - Past, present, and future. Plant Disease 94: 1076-1087.

Guthrie, E.J. 1959. The occurrence of *Pezicula alba* sp.nov. and *P. malicorticis*, the perfect states of *Gloeosporium album* and *G. perennans*, in England. Transactions of the British Mycological Society 42: 502-506.

Hahn, M. 2014. The rising threat of fungicide resistance in plant pathogenic fungi : *Botrytis* as a case study. Journal of Chemical Biology 7: 133-141.

Henriquez, J.L., Sugar, D. and Spotts, R.A. 2004. Etiology of bull's eye rot of pear caused by *Neofabraea* spp. in Oregon, Washington, and California. Plant Disease 88: 1134-1138.

Henriquez, J.L., Sugar, D. and Spotts, R.A. 2006. Induction of cankers on pear tree branches by *Neofabraea alba* and *N. perennans*, and fungicide effects on conidial production on cankers. Plant Disease 90: 481-486.

Henriquez, J.L., Sugar, D. and Spotts, R. A. 2008. Effects of environmental factors and cultural practices on bull's eye rot of pear. Plant Disease 92: 421-424.

Hortova, B., Novotny, D. and Erban, T. 2014. Physiological characteristics and pathogenicity of eight *Neofabraea* isolates from apples in Czechia. European Journal of Horticultural Science 79: 327-334.

Ippolito, A. and Nigro, F. 2000. Impact of preharvest application of biological control agents on postharvest diseases of fresh fruits and vegetables. Crop Protection 19: 715-723.

Janisiewicz, W.J., Leverentz, B., Conway, W.S., Saftner, R.A., Reed, A.N. and Camp, M.J. 2003. Control of bitter rot and blue mold of apples by integrating heat and antagonist treatments on 1-MCP treated fruit stored under controlled atmosphere conditions. Postharvest Biology and Technology 29: 129-143.

Johnston, P.R., Seifert, K.A. and Stone, J.K. 2014. Recommendations on generic names competing for use in *Leotiomycetes* (*Ascomycota*). International Mycological Association 5: 91-120.

Kienholz, J.R. 1939. Comparative study of the apple anthracnose and perennial canker fungi. Journal of Agricultural Research 59: 635-665.

Kienholz, J.R., 1956. Control of bull's eye rot on apple and pear fruits. Plant Disease Reporter 40: 872-877.

Kingsnorth, J., Perrine, J., Berrie, A. and Saville, R. 2017. First report of *Neofabraea kienholzii* causing bull's eye rot of apple in the UK. New Disease Reports 36: page 15.

Köhl, J., Wenneker, M., Groenenboom-de Haas, B.H., Anbergen, R., Goossen-van de Geijn, H.M., Lombaers-van der Plas, C.H., Pinto, F.A.M.F. and Kastelein, P. 2018. Dynamics of postharvest pathogens *Neofabraea* spp. and *Cadophora* spp. in plant residues in Dutch apple and pear orchards. Plant Pathology 67: 1264-1277.

Lattanzio, V., Di Venere, D., Linsalata, V., Bertolini, P., Ippolito, A. and Salerno, M. 2001. Low temperature metabolism of apple phenolics and quiescence of *Phlyctema vagabunda*. Journal of Agricultural and Food Chemistry 49: 5817-5821.

Leibinger, W., Breuker, B., Hahn, M. and Mendgen, K. 1997. Control of postharvest pathogens and colonization of the apple surface by antagonistic microorganisms in the field. Phytopathology 87: 1103-1110.

Lennox, C.L., Spotts, R.A. and Booyse, M. 2004. Incidence of postharvest decay of 'd'Anjou' pear and control with a thiabendazole drench. Plant Disease 88: 474-478.

Lockhart, C.L. 1967. Influence of controlled atmospheres on the growth of *Gloeosporium album* in vitro. Canadian Journal of Plant Science 47: 649-651.

Lolas, M., Díaz, G., Mendez, R., Cáceres, M. and Neubauer, L. 2016. Evaluation of the efficacy of fungicide fludioxonil in the postharvest control of bull's eye rot (*Neofabraea alba*) in Chile. Acta Horticulturae 1144: 461-464.

Lurie, S., Othman, S. and Borochov, A. 1995. Effects of heat treatment on plasma membrane of apple fruit. Postharvest Biology and Technology 5: 29-38.

Mari, M., Di Francesco, A. and Bertolini, P. 2014. Control of fruit postharvest diseases: old issues and innovative approaches. Stewart Postharvest Review 10: 1-4.

Maxin, P., Huyskens-Keil, S., Klopp, K. and Ebert, G. 2005. Control of postharvest decay in organic grown apples by hot water treatment. Acta Horticulturae 682: 2153-2158.

Maxin, P., Weber, R.W.S., Pedersen, H.L. and Williams, M. 2012. Control of a wide range of storage rots in naturally infected apples by hot-water dipping and rinsing. Postharvest Biology and Technology 70: 25-31.

Mbili, N. 2015. Management of postharvest diseases of apples using essential oils. PhD Thesis, Stellenbsoch University, Stellenbosch, South Africa. Thesis number 97691.

McCallan, S.E.A. 1949. The nature of the fungicidal action of copper and sulfur. The Botanical Review 15: 629-643.

Michalecka, M., Bryk, H., Poniatowska, A. and Puławska, J. 2015. Identification of *Neofabraea* species causing bull's eye rot of apple in Poland and their direct detection in apple fruit using multiplex PCR. Plant Pathology 65: 643-654.

Michalecka, M., Bryk, H., Poniatowska, A., Seliga, P. and Puławska, J. 2016. Identification and characterization of *Neofabraea* fungi causing Bull's eye rot on apple in Poland. Acta Horticulturae 1144:183-188.

Milling, R.J. and Richardson, C.J. 1995. Mode of action of the anilino-pyrimidine fungicide pyrimethanil. 2. Effects on enzyme secretion in *Botrytis cinerea*. Pesticide Science 45: 43-48.

Neri, F., Cappellin, L., Aprea, E., Biasioli, F., Gasperi, F., Spadoni, A. and Cameldi, I. 2019. Interplay of apple volatile organic compounds with *Neofabraea vagabunda* and other postharvest pathogens. Plant Pathology 68: 1033-1232.

Pešicová, K., Kolařík, M., Hortová, B. and Novotný, D. 2017. Diversity and identification of *Neofabraea* species causing bull's eye rot in the Czech Republic. European Journal of Plant Pathology 147: 683-693.

Powell, D., Janson, B. and Sharvelle, E.G. 1965. Page 26 in: Diseases of apples and pears in the Midwest. North Central Regional Extension Publication No. 16. Cooperative Extension Service Circular 909 University of Illinois Urbana, USA.

Putnam, M.L. and Adams, G.C. 2005. *Phlyctema vagabunda* causes coin canker of ash (*Fraxinus* spp.) in North America. Plant Disease 89: 773.

Rochefort, J. 2015. Epidemiology and etiology of *Neofabraea* species causing lenticel decay of pome fruit in the Western Cape of South Africa. MSc Thesis, Stellenbosch University, Stellenbosch, South Africa. Thesis number 100634.

Romero, J., Raya, M.C., Roca, L.F., Moral, J. and Trapero, A. 2016. First report of *Neofabraea vagabunda* causing branch cankers on olives in Spain. Plant Disease 100: 527-527.

Rooney-Latham, S., Gallegos, L.L., Vossen, P.M. and Gubler, W.D. 2013. First Report of *Neofabraea alba* causing fruit spot on olive in North America. Plant Disease 97: 1384.

Ross, R.G. and Lockhart, C.L. 1960. *Gloeosporium album* rot and storage scab of apples. Canadian Plant Disease Survey 40: 14-15.

Rosslenbroich, H.J. and Stuebler, D. 2000. *Botrytis cinerea* - history of chemical control and novel fungicides for its management. Crop Protection 19: 557-561.

Rossman, A.Y., Castlebury, L.A., Adams, G.C. and Putnam, M.L. 2002. *Phlyctema vagabunda* isolated from coin canker of ash trees in Michigan. Plant Disease 86: 442.

Russell, P.H. 1995. Fungicide resistance: occurence and management. The Journal of Agricultural Science 124: 317-323.

Saftner, R.A., Bai, J., Abbott, J.A. and Lee, Y.S. 2003. Sanitary dips with calcium propionate, calcium chloride, or a calcium amino acid chelate maintain quality and shelf stability of fresh-cut honeydew chunks. Postharvest Biology and Technology 29: 257-269.

Sharples, R.O. 1959. Observations on the perfect state of *Gloeosporium perennans* in England. Transactions of the British Mycological Society 42: 507-512.

Sholberg, P.L., Bedford, K. and Stokes, S. 2005. Sensitivity of *Penicillium* spp. and *Botrytis cinerea* to pyrimethanil and its control of blue and gray mold of stored apples. Crop Protection. 24: 127-134.

Soto-Alvear, S., Lolas, M., Rosales, I.M., Chavez, E.R. and Latorre, B.A. 2013. Characterization of the bull's eye rot of apple in Chile. Plant Disease 97: 485-490.

Spotts, R.A. 1985. Effect of preharvest pear fruit maturity on decay resistance. Plant Disease 69: 388-390.

Spotts, R.A., Cervantes, L.A. and Mielke, E.A. 1999. Variability in postharvest decay among apple cultivars. Plant Disease 83: 1051-1054.

Spotts, R.A., Sholberg, P.L., Randall, P., Serdani, M. and Chen, P.M. 2007. Effects of 1-MCP and hexanal on decay of 'd'Anjou' pear fruit in long-term cold storage. Postharvest Biology and Technology 44: 101-106.

Spotts, R.A., Seifert, K.A., Wallis, K.M., Sugar, D., Xiao, C.L., Serdani, M. and Henriquez, J.L. 2009. Description of *Cryptosporiopsis kienholzii* and species profiles of *Neofabraea* in major pome fruit growing districts in the Pacific Northwest USA. Mycological Research 113: 1301–1311.

Tahir, I.I., Johansson, E. and Olsson, M.E. 2009. Improvement of apple quality and storability by a combination of heat treatment and controlled atmosphere storage. HortScience 44: 1648-1654.

Tahir, I.I., Ahmadi-Afzadi, M., Nybom, H. and Dey, E.S. 2014. Rye bran alkylresorcinols inhibit growth of *Penicillium expansum* and *Neofabraea perennans in vitro* and *in vivo* on different apple cultivars. European Journal of Horticultural Science 79: 218-225.

Tan, A.M. and Burchill, R.T. 1972. The infection and perennation of bitter rot fungus, *Gloeosporium album*, on apple leaves. Annals of Applied Biology 70: 199-206.

Van Bodegom, P., Hurndall, R., Van Der Merwe, J.A., Perks, A., Findlay, J.S. and Combrink, J.C. 2013. South African controlled atmosphere storage operator's manual. SA Apple and Pear Producers' Association.

Vanwalleghem, T., Dekeyser, D., Nuyttens, D., Tsige, A., Verboven, P., Van Hemelrijck, W. and Bylemans, D. 2016. Vaporization of biological control organisms in cold storage rooms to control postharvest diseases. Acta Horticulturae 1144: 121-127.

Verkley, G.J.M. 1999. A monograph of the genus *Pezicula* and its anamorphs. Studies in Mycology 44: 1-180

Weber, R.W.S. and Palm, G. 2010. Resistance of storage rot fungi *Neofabraea perennans*, *N. alba*, *Glomerella acutata* and *Neonectria galligena* against thiophanate-methyl in Northern German apple production. Journal of Plant Diseases and Protection 117: 185-191.

Wenneker, M. and Köhl, J. 2014. Postharvest decay of apples and pears in the Netherlands. Acta Horticulturae 1053: 107-112.

White, L.J. and Wilkinson, E.H. 1962. Pathogenicity of *Gloeosporium album* to maiden shoots of Cox's orange pippin. Plant Pathology 11: 110-114.

Wilkinson, E.H. 1945. Observations on the perennial canker fungus. Transactions of the British Mycological Society 28: 77-85.

Wood, P.N. and Fisher, B.M. 2017. The effect of fungicides on spore germination, mycelial growth and lesion development of *Phlyctema vagabunda* (syn: *Neofabraea alba*) (bull's eye rot of apples). New Zealand Plant Protection 70: 112-119.

Yang, C., Hamel, C., Vujanovic, V. and Gan, Y. 2011. Fungicide: modes of action and possible impact on nontarget microorganisms. ISRN Ecology 2011: 1-8.

Zeller, S.M. and Childs, L. 1925. Perennial canker of apple trees. Oregon Agriculture Experiment Station Bulletin 217.

Zhang, Z., Tian, S., Zhu, Z., Xu, Y. and Qin, G. 2012. Effects of 1-methylcyclopropene (1-MCP) on ripening and resistance of jujube (*Zizyphus jujuba* cv. Huping) fruit against postharvest disease. LWT - Food Science and Technology 45: 13-19.

CHAPTER 2

Identifying bull's eye rot of apple causal organisms and evaluating cultivar susceptibility for management[1]

ABSTRACT

Bull's eye rot is a postharvest lenticel decay disease of pome fruit. Four *Neofabraea* species are responsible for the disease and identification of the causal organism is vital for management of the disease. Cultivars vary in their susceptibility due to differences in their physiological and biochemical characteristics. To identify the causal organism of bull's eye rot, isolations were made from symptomatic fruit received from the storage of two commercial apple packhouses (Barrydale and Elgin) in the Western Cape of South Africa. The possible *Neofabraea* isolates were identified using a multiplex PCR. In total, 91 isolates from fruit were successfully identified as *N. vagabunda*. No other *Neofabraea* spp. were identified from either packhouse. The susceptibility of key apple cultivars was evaluated towards *Neofabraea* pathogens isolated from different apple production areas throughout the Western Cape province. Cultivars 'Cripps Pink', 'Fuji' and 'Golden Delicious' were examined for their susceptibility to seven *N. vagabunda* isolates. Inoculated 'Cripps Pink' and 'Fuji' apple fruit was found to be significantly more susceptible to *N. vagabunda*, than cv. Golden Delicious. Management practices aimed at controlling *N. vagabunda* would reduce bull's eye rot incidence in the Western Cape, and control should especially be focussed on highly susceptible cultivars 'Cripps Pink' and 'Fuji'.

INTRODUCTION

Pathogens that cause latent infections are problematic to the fruit industry because disease detection occurs only when symptoms are expressed several months into storage. Bull's eye rot is a sporadic disease on pome fruit that has been prevalent in South Africa for many years in apple orchards (Matthee, 1982; Den Breeyen and Lennox, 2012). Internationally, the disease is caused by a complex of species that belonging to the genus *Neofabraea*, they are *N. malicorticis* Jacks, *N. perennans* Kienholz, *N. vagabunda* Guthrie and *N. kienholzii* Seifert, Spotts and Levesque (Verkley, 1999; Spotts *et al.,* 2009). The pathogen infects the lenticels of fruit in the orchard where it remains dormant for 3-5 months after storage before disease symptoms become visible (Edney, 1964; Spotts *et al*., 2009).

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[1]Den Breeyen, A., Rochefort, J., Meitz-Hopkins, J.C. Russouw, A., Lennox, C.L. 2019. Preharvest detection and postharvest incidence of *Neofabraea vagabunda* on 'Cripps Pink' Apples in South Africa. Plant Disease [In press].

Knowledge of the causal species in an orchard is vital for the management of the disease in that orchard. *Neofabraea malicorticis* and *N. perennans* are aggressive canker forming species on apple and pear tree branches (Kienholz, 1939; Henriquez *et al.*, 2004, 2006). These two pathogens sporulate on cankers where the inoculum gets splash-dispersed with rain to healthy plant material (Grove *et al.,* 1992). Removal of these cankers and application of fungicides in disease conducive conditions are thus essential for disease risk management (Powell *et al*., 1965; Grove, 1990; Henriquez *et al.*, 2006). *Neofabraea vagabunda* has occasionally been found to cause small cankers on pear trees. This species is a successful saprophyte, and the main sources of inoculum include dead plant litter from which pathogen spores are dispersed (Sharples, 1959; Tan and Burchill, 1972). Therefore, orchard sanitation practices like removing and destroying dead plant material as well as a focussed fungicide spray programme will help mitigate disease incidence by *N. vagabunda* (Grove, 1990). The ability of *N. vagabunda* to sporulate on cankers is still under speculation, although it has been found to sporulate on artificially induced cankers on pear tree branches (Henriquez *et al.*, 2006). *Neofabraea kienholzii* is the most recently described species (Spotts *et al.*, 2009) and not much information is available on its epidemiology. However, it is found in similar geographic regions as *N. perennans* and shares similar canker and sporulation characteristics as *N. perennans* (Spotts *et al.*, 2009; Aguilar *et al.,* 2019).

Identification of the causal bull's eye rot species generally relies on symptom expression before pathogen identity can be verified. However, several methods of detection have been developed to identify *Neofabraea* pathogens from diseased and healthy plant material. The first molecular technique for identification was the sequencing of the β-tubulin gene which could differentiate between *N. malicorticis* and *N. perennans* (de Jong *et al.,* 2001). Gariépy *et al.* (2003) designed a species-specific multiplex PCR that could successfully distinguish *N. vagabunda, N. malicorticis* and *N. perennans* from one another after DNA was sampled from pure cultures. Since the discovery of the novel species *N. kienholzii*, Michalecka *et al.* (2015) adapted the multiplex PCR by Gariépy *et al.* (2003) to also identify this species. Not only could they successfully distinguish all four *Neofabraea* species in one reaction, but they could identify them from DNA collected from both bull's eye rot symptomatic and asymptomatic plant material (Michalecka *et al.*, 2015).

Recently, *N. vagabunda* has been identified in Dutch orchards causing bull's eye rot postharvest and subsequently, Köhl *et al.* (2018) designed a species-specific TaqMan PCR assay that could identify *N. vagabunda* from inoculum sources that include cankers, mummies, pruning's, fruit spurs and leaf litter. Pešicová *et al.* (2017) developed a cheap and reliable PCR-fingerprinting method for identifying and distinguishing all four species. Adamiak *et al.* (2012) used the biospeckle technique and found biological activity to be a means to monitoring bull's eye rot pathogen development during storage before symptoms are expressed.

Not only do bull's eye rot causing *Neofabraea* spp. differ in their epidemiology, but they also vary in their pathogenicity towards different apple cultivars. However, there is also contradiction among cultivar susceptibility studies where one study would find tolerance in a cultivar towards a particular *Neofabraea* species (Spotts *et al*., 1999), but a different study finds relative levels of susceptibility within the same cultivar (Spotts *et al.,* 1999; Maxin *et al.,* 2005). This is possibly due to variability in different *Neofabraea* population's characteristics in terms of preferred pH and nutrient composition of the host. An important factor in the susceptibility of cultivars is the harvest date. Although bull's eye rot infection of apple fruit in the orchard can occur as early as one month after bloom, fruit susceptibility increases with maturity (Edney *et al.*, 1977; Grove *et al.*, 1992). Cold and rainy conditions are more prevalent in the late season which is conducive to pathogen dispersal and infection (Edney, 1964, 1974). The cultivar 'Cripps Pink' has been of particular importance in bull's eye rot studies due to its late harvest times and reported high levels of susceptibility (Soto-Alvear *et al.*, 2013).

The aim of this study was to identify the species responsible for bull's eye rot postharvest decay of apple fruit in the Western Cape of South Africa, and to compare the susceptibility of key apple cultivars to lesion development by the pathogens in the Western Cape of South Africa.

MATERIAL AND METHODS

Collection of *Neofabraea* isolates and identification

Neofabraea reference isolates (N= 4), *N. vagabunda* (CBS 304.62)*, N. kienholzii* (CBS 355.72)*, N. malicorticis* (CBS 141.22) and *N. perennans* (CBS 453.64), as well as *N. vagabunda* isolates BER208, BER209 and BER867 were revived from the STE-U culture collection at the Department of Plant Pathology, Stellenbosch University. Isolates BER208 and BER209 are from the Witzenberg Valley and BER867 from Vyeboom (Rochefort, 2015). These isolates were grown on malt extract agar amended with 0,04 mg/L Streptomycin (MEA^+). Possible *Neofabraea* sp. isolates (N= 87) were obtained from symptomatic fruit (Fig.1A) received from two pack houses in the Western Cape of South Africa in the 2017 season (Fig. 1A). 'Cripps Pink' apples were obtained from Barrydale and 'Cripps Red' apples from Elgin. From the symptomatic fruit, four isolations were made from the inside of fruit at the margin of the lesions (Fig. 1B) and subsequently placed on MEA^+. After *Neofabraea*-like growth was observed, mycelial plugs were taken from the margin of colonies and placed on 2% water agar (WA) and incubated for 3-4 days at 24˚C after which hyphal tipping was conducted to purify cultures.

DNA extraction

Fungal mycelium was scraped off of 4-week old cultures grown on MEA$^+$ and placed in 2 mL Eppendorf tubes before being stored at -20˚C until DNA extraction. Firstly, 2 mm glass beads were added to the 2 mL Eppendorf tubes containing the mycelia and the tubes were then placed in liquid nitrogen. After quickly freezing samples in the liquid nitrogen the tubes were transferred to a Mixer Mill MM404 beater (Retsch, Haan, Germany) and shaken for 3 min at 30/s, after which 1 mL of warm extraction buffer was added. The extraction buffer consisted of 5 mL 0.5 M ethylene-diamine-tetra-acetic acid (EDTA) pH 8.0, 5 mL 1 M Tris-HCl pH 8.0, 8.3 mL 3 M NaCl, 1.25 mL 10% sodium dodecyl sulphate, 1 g 100% PVP-40 (Sigma-Aldrich, PVP40) and 0.35 mL 100% β-mercaptoethanol (Sigma-Aldrich, M6250). The mixture was then vortexed and placed into a 65 ˚C water bath for 1 h, inverting the samples every 15 min. Next, 333 µL 5 M potassium acetate was added, samples vortexed and placed on ice for 30 min. Following the incubation on ice, samples were centrifuged at 14 000 rpm for 10 min after which the supernatant was removed, carefully keeping the pellet and drying it upside down for 20 min. After drying the pellets, 200 µL of 70% ethanol was added and centrifuged for 2 min (14 000 rpm) to wash the pellet. Finally, after the supernatant was removed and the pellet dried off once more, the pellet was resuspended in 100 µL 0.1 M TE buffer (100 µL Tris-HCL pH 8.0, 2 µL EDTA 0.5 M pH 8.0 made up to 10 mL with sterile deionised H_2O).

Species-specific multiplex polymerase chain reaction (PCR)

Neofabraea species-specific primers developed by Michalecka *et al.* (2015) were used to identify isolates. Five primer sets were used: Neo_mal-loTub-262 (5'-GACAGCCAACTTGCGG-3'), Neo_per-loTub-328 (5'-GGGTCGAACATCTGTTGT-3'), Neo_spnov-loTub-319 (5'-TGGTGAGAGGAGCGAAC-3'), Neo_alba3 (5'-AATATTAGCAGGATATCTCTTCAAG-3') and Neofab_uni (5'-AACTTTCTCCGTTGTCCCATC-3'). Each reaction contained 0.2 mM dNTP's, 3.0 mM $MgCl_2$, 0.1 µM of each primer, 0.5 mg/mL of bovine serum albumin (BSA), 0.5 U Bioline Taq (5 U/µL; Bioline, London, UK) and 2 µL of a 10-100 ng DNA template. All reactions were made up to a final volume of 25 µL with sterile distilled H_2O. Amplification conditions were as follows: an initial denaturation step at 94 ˚C for 3 min, annealing at 58 ˚C for 30 s and then 35 cycles of denaturation at 94 ˚C for 30 s, annealing at 58 ˚C for 30 s and 72 ˚C for 45 s with a final extension step at 72 ˚C for 5 min. PCR products were separated by electrophoresis using a 2% (w/v) agarose gel, stained with ethidium bromide and visualised under UV light.

Cultivar susceptibility

A total of 7 isolates obtained from different areas throughout the Western Cape were grown on MEA$^+$ and tested for their pathogenicity on 'Cripps Pink', 'Fuji' and 'Golden Delicious'. The

origin of the isolates included Barrydale (AN001 and AN004), Elgin (AN020 and AN091), the Witzenberg Valley (BER208 and BER209) and one Vyeboom (BER867). Before inoculation, the fruit were surface sterilised by washing with 70% ethanol for 30 s and then left to air dry inside a laminar flow cabinet. After drying the fruit, the fruit were inoculated by puncturing a 5 to 10 mm hole in the fruit using a 6mm diameter sterile cork borer. Subsequently, a 6 mm agar plug containing mycelia was taken from the margin of an actively growing colony and placed in the wound hole. The wounds were then sealed with petroleum jelly and placed in a clear Perspex moisture chamber with high relative humidity (>90%) at ambient room temperature (24˚C). Control fruit were also included for each cultivar and the fruits were inoculated with a clean MEA^{+} plug only. Lesion diameters were measured after 14 days using a digital calliper and re-isolations were made to confirm the causal organism as *N. vagabunda*. The trial was repeated once.

Statistical analysis

Statistical analysis was performed using Statistica V13.5 (TIBCO Software, California, United States). The cultivar susceptibility to *N. vagabunda* isolates data was analysed using a factorial analysis of variance (ANOVA). P-values were generated with a 95% confidence interval to determine significant differences using Duncan's Multiple Range test. Average susceptibility between cultivars was analysed with a one-way ANOVA.

RESULTS

Identifying *Neofabraea* species causing bull's eye rot

All of the reference isolates were successfully revived from storage. From the packhouses, a total of 104 fruit had bull's eye rot symptoms (AN001-AN104). Out of the 104 fruit obtained, seven fruit were from Barrydale (AN001-007) and the rest from the Elgin area (AN008-104). Seven isolates were obtained from the Barrydale fruit and 84 isolates from the Elgin fruit. Two fruit isolations, AN016 and AN019, produced two different mycelial growth morphologies from their respective isolations. Each growth type was sub-plated and re-labelled accordingly (AN016a, AN016b, AN019a and AN019b). Multiplex-PCR amplification of the reference isolates produced product sizes of 499 bp for *N. vagabunda*, 400 bp for *N. perennans,* 336 bp for *N. kienholzii* and 270 bp for *N. malicorticis* (Fig. 2). A 500 bp PCR product was obtained for all the *Neofabraea* isolates from fruit (Figs. 2 and 3). Amplification of a second band sized 800 bp, was produced by 38 of the newly identified *N. vagabunda* isolates. Some of the isolates that produced an 800 bp band also produced a faint 300 bp product. The bands obtained for isolates AN018, AN037, AN041 and AN062 were light yet still distinguishable.

Cultivar susceptibility to different *N. vagabunda* isolates

All the isolates were pathogenic on all three cultivars with low variability in lesion diameter between isolates (Fig. 4). Isolate BER209 had similar pathogenicity across all three cultivars with no significant difference between the average lesion diameters. The largest lesion diameter, although not significantly different from other isolates, was produced by AN001 on cv. Cripps Pink averaging 11.12 mm. The smallest lesion diameter was produced by AN091, which was on cv. Golden Delicious measuring only 6.16 mm. For all the isolates, except BER867, pathogenicity on cultivars 'Fuji' and 'Cripps Pink' was not significantly different. BER867 caused lesions of 10.3 mm on 'Fuji' but only 7.33 mm on 'Cripps Pink'.

The smallest lesion diameter for every isolate was on 'Golden Delicious', averaging between 6.0-7.5 mm, except for BER209, which measured 10.14 mm. The Witzenberg isolates produced the largest lesion diameter development on 'Fuji' and 'Cripps Pink' when compared to the isolates from other regions. Per cultivar, the average lesion diameter for all the isolates was larger on 'Fuji' at 8.36 mm, with 'Cripps Pink' second at 8.15 mm (Fig. 5). There is, however, no significant difference in susceptibility between these two cultivars. 'Golden Delicious' is significantly less susceptible, with lesion development averaging 6.28 mm. The disease incidence recorded for the inoculated fruit was 90-100% incidence across all of the tested isolates on all cultivars (data not shown). Except for isolate AN091, which had 87% disease incidence on 'Fuji' and 83% on 'Cripps Pink', and isolate BER867 with 83% disease incidence on 'Cripps Pink'.

DISCUSSION

The multiplex-PCR adapted by Michalecka *et al.* (2015) successfully distinguished between *N. vagabunda, N. perennans, N. kienholzii* and *N. malicorticis*. A total of 91 *Neofabraea* isolates were successfully obtained from the inside of fruit lesions. All the isolates were identified as *N. vagabunda* using the multiplex-PCR by Michalecka *et al.* (2015). This supports previous findings by Den Breeyen and Lennox (2012) that *N. vagabunda* is the causal species of bull's eye rot on apple fruit in the Western Cape of South Africa. Interestingly, *N. perennans* was identified on the surface of 'Cripps Pink' apples collected from an orchard preharvest, but was not found to cause decay (Rochefort, 2015). *Neofabraea kienholzii* has also previously been found to cause decay of a single pear fruit postharvest (Rochefort, 2015). Both *N. perennans* and *N. kienholzii* was isolated from an orchard in the Grabouw area of the Western Cape. Although Rochefort (2015) discussed that even though *N. perennans* and *N. kienholzii* were found, the numbers were very low and that it is possibly of little importance to disease incidence. In the current study no new *N. perennans* or *N. kienholzii* isolates were detected from the sampled regions (Barrydale and Elgin).

Extra unexpected PCR bands were produced by several of the *N. vagabunda* isolates. The bands were respectively 300 bp and 800 bp in size. The extra bands are possibly due to non-specific binding of the multiple primers used in the multiplex-PCR. *Neofabraea vagabunda* shares >99% of its genome with its bull's eye rot counterparts: *N. malicorticis*, *N. perennans* and *N. kienholzii* (www.ncbi.nlm.nih.gov). These extra bands do not match band sizes that would have been produced for other bull's eye rot species and were therefore considered as negligible. However, as previously mentioned, the other bull's eye rot species have been found in the Western Cape, with a single isolate of both *N. perennans* and *N. kienholzii* obtained from decaying pear fruit in the Witzenberg Valley in 2012. Subsequently, only *N. vagabunda* was isolated from the same orchard the following year (Rochefort, 2015).

Both *N. perennans* and *N. kienholzii* are found in more semi-arid apple-growing regions where the fungus can withstand hot, dry summers and freezing winter conditions, meaning they prefer similar environmental conditions (Kienholz, 1939; De Jong *et al.*, 2001; Henriquez *et al.*, 2004; Spotts *et al.*, 2009; Aguilar *et al*., 2018). The apple-growing regions in the Western Cape have a Mediterranean climate, which has more moderate winter temperatures rather than freezing temperatures. *Neofabraea perennans* and *N. kienholzii* might require more extreme winter temperatures for successful infection of apple trees which is absent from the South African apple-growing areas. Henriquez *et al*. (2004) found that *N. vagabunda, N. perennans* and *N. kienholzii* to coexist in orchards. However, the levels of individual species fluctuated and Henriquez *et al*. (2004) proposed that the importance of each species was influenced by the pathogen's favoured environmental conditions.

Neofabraea perennans requires wounds to infect apple tree branches and twigs. Once the pathogen has infected an apple tree, it causes cankers from where spores are dispersed. Trees respond to the canker formation by producing callus tissue around the canker margin, with the callus tissue serving as a physical barrier to impede on the further spread of the pathogen (Grove *et al*., 1992). In freezing winter temperatures, the callus tissue cracks and these cracks in the bark serve as new infection sites for *N. perennans* (Dugan *et al*., 1993). Moreover, woolly apple aphids feed on the canker callus tissue, producing wounds and subsequently, more infection sites (Dugan *et al*., 1993). The absence of freezing winter temperatures in the Western Cape could account for the inability of *N. perennans* to establish in orchards and induce disease due to no new infection sites being produced with cracked bark tissue. Although little knowledge is available on the epidemiology of *N. kienholzii*, Aguilar *et al*. (2019) proposed feeding galls of woolly apple aphids to be of importance to the bull's eye rot disease as they found the aphid to colonize both *N. perennans* and *N. kienholzii* produced cankers.

The objective of this study was to identify which of the three cultivars are most susceptible to the pathogen. Cultivars Fuji and Cripps Pink proved to have similar levels of susceptibility

to lesion diameter caused by *N. vagabunda*. 'Golden Delicious' was significantly less susceptible to *N. vagabunda* disease development. *Neofabraea vagabunda* has the widest apple host range of all the bull's eye rot species and has previously been found pathogenic on all three of the tested cultivars (Gualanduzzi *et al*., 2005; Spotts *et al.*, 2009; Hortova *et al.*, 2014). Although 'Golden Delicious' was less susceptible than the other cultivars in this study, it has been found susceptible to all of the bull's eye rot causing species including the novel species *N. kienholzii* (Spotts *et al.*, 1999, 2009; Hortova *et al.*, 2014). *Neofabraea kienholzii* has also been identified as pathogenic on cv. Fuji (Spotts *et al.*, 2009). Generally, 'Cripps Pink' and 'Fuji' are more prone to infection preharvest due to their harvest times. These two cultivars are harvested later in the season than most other cultivars, and later harvest means apple fruit are more susceptible, as well as more inoculum being present in *Neofabraea* sp. infected orchards (Henriquez *et al*., 2008; Børve *et al.*, 2013; Cameldi *et al*., 2016).

In the case of 'Cripps Pink', the Pink Lady® Association set colour standards for the commercialisation of 'Cripps Pink' apples. These standards force growers to delay their harvest for the fruit to be the correct colour for the export market. In cool conditions the necessary pink colour can likely be obtained by harvest, but warmer weather prior to harvest delays the colour development (Lin-Wang *et al*., 2011). Thus, when conditions are not favourable, the harvest is delayed and more *Neofabraea* inoculum is present under optimal infection conditions for the pathogen, with fruit being more mature and more susceptible (Henriquez *et al.*, 2006, 2008; Aguilar *et al.*, 2017). Moreover, Durić *et al*. (2012) found 'Cripps Pink' to have an average number of 8.01 ±0.77 lenticels per 1 cm^3 fruit peel, the second highest number out of the ten cultivars evaluated. The highest was 'Granny Smith' with an average of 12.60 ±1.55 lenticels per 1 cm^3 fruit peel. The high number of lenticels on 'Cripps Pink' apple fruit, provides abundant infection sites for bull' eye rot pathogens and increases the pathogen's chances of successful infection. 'Cripps Pink' is also higher in some volatile organic compounds compared to other cultivars. The bull's eye rot fungi utilizes these compounds and thus favours colonisation of the 'Cripps Pink' host (Neri *et al.*, 2019).

The results obtained in this study contributes to the development of an integrated management strategy. *Neofabraea vagabunda* was identified as the sole causal pathogen of bull's eye rot in most regions and management strategies should be applied to focus on control of this species. The pathogen is a known saprophyte and management of the disease would benefit from a thorough orchard sanitation programme. *Neofabraea vagabunda* survives on fruit mummies, senescent or decaying leaves and plant tissue such as twigs and branches on the orchard floor (Tan and Burchill, 1972; Verkley, 1999; Henriquez *et al.*, 2008). These survival structures serve as inoculum sources from which conidia splash-disperse with water to infect fruit in the current or the next season (Tan and Burchill, 1972; Edney, 1974).

Cleaning up the orchard floor during pruning in autumn and disposing of plant litter properly reduces inoculum sources (Tan and Burchill, 1972; Grove, 1990). Use of under-tree irrigation system should be implemented to minimise or avoid dispersal of conidia from the orchard floor throughout the season (Henriquez *et al.*, 2008). Reducing the infection pressure from the pathogen can be achieved by making conditions unfavourable through pruning of trees to minimise orchard density. Low density orchards aid in keeping humidity levels low and have the additive benefit of increased radiation exposure (Tahir and Nybom, 2013). Harvesting cultivars 'Fuji' and 'Cripps Pink' earlier in the season can effectively reduce the risk of fruit infection by *Neofabraea* species but could compromise fruit colour and taste (Cameldi *et al.*, 2016).

A judicious preharvest spray programme adapted for *N. vagabunda* will impede further infection and disease development in the orchard. It is also important to make use of a disease risk model to predict high disease pressure events. Adequate application of fungicides before the occurrence of high rainfall and high relative humidity periods can significantly reduce the number of viable spores dispersed as well as possible infection sites (Henriquez *et al*., 2008). Fungicides registered in South Africa that have been shown to be effective against *N. vagabunda* include benomyl, captan, mancozeb, thiram and trifloxystrobin (Powell, 1965; Spotts *et al*., 2009; Wood and Fisher, 2017). Applying captan, mancozeb or thiram from early on in the season in weekly intervals or as instructed, reduces inoculum and prevents infection throughout the season (Powell, 1965; Burchill and Edney, 1972; Spotts *et al.*, 2009).

Benomyl and trifloxystrobin are systemic fungicides, and applying these fungicides before or after high disease pressure events (high rainfall, high humidity, before harvest) can reduce bull's eye rot incidence postharvest (Henriquez *et al.*, 2006, 2008; Wood and Fisher, 2017). It is important that the latter two fungicides not be overused and used in conjunction with other fungicides as resistance development by *N. vagabunda* has been reported (Weber and Palm, 2010; Wood and Fisher, 2017). For late harvest cultivars such as 'Fuji', 'Cripps Pink', 'Cripps Red' and 'Granny Smith', applying fungicides close to harvest will reduce risk of infection as fruit are most susceptible at this time and environmental conditions favour the pathogen (Henriquez *et al.*, 2006; Spotts *et al.*, 2009; Aguilar *et al*., 2018).

Treating fruit with fungicides postharvest as a curative action before going into storage can significantly reduce bull's eye rot incidence after storage. Fludioxonil and pyrimethanil have been shown to be effective as a dip, drench and thermo-fog treatment (Spotts *et al.*, 2009; Lolas *et al.*, 2016; Aguilar *et al*., 2018). Previous studies showed thiabendazole to reduce incidence when applied as a thermo-fog treatment as well as when applied in combination with fludioxonil as a drench (Bertolini *et al.*, 1995; Lolas *et al.*, 2016).

In conclusion, bull's eye rot can be controlled when management practices are adapted to the pathogen and performed correctly. In the Western Cape of South Africa, focussing

management of bull's eye rot on *N. vagabunda* would be beneficial if disease has been found present in an orchard. Management practices should especially be implemented in late harvested cultivar orchards such as 'Cripps Pink' and 'Fuji'.

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REFERENCES

Adamiak, A., Zdunek, A., Kurenda, A. and Rutkowski, K. 2012. Application of the biospeckle method for monitoring bull's eye rot development and quality changes of apples subjected to various storage methods - preliminary studies. Sensors 12: 3215-27.

Aguilar, C.G., Mazzola, M. and Xiao, C.L. 2017. Timing of apple fruit infection by *Neofabraea perennans* and *Neofabraea kienholzii* in relation to bull's-eye rot development in stored apple fruit. Plant Disease 101: 800-806.

Aguilar, C.G., Mazzola, M. and Xiao, C.L. 2019. Control of bull's eye rot of apple caused by *Neofabraea perennans* and *Neofabraea kienholzii* uisng pre- and postharvest fungicides. Plant Disease 102: 905-910.

Bertolini, P., Guarnieri, A. and Venturi, P. 1995. Postharvest fog treatment of apples: deposition patterns and control of *Phlyctema vagabunda* and superficial scald. Crop Protection 14: 345-348.

Børve, J., Røen, D. and Stensvand, A. 2013. Harvest time influences incidence of storage diseases and fruit quality in organically grown "Aroma" apples. European Journal of Horticultural Science 78: 232-238.

Burchill, R.T. and Edney, K.L. 1972. An assessment of some new treatments for the control of rotting of stored apples. Annals of Applied Biology 72: 249-255.

Cameldi, I., Neri, F., Ventrucci, D., Ceredi, G., Muzzi, E. and Mari, M. 2016. Influence of harvest date on bull's eye rot of 'Cripps Pink' apple and control chemical strategies. Plant Disease 100: 2287-2293.

De Jong, S.N., Lévesque, C.A., Verkley, G.J.M., Abeln, E.C.A., Rahe, J.E. and Braun, P.G. 2001. Phylogenetic relationships among *Neofabraea* species causing tree cankers and bull's-eye rot of apple based on DNA sequencing of ITS nuclear rDNA, mitochondrial rDNA, and the β-tubulin gene. Mycological Research 105: 658-669.

Den Breeyen, A. and Lennox, C.L. 2012. Towards the development of a rapid detection method for bull ' s eye rot in apples. Proceedings of the 9th International IOBC-WPRS Workshop on Pome Fruit Diseases 84: 5-8.

Dugan, F.M., Grove, G.G. and Rogers, J.D. 1993. Comparative studies of *Cryptosporiopsis curvispora* and *C. perennans*. I. Morphology and pathogenic behaviour. Mycologia 85: 551-564.

Durić, G., Mićić, N. and Pasalić, B. 2012. Lenticels as pomological characteristic of apple and pear fruits. 2nd International Symposium on Horticulture in Europe 1099: 771-775.

Edney, K.L. 1964. A comparison of the production of extracellular enzymes and rotting of apples by *Pezicula alba* and *P. malicorticis*. Transactions of the British Mycological Society 47: 215-225.

Edney, K.L. 1974. *Pezicula malicorticis*: Factors affecting spore germination and invasion of apple fruit. Transactions of the British Mycological Society 62: 25-34.

Edney, K.L., Tan, A.M., and Burchill, R.T. 1977. Susceptibility of apples to infection by *Gloeosporium album*. Annals of Applied Biology 86: 129-132.

Gariépy, T.D., Lévesque, C.A., de Jong, S.N. and Rahe, J.E. 2003. Species specific identification of the *Neofabraea* pathogen complex associated with pome fruits using PCR and multiplex DNA amplification. Mycological Research 107: 528-536.

Grove, G.G. 1990. Anthracnose and perennial canker. Pages 36-38 in: Compendium of Apple and Pear Diseases. (A. L. Jones and H. S. Aldwinckle, eds.) St. Paul, Minnesota: The American Phytopathological Society.

Grove, G.G., Dugan, F. M. and Boal, R. J. 1992. Perennial canker of apple: seasonal host susceptibility, spore production, and perennation of *Cryptosporiopsis perennans* in infected fruit in eastern Washington. Plant Disease 76: 1109-1114.

Gualanduzzi, S., Neri, F., Brigati, S. and Folchi, A. 2005. Storage of “Pink Lady®” apples: Quality and bio-pathological aspects. Acta Horticulturae 682: 2077-2084.

Henriquez, J.L., Sugar, D. and Spotts, R.A. 2004. Etiology of bull’s eye rot of pear caused by *Neofabraea* spp. in Oregon, Washington, and California. Plant Disease 88: 1134-1138.

Henriquez, J.L., Sugar, D. and Spotts, R.A. 2006. Induction of cankers on pear tree branches by *Neofabraea alba* and *N. perennans*, and fungicide effects on conidial production on cankers. Plant Disease 90: 481-486.

Henriquez, J.L., Sugar, D. and Spotts, R.A. 2008. Effects of environmental factors and cultural practices on bull’s eye rot of pear. Plant Disease 92: 421-424.

Hortova, B., Novotny, D. and Erban, T. 2014. Physiological characteristics and pathogenicity of eight *Neofabraea* isolates from apples in Czechia. European Journal of Horticultural Science 79: 327-334.

Kienholz, J.R. 1939. Comparative study of the apple anthracnose and perennial canker fungi. Journal of Agricultural Research 59: 635-665.

Köhl, J., Wenneker, M., Groenenboom-de Haas, B.H., Anbergen, R., Goossen-van de Geijn, H.M., Lombaers-van der Plas, C.H., Pinto, F.A.M.F. and Kastelein, P. 2018. Dynamics of postharvest pathogens *Neofabraea* spp. and *Cadophora* spp. in plant residues in Dutch apple and pear orchards. Plant Pathology 67: 1264–1277.

Lin-Wang, K., Micheletti, D., Palmer, J., Volz, R., Lozano, L., Espley, R., Hellens, R.P. and Chagnè, D. 2011. High temperature reduces apple fruit colour via modulation of the anthocyanin regulatory complex. Plant, Cell and Environment 34: 1176-1190.

Lolas, M., Díaz, G., Mendez, R., Cáceres, M. and Neubauer, L. 2016. Evaluation of the efficacy of fungicide fludioxonil in the postharvest control of bull’s eye rot (*Neofabraea alba*) in Chile. Acta Horticulturae 1144: 461-464.

Maxin, P., Huyskens-Keil, S., Klopp, K. and Ebert, G. 2005. Control of postharvest decay in organic grown apples by hot water treatment. Acta Horticulturae 682: 2153-2158.

Matthee, F.N. 1982. Rypverrotting by apples: siekte wat opgebergde appels laat vrot. Landbouweekblad 60: 25-27.

Michalecka, M., Bryk, H., Poniatowska, A. and Puławska, J. 2015. Identification of *Neofabraea* species causing bull's eye rot of apple in Poland and their direct detection in apple fruit using multiplex PCR. Plant Pathology 65: 643-654.

Neri, F., Cappellin, L., Aprea, E., Biasioli, F., Gasperi, F., Spadoni, A. and Cameldi, I. 2019. Interplay of apple volatile organic compounds with *Neofabraea vagabunda* and other postharvest pathogens. Plant Pathology 68: 1033-1232.

Pešicová, K., Kolařík, M., Hortová, B. and Novotný, D. 2017. Diversity and identification of *Neofabraea* species causing bull's eye rot in the Czech Republic. European Journal of Plant Pathology 147: 683-693.

Powell, D., Janson, B. and Sharvelle, E.G. 1965. Diseases of apples and pears in the Midwest. North Central Regional Extension Publication No. 16. Cooperative Extension Service Circular 909. University of Illinois Urbana, USA.

Rochefort, J. 2015. Epidemiology and etiology of *Neofabraea* species causing lenticel decay of pome fruit in the Western Cape of South Africa. MSc Thesis, Stellenbosch University, Stellenbosch, South Africa. Thesis number 100634.

Sharples, R.O. 1959. Observations on the perfect state of *Gloeosporium perennans* in England. Transactions of the British Mycological Society 42: 507-512.

Soto-Alvear, S., Lolas, M., Rosales, I.M., Chavez, E.R. and Latorre, B.A. 2013. Characterization of the bull's eye rot of apple in Chile. Plant Disease 97 (4): 485-490.

Spotts, R.A., Cervantes, L.A. and Mielke, E.A. 1999. Variability in postharvest decay among apple cultivars. Plant Disease 83: 1051-1054.

Spotts, R.A., Seifert, K.A., Wallis, K.M., Sugar, D., Xiao, C.L., Serdani, M. and Henriquez, J.L. 2009. Description of *Cryptosporiopsis kienholzii* and species profiles of *Neofabraea* in major pome fruit growing districts in the Pacific Northwest USA. Mycological Research 113: 1301-1311.

Tahir, I.I. and Nybom, H. 2013. Tailoring organic apples by cultivar selection, production system, and postharvest treatment to improve quality and storage life. HortScience 48: 92-101.

Tan, A.M. and Burchill, R.T. 1972. The infection and perennation of bitter rot fungus, *Gloeosporium album*, on apple leaves. Annals of Applied Biology 70: 199-206.

Verkley, G.J.M. 1999. A monograph of the genus *Pezicula* and its anamorphs. Studies in Mycology 44: 1-180.

Weber, R.W.S. and Palm, G. 2010. Resistance of storage rot fungi *Neofabraea perennans*, *N. alba*, *Glomerella acutata* and *Neonectria galligena* against thiophanate-methyl in Northern German apple production. Journal of Plant Diseases and Protection 117: 185-191.

Wood, P.N. and Fisher, B.M. 2017. The effect of fungicides on spore germination, mycelial growth and lesion development of *Phlyctema vagabunda* (syn: *Neofabraea alba*) (bull's eye rot of apples). New Zealand Plant Protection 70: 112–119.

TABLES AND FIGURES

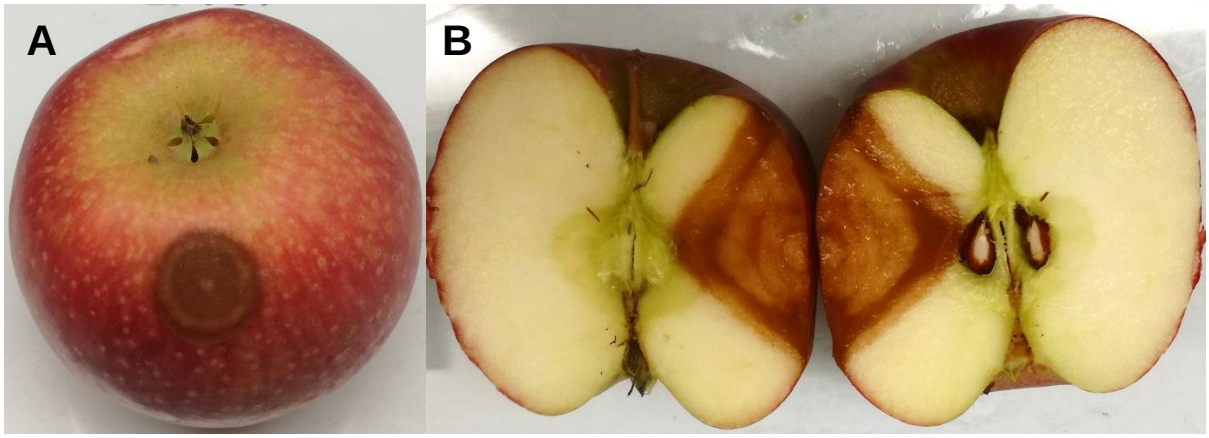

Figure 1. External (**A**) and internal (**B**) symptoms of bull's eye rot caused by *Neofabraea vagabunda* on 'Cripps Red' apple. Externally, decay spreads outward from infected lenticel. As lesions develop light and dark brown concentric rings form which gives bull's eye rot its distinctive lesion trait. Internally, the pathogen spreads inward in a conical manner, leading to discolouration of disintegrating tissue

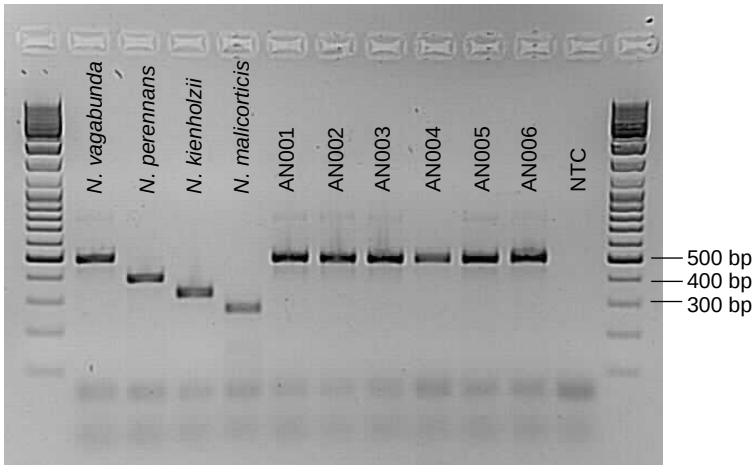

Figure 2. Multiplex PCR amplification of reference positive control *Neofabraea* isolates obtained from the Westerdijk Fungal Biodiversity Institute Centraalbureau voor Schimmelculture (CBS), a non-template control (NTC) and isolates obtained from symptomatic apple fruit (AN001-AN006). PCR products were separated by electrophoresis using a 2% (w/v) agarose gel stained with ethidium bromide and visualized under ultra-violet light. Amplicons of reference isolates were 499 bp for *N. vagabunda* (CBS 304.62), 400 bp for *N. perennans* (CBS 453.64), 336 bp for *N. kienholzii* (CBS 355.72) and 270 bp for *N. malicorticis* (CBS 141.22). Isolates AN001-006 produced amplicons of 499bp in size. Lanes 1 and 13 contain GeneRuler™ 100 bp Plus DNA ladder (Thermo Scientific).

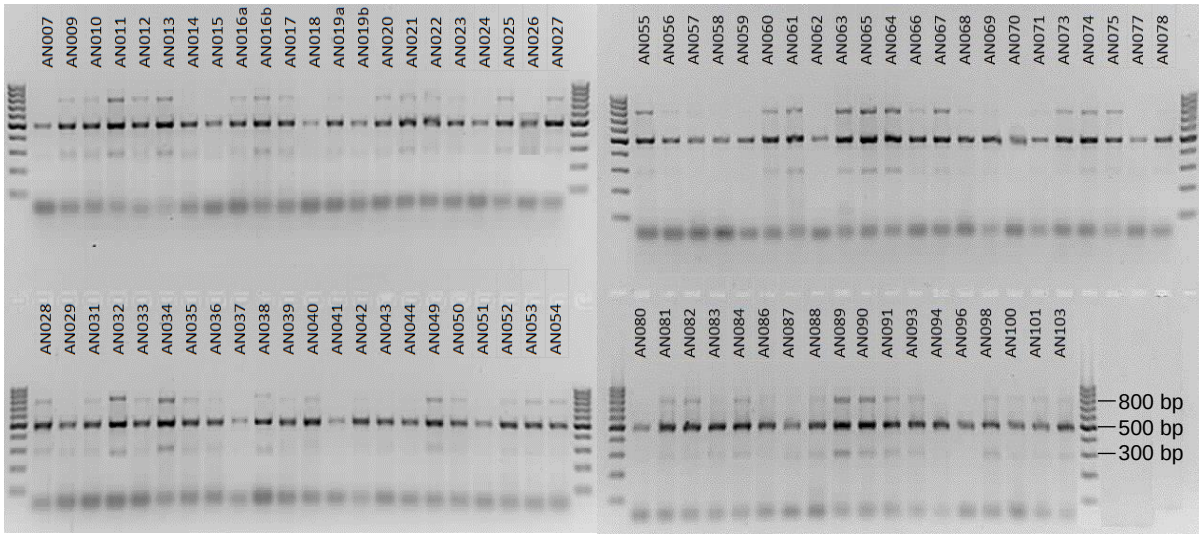

Figure 3. Amplicons produced by a multiplex PCR of *Neofabraea* sp. isolates (AN007-AN103) obtained from symptomatic fruit. The 499 bp products represent positive *N. vagabunda* identification. PCR products were separated by electrophoresis using a 2% (w/v) agarose gel stained with ethidium bromide and visualized under ultra-violet light. A GeneRuler™ 100 bp Plus DNA ladder (Thermo Scientific) was used for band size identification. The amplicons produced by the isolates AN007-AN103 matched the amplicon size of the reference CBS *N. vagabunda* (CBS 304.62) isolate which was also 499 bp.

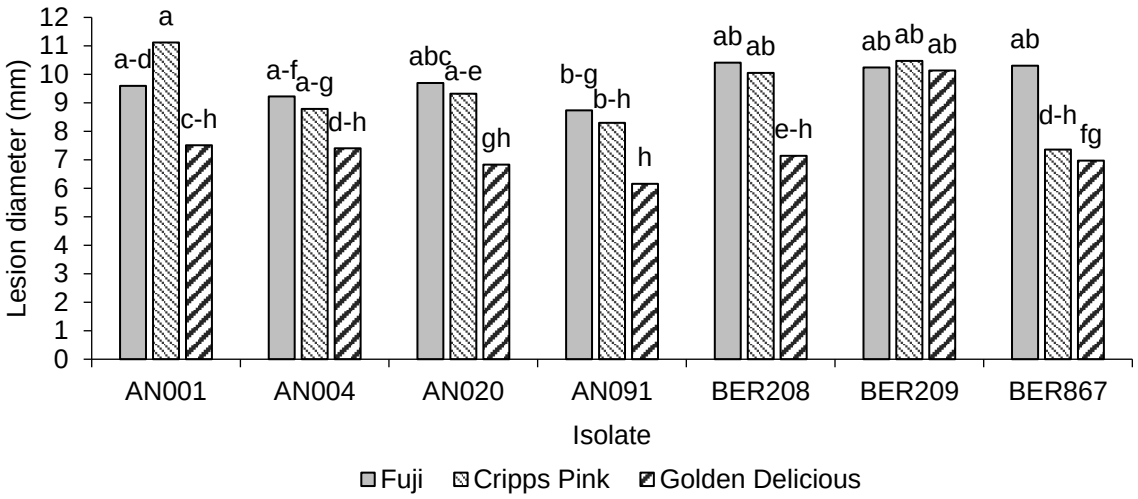

Figure 4. The average bull's eye rot lesion diameters caused by *Neofabraea vagabunda* isolates obtained from the Barrydale (AN001 and AN004), Elgin (AN020 and AN091), Witzenberg (BER208 and BER209) and Vyeboom (BER867) areas on three apple cultivars. Fruit was incubated for 14 days after inoculation The Duncan's Multiple Range Test was used to identify significant differences between lesion diameters. Means with the same letter are not significantly different (P<0.05).

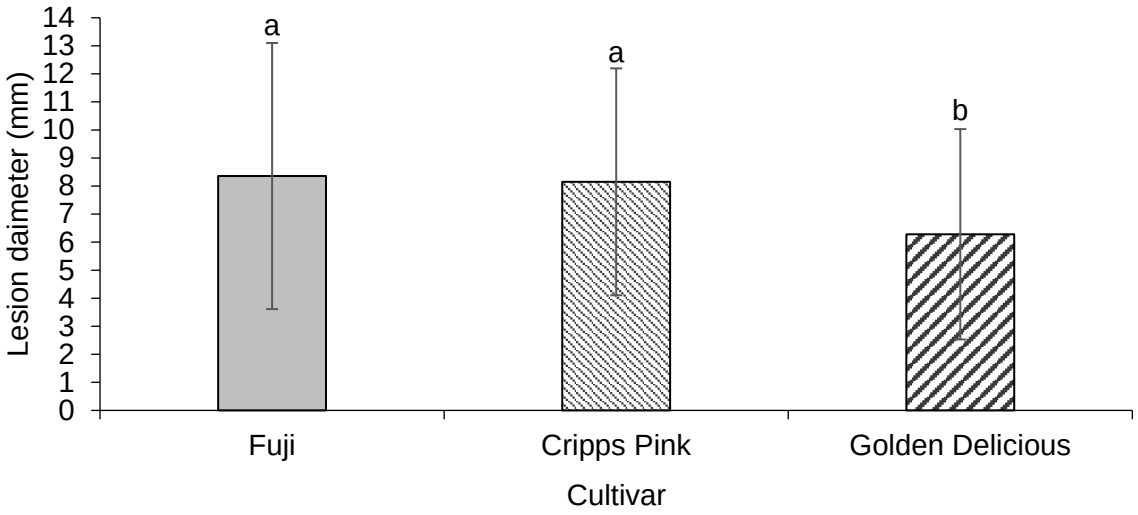

Figure 5. Mean combined bull's eye rot lesion diameters caused by different *Neofabraea vagabunda* isolates obtained in South Africa on apple fruit. The Duncan's Multiple Range Test was used to identify significant differences between average lesion diameters. Means with the same letter are not significantly different (P<0.05). Error bars indicate standard deviation.

CHAPTER 3

Postharvest application of fludioxonil and pyrimethanil to control bull's eye rot on apple caused by *Neofabraea vagabunda*

ABSTRACT

Chemical control is a valuable method to manage postharvest diseases on fruit. Bull's eye rot is a postharvest disease of pome fruit caused by *Neofabraea* spp. and infects the lenticels of fruit in the orchard with the disease only becoming apparent months into storage. There is, however, no fungicide registered specifically against bull's eye rot in South Africa. In this study the efficacy of the fungicides fludioxonil (299 mg/L) and pyrimethanil (500 mg/L) was tested on *N. vagabunda* bull's eye rot of apple. Both fungicides are registered on pome fruit against other fungal pathogens. Fruit trials involved curative dip, drench and thermo-fogging applications of the fungicides on *N. vagabunda* inoculated 'Cripps Pink' and 'Fuji' apple fruit. Furthermore, the variation of pyrimethanil (500 and 1000 mg/L) sensitivity *in planta* in a dip application was evaluated on six different *N.* vagabunda isolates from the Western Cape of South Africa. The effect of incubation time before treatment was tested in relation to the curative efficacy of the fungicides fludioxonil and pyrimethanil.

Fludioxonil was highly effective in controlling bull's eye rot disease incidence as a dip application and moderately effective as a drench or thermo-fog application. Pyrimethanil had moderate efficacy on both cultivars as a thermo-fog application. The pyrimethanil dip application was ineffective in controlling disease incidence and the drench had low efficacy on 'Fuji' apples and no efficacy on 'Cripps Pink' inoculated fruit. There no sign of variation in efficacy of pyrimethanil to *N. vagabunda* isolates. Incubation time had a significant effect on pyrimethanil efficacy. Delaying pyrimethanil application after inoculation, significantly decreased the efficacy of the fungicide in controlling bull's eye rot incidence. The longer fruit pathogen inoculations were incubated for, the less effective the fungicide became. Fludioxonil can control *N. vagabunda* on apple fruit when applied as a postharvest treatment. However, the artificial inoculation method used in this study does not adequately mimic natural infection of the fruit and pyrimethanil will thus have to be evaluated under natural infection conditions in the future.

INTRODUCTION

Postharvest decay leads to significant losses of fruit and vegetables either in or after storage. Fungal pathogens that cause postharvest diseases can infect fruit anytime during the developmental stage of the fruit as well as during or after harvest. Two types of fungi cause

postharvest diseases on pome fruit, wound fungi such as *Botrytis* and *Penicillium*, and latent fungi such as *Alternaria*, *Colletotrichum, Monilinia* and *Neofabraea* (Creemers, 1989). There is currently no method to accurately predict how much decay will occur throughout the fruit commercialisation process. Thus, the application of multiple fungicides before and after harvest is used to mitigate the risk of postharvest decay (Köhl *et al*., 2018). However, for the correct fungicides to be applied, the disease that is being targeted and its nature must be considered as well as the residues and regulations of the fungicide. There are two types of fungicides used against pathogens, preventative and curative. Preventative fungicides are present on the plant before the pathogen arrives and prevents infection of the plant. Curative fungicides stop the early growth or development of a pathogen already present in plant tissue (Ivic, 2010).

Bull's eye rot of pome fruit is a latent disease that occurs sporadically from season to season and is caused by four species of the genus *Neofabraea* (Grove, 1990). Latent or 'quiescent' infections are caused by pathogens during fruit development, but remain inactive until the host physiology changes through the ripening process and eventually allowing the pathogen's development to continue and disease symptoms appear (Coates and Johnson, 1997). Control of the bull's eye rot pathogens *N. malicorticis*, *N. perennans, N. vagabunda* and *N. kienholzii* is difficult to achieve because infections can occur anytime during the growing season, from flowering to harvest (Grove *et al*., 1992). The fungi infect the lenticel of fruit in the orchard where it remains dormant for several months after harvest (Edney, 1964). The fungus hyphae survive in the infected lenticel cavity and only continue to develop when it can overcome the host's defence mechanisms (Edney, 1958; Neri *et al.*, 2019). Apple physiological and biochemical characteristics change drastically when fruit senesce. This is especially noticeable in a reduction of fungistatic compounds and physical resistance (Lattanzio *et al*., 2001). The repeated application of preventative fungicides during the growing season prevents new infections of *Neofabraea* spp. in the orchard (Coates and Johnson, 1997). However, infection can still occur because the disease is highly sporadic and flourishes under optimal infection conditions (Bompeix, 1978). Application of curative fungicides postharvest, before fruit is transferred into storage, can reduce bull's eye rot incidence after storage (Spotts *et al*., 2009).

Applying fungicides postharvest does not only allow for a more targeted approach to pathogen control but also has a reduced impact on the environment due to less run-off and thus a smaller carbon footprint (Moggia *et al*., 2003). Several postharvest fungicides have been tested against the *Neofabraea* spp. however, the fungicides differ in their efficacy between the species. Several studies have shown that fungicides which control one species do not necessarily control the other (Henriquez *et al*., 2006; Spotts *et al.*, 2009; Wood and Fisher, 2017). Fludioxonil and pyrimethanil are two fungicides that have potential in managing

bull's eye rot as a postharvest application. Fludioxonil is a phenylpyrrole fungicide that inhibits spore germination, germ-tube elongation and mycelial growth of fungi. Pyrimethanil is an aminopyrimidine fungicide that inhibits germ-tube elongation and mycelial growth (Rosslenbroich and Stuebler, 2000).

Currently no fungicides are registered specifically against bull's eye rot in South Africa. Both fludioxonil and pyrimethanil have shown the ability to control *Neofabraea* species causing bull's eye rot when applied postharvest in the United States and Chile (Henriquez *et al*., 2008; Spotts *et al.*, 2009; Lolas *et al*., 2016; Aguilar *et al*., 2018). These two fungicides are also ideal resistance management partners, because they belong to different FRAC codes. Rotation of these two fungicides in a packhouse could ensure effective management of bull's eye rot (Serfontein, 2018). Postharvest fungicides can be applied in various methods postharvest including dipping, drenching and thermo-fogging. Dip treatment is the submerging of fruit in a fungicide solution, that is at a specific fungicide concentration, for a specified amount of time to ensure loading of optimum fungicide residue. Drench treatment is when fruit in field bins are passed through a shower of re-circulating fungicide solution immediately after harvest. Thermo-fog treatment entails the vaporisation of fungicide in a sealed room containing fruit in bins. The fruit is then left in the sealed room for an extended period.

The aim of this study was to confirm if fludioxonil and pyrimethanil could effectively control bull's eye rot on apple in South Africa as a postharvest treatment against the prevalent pathogen *N. vagabunda*. Moreover, different application methods of postharvest fungicides were tested for their efficacy in controlling *N. vagabunda* on apple. Lastly, *N. vagabunda* sensitivity to pyrimethanil and the effect of incubation time on fungicide efficacy was investigated.

MATERIALS AND METHODS

Dip application

The two fungicides fludioxonil (Teacher 230SC, ICA International, South Africa) and pyrimethanil (Protector 400SC, ICA International, South Africa) were tested as a postharvest dip against bull's eye rot on cultivars 'Cripps Pink' and 'Fuji'. Both fungicides were tested at four different concentrations: 200%, 100%, 50%, 0.25% and a 0% (control) of the recommended concentration (fludioxonil 299 mg/L; pyrimethanil 500 mg/L).

For each concentration, six fruit in replicates of five (N=30) were inoculated. Fruit were surface sterilised by washing with 70% ethanol for 30 s and then left to dry inside a laminar flow cabinet. After the fruit was dry, they were inoculated by punching a hole in the fruit with a sterile cork borer, the holes were 6 mm in diameter and approximately 5 mm deep.

Subsequently, a 6 mm plug was taken from the margin of an actively growing *N. vagabunda* (AN020) culture and placed in the wound hole.

Each fruit was inoculated two times, with each inoculation on opposite sides of the equatorial region of the fruit. Inoculated fruit were then incubated at 25°C for 24 hours in a clear Perspex moisture chamber with high relative humidity (RH) (>90%) to allow pathogen establishment. After incubation, the fruit were dipped for the recommended exposure time (fludioxonil, 30s; pyrimethanil, 1 min) in the different fungicide concentration solutions. After dipping, the fruit were left to dry, the wounds sealed with petroleum jelly and stored for 14 d in a moisture chamber (>90% RH) at ambient room temperature (24°C). After the 14 days, incidence of successful infection at the wound site was measured by evaluating symptom expression. The trial was repeated once. Residue analysis was performed on the one- and two-time recommended concentration treatments on both cultivars. Six fruit were used per treatment per cultivar for the analysis. The fruit were frozen at -20°C before being put through a food processor and processed to a pulp. The samples were then kept at -20°C before being sent to Hortec Analytical (Pty) Ltd., Somerset West, South Africa, for the residue analysis using liquid chromatography-mass spectrometry (LC/MS).

Drench application

Two fungicides were tested against bull's eye rot, namely fludioxonil (Teacher 230SC, ICA International, South Africa) and pyrimethanil (Protector 400SC, ICA International, South Africa), on two apple cultivars 'Cripps Pink' and 'Fuji'. Apple fruit were washed using 70% ethanol for 30 s, let to dry and then wounded and inoculated with a *N. vagabunda* mycelial agar plug following the same method mentioned with the postharvest fungicide dip application. Twelve fruit was tested in replications of three for each treatment (N=36). After 24 h of incubation at 24°C, fruit was put through a drench system with the recommended treatment concentration for fludioxonil (299 mg/L) or pyrimethanil (500 mg/L). The drenching mechanism consisted of the fungicides being made up to a 100 L and pumped out of a plastic tub (fungicide reservoir) into a plastic vented crate (L540 x W350 x H295 mm) at a rate of 55 L/min. The bottom of the crate had evenly spaced holes through which the fungicide could flow. The crate was then placed on top of another crate containing the inoculated and some uninoculated fruit (imitating industry drench application scenario). These crates were then put on a wire mesh on top of the fungicide reservoir so that the fungicide could re-circulate. Control fruit was inoculated but left untreated. Drenching time was 30 s for fludioxonil and 1 min for pyrimethanil. After drenching, fruit was left to dry and then stored at ambient room temperature for 14 d in a moisture chamber, thereafter disease incidence was measured. The trial was repeated once.

Thermo-fog application

Fludioxonil and pyrimethanil were applied as a thermo-fog treatment. For fludioxonil, eFog®-80 FDL (Pace International, Wapato, Washington, United States) and for pyrimethanil, eFog®-160 PYR (Pace International) were tested on 'Fuji' and 'Cripps Pink' apple cultivars. The fruit was washed and inoculated according to the same method used for the drench application trial. Twelve fruit was tested in replications of three for each treatment (N=36). The thermo-fog treatment was conducted 24 h after inoculation. Inoculated fruit were placed in vented plastic crates (L540 x W350 x H295 mm) and placed inside a sealed 5.32 m^3 container. An Electrofog EWH10000 fog machine (Chempac (Pty) Ltd., Simondium, South Africa) was used to apply the fungicides, following the operating instructions accordingly.

Both fungicides were applied at recommended concentrations of 60 mL per ton of apple fruit. After the fungicides were applied, the fruit were left exposed for 30 min before releasing the fog. Inoculation wounds were sealed with petroleum jelly and fruit was incubated for 14 d in a moisture chamber at 24°C, after which incidence of successful infection was determined. Although the trial was only done once, the number of fruits used was doubled to supply the results with more statistical robustness.

***Neofabraea vagabunda* sensitivity to pyrimethanil**

A total of six *N. vagabunda* isolates randomly selected (AN004, AN020, AN024, AN038, AN049 and AN098) were tested for their sensitivity to pyrimethanil *in vivo*, and to identify possible resistance. Only the cv. Cripps Pink was used in this trial. The recommended concentration (500 mg/L) and two times the recommended concentration (1000 mg/L) were tested. The same methodology was followed for the fruit inoculation and treatment as for the dip application trial including two inoculations per fruit. Disease severity was determined by measuring lesion diameters 14 days after incubation with a digital calliper. The trial was repeated once.

Influence of pathogen incubation times on fungicide efficacy

The effect of incubation time before treatment on fungicide efficacy was tested for both fludioxonil and pyrimethanil. 'Cripps Pink' apple fruit was washed with 70% ethanol and inoculated as per the dip application trial. However, treatment of the fruit was done at 6, 12 and 24 h after inoculation respectively. Twelve fruit was tested in replications of three for each treatment (N=36). After inoculation, the fruit were placed in a Perspex moisture chamber (>90% RH) at ambient room temperature (24°C) until treatment. The fruit was treated at the recommended concentrations of 299 mg/L fludioxonil and 500 mg/L pyrimethanil. After treatment, the inoculation wounds were sealed with petroleum jelly and the fruit were placed back into the moisture chamber. Successful infection was determined by evaluating symptom

expression, and lesion diameters were measured with a digital calliper 14 d after treatment. The trial was repeated once.

Statistical analysis

Statistical analysis was performed using Statistica V13.5 (TIBCO Software, California, USA). Data was analysed using either a one-way analysis of variance (ANOVA) or factorial ANOVA depending on the data set. Probability values (P-values) were generated with a 95% confidence interval to determine significant differences using Duncan's Multiple Range test.

RESULTS

Dip application

Fludioxonil and pyrimethanil, were applied at four different concentrations to investigate their ability to inhibit bull's eye rot incidence and concentration efficacy. The untreated fruit of both cultivars had high levels of bull's eye rot incidence. The incidence levels for the untreated fruit was 'Fuji' with 97% incidence in bull's eye rot and 'Cripps Pink' with 99% (Fig. 1A).

For the fludioxonil treated fruit, a steady decrease in disease incidence was obtained with increasing fludioxonil concentrations. At the lowest concentration of 74.75 mg/L active ingredient (a.i.) (25% of the recommended concentration), fludioxonil reduced incidence levels significantly for both cultivars compared to the untreated fruit. Incidence was reduced to 53% on 'Fuji" and 43% on 'Cripps Pink'. The incidence between the cultivars differed significantly. The 149.5 mg/L fludioxonil concentration (50% recommended concentration) yielded incidence levels of 31% on 'Fuji' and 26% on 'Cripps Pink'. The incidence reported with the 149.5 mg/L treatment was significantly less than that of the 74.75 mg/L treatment (Fig. 1A).

Although the recommended concentration of 299 mg/L fludioxonil reduced incidence on both 'Cripps Pink' and 'Fuji' to 16%, only the incidence on 'Fuji' was significantly different from the 149.5 mg/L treatment. The disease incidence levels obtained for the 1- and 2-times recommended concentration of 298 mg/L and 598 mg/L fludioxonil were not significantly different between the two cultivars. The 299 mg/L fludioxonil treatment reduced disease incidence to 16% on both cultivars, while the 598 mg/L fludioxonil treatment reduced incidence on 'Fuji' to 17% and 'Cripps Pink' to 8% (Fig. 1A).

None of the pyrimethanil treatments effectively reduced bull's eye rot disease incidence (Fig. 1B). Only the 2-times recommended concentration at 1000 mg/L on cv. Fuji significantly reduced disease incidence compared to the untreated fruit. On 'Cripps Pink', incidence levels for all the fungicide concentrations were above 95%. Although the 1000 mg/L treated 'Fuji' differed significantly from the untreated fruit, 82% incidence was still observed. The other treatments on 'Fuji' had incidences of 90% for 125 mg/L, 93% for 250 mg/L and 90% for 500 mg/L.

Residue measurements on fludioxonil treated ‘Fuji’ were 2.5 mg/kg for the 299 mg/L treatment and 3.6 mg/kg for the 598 mg/L treatment. Pyrimethanil treated ‘Fuji’ had 0.041 mg/kg for the 500 mg/L treatment and 1.7 mg/kg for the 1000 mg/L treatment. Fludioxonil residues on ‘Cripps Pink’ was 4.7 mg/kg for the 299 mg/L treatment and 7.1 mg/kg for the 599 mg/L treatment. Pyrimethanil on ‘Cripps Pink’ had 1.9 mg/kg and 2.5 mg/kg residue levels for the 500 mg/L and 1000 mg/L treatment respectively.

Drench application

For the drench treatment fludioxonil and pyrimethanil were both applied at recommended concentrations as a postharvest application. The untreated fruit yielded high disease incidence levels on ‘Fuji’ and ‘Cripps Pink’ (Fig. 2). ‘Fuji’ had 97% incidence which was not significantly different from cv. Cripps Pink’s 96%. The fludioxonil treatments performed significantly better than the pyrimethanil treatments. Incidence on ‘Fuji’ was reduced to 26% and, although significantly less than the disease incidence for ‘Fuji’, reduced incidence on ‘Cripps Pink’ to 57%. Pyrimethanil had no significant effect in reducing disease incidence on ‘Cripps Pink’ with a reported 95% incidence level. On ‘Fuji’ however, incidence was significantly reduced to 71%.

Thermo-fog application

Both the fludioxonil and pyrimethanil treatments significantly reduced disease incidence on both cultivars compared to the untreated control with 100% incidence on ‘Fuji’ and 99% on ‘Cripps Pink’ (Fig. 3). The fludioxonil thermo-fog application had better incidence reduction on ‘Fuji’ with 53% incidence compared to ‘Cripps Pink’ with 71% disease incidence. Each treatment on each cultivar differed significantly. The pyrimethanil treated ‘Fuji’ had the lowest incidence of all the treatments with only 41% disease incidence. However, pyrimethanil treated ‘Cripps Pink’ was the least successful treatment with 81% incidence. This was significantly less than for the untreated controls, but significantly more than the incidence obtained by the other treatments.

Effective application methods

There was great variance between the fungicides and the method of application (Figs. 4A and B). The dip application of fludioxonil produced the best overall results for this fungicide by reducing bull’s eye rot incidence on both ‘Fuji’ and ‘Cripps Pink’ to only 15%. The second-best control was obtained with the drench treatment on ‘Fuji’. Although, this treatment did not significantly differ from the fludioxonil dip on ‘Cripps Pink’. Thermo-fogging with fludioxonil was less effective at reducing disease incidence on ‘Cripps Pink’ compared to the other application methods. However, disease incidence for fludioxonil fogged ‘Fuji’ did not differ significantly from the fludioxonil drench treated ‘Cripps Pink’.

Pyrimethanil applied as a thermo-fog treatment had the best effect on 'Fuji' with 41% disease incidence. This was significantly less incidence recorded than the pyrimethanil drench which had 30% more disease incidence recorded on 'Fuji' with 71%. Pyrimethanil had low inhibition ability of incidence on cv. Cripps Pink. The pyrimethanil dip treated fruit still produced 99% incidence and the drench 95%. However, the pyrimethanil thermo-fog treatment significantly reduced incidence, compared to the untreated control, with 81% disease incidence.

***Neofabraea vagabunda* sensitivity to pyrimethanil**

Significant differences were observed in sensitivity to pyrimethanil applied as a dip treatment between the six *N. vagabunda* isolates (Fig. 5), with lesions diameters produced averaging between 8 and 15 mm. Isolate AN020 produced the largest lesion diameter (14.6 mm) with the 500 mg/L treatment. The smallest lesion diameter produced was by isolate AN024 (8.1 mm) with the 1000 mg/L treatment. Although there were differences in the average lesion diameter between the six *N. vagabunda* isolates, no significant difference was observed between the 500 mg/L and 1000 mg/L treatments for an isolate.

Influence of pathogen incubation times on fungicide efficacy

In the first repeat of the incubation effect trial, no *N. vagabunda* incidence was observed on fludioxonil treated fruit at the incubation times tested (data not shown). However, incidence was obtained in the repeat trial. Generally, all the fludioxonil treatments successfully controlled incidence on 'Cripps Pink' fruit and they all differed significantly from the untreated control (Fig. 6A). The incidence levels for the 6 h and 24 h incubation times did not differ significantly from each other with 15% and 14% respectively. However, the 12 h incubation time had significantly lower disease incidence of 1%. For the pyrimethanil treatment, an increase in *N. vagabunda* incidence was observed the longer fruit was incubated before the fungicide treatment was applied. Disease incidence did not differ significantly between the 24 h incubated and untreated fruit significantly with 96% and 100% being obtained respectively. The incidence levels for both the 12 h and 6 h incubation were both significantly lower compared to the untreated control and 24 h incubated fruit. For the 12 h incubation, 65% incidence was observed, and only 50% for the 6 h incubation. The pyrimethanil treatment had significantly higher disease incidence compared to the fludioxonil treatments.

The severity (lesion diameter) of disease obtained, on fruit that did have bull's eye rot incidence, for the pyrimethanil treated fruit after 6 h of incubation, did not differ significantly from that of the lesion diameters obtained for the fludioxonil treated fruit (Fig. 6B). The fludioxonil treated fruit had lesion diameters of 6.3 mm, 5.9 mm and 6.3 mm for the 6 h, 12 hr and 24 hr incubation times respectively. The lesion diameters obtained for fludioxonil treated

fruit were significantly different compared to the untreated control fruit, but not from each other. Only the 6 h incubation time pyrimethanil treated fruit, with an average lesion diameter of 8 mm, was significantly different from the untreated control fruit lesion diameter of 12.2 mm. The 12 h and 24 h pyrimethanil treated fruit had lesion diameters of 8.2 mm and 8.6 mm respectively, which did not differ significantly from the untreated control or the fludioxonil treated lesion diameters.

DISCUSSION

The most successful fungicide treatment was fludioxonil applied as a dip. Interestingly the recommended concentration treatment and double the recommended concentration treatment did not differ significantly. This suggests that fludioxonil, at the recommended concentration, is achieving optimal efficacy with an 84% reduction in *N. vagabunda* incidence on both 'Fuji' and 'Cripps Pink' apples when compared to the untreated control fruit.

The fludioxonil drench yielded promising results by reducing *N. vagabunda* incidence on 'Fuji' by 74% and 'Cripps Pink' by 41%. Lolas *et al*. (2016) found similar results with a 230 mg/L fludioxonil (Scholar 230 SC, Syngenta, Canada) drench treatment of 'Cripps Pink' apple fruit, naturally infected with *N. vagabunda*. The treatment reduced *N. vagabunda* incidence by 50% compared to the untreated control fruit. Aguilar *et al*. (2018) tested fludioxonil's curative ability against naturally infected *N. perennans* and *N. kienholzii* 'Fuji' fruit. They found that a postharvest dip application of fludioxonil at 285 mg/L was unable to significantly reduce *N. kienholzii* disease incidence when infection occurred 18-, 5- or 2-weeks before harvest. However, fludioxonil was capable of reducing *N. perennans* incidence when infection occurred 2 weeks before harvest (Aguilar *et al*., 2018).

Interestingly, Spotts *et al*. (2009) tested fludioxonil as a protective application against all four *Neofabraea* species on 'd'Anjou' pear fruit and found that a 150 mg/L fludioxonil treatment to significantly reduced disease incidence of *N. vagabunda* and *N. malicorticis*, but not *N. perennans* and *N. kienholzii.* Protective application of fungicides would, however, only be applicable in a preharvest treatment to protect orchard fruit from infection. Nonetheless, the findings by Spotts *et al*. (2009) and Aguilar *et al*. (2018) suggest that the fungicide fludioxonil is effective against *N. vagabunda* and *N. malicorticis*, but further research is required for a definitive answer. Thermo-fog treatment with fludioxonil delivered significant reduction of *N. vagabunda* incidence on both 'Fuji' and 'Cripps Pink' fruit, with a reduction of 47% and 28% respectively. Fludioxonil as a postharvest thermo-fog treatment was effective against *N. perennans* on cv. Fuji fruit inoculated two weeks before harvest with the treatment significantly reducing incidence of bull's eye rot (Aguilar *et al*., 2018).

The pyrimethanil dip treatment did not successfully control *N. vagabunda* disease incidence on either apple cultivar. Although both the one- and two-times recommended concentration significantly reduced incidence on cv. Fuji, high incidence was still obtained. Interestingly, the pyrimethanil drench significantly reduced disease incidence on both cultivars compared to the dip application, which is in contrast with the results from the fludioxonil dip and drench applications.

Other studies testing pyrimethanil against the bull's eye rot fungi found the fungicide much more successful in controlling bull's eye rot than the current study. Aguilar *et al.* (2018) tested pyrimethanil (500 mg/L) against *N. perennans* and *N. kienholzii* as a curative dip treatment. Pyrimethanil controlled bull's eye rot on cv. Fuji with incidence levels of inoculated fruit less than 10% for both *N. perennans* and *N. kienholzii*. Only in one of the five trials they conducted, did pyrimethanil not significantly reduce *N. perennans* incidence relative to the control (Aguilar *et al*., 2018). Spotts *et al*. (2009) found pyrimethanil (500 mg/L) to prevent bull's eye rot infection by all four *Neofabraea* species on 'd'Anjou' pear fruit with great success. Pyrimethanil restricted incidence levels to 0 to 4.2% when applied as a protective treatment. Application of pyrimethanil as a fog was the best method for controlling *N. vagabunda* in this study. Although incidence on 'Cripps Pink' was only reduced by 18%, there was still significantly lower incidence than was recorded for the other fungicide treatments. Incidence on 'Fuji' was reduced by 53% which was the most control achieved with pyrimethanil for any application method in the current study.

The results obtained with the pyrimethanil in the dip and drench applications differed from the results found in other studies testing pyrimethanil against the bull's eye rot fungi. These other studies found some degree of control with the same application methods as well as similar pyrimethanil products (Pentobec 400SC, Pace International) registered against bull's eye rot fungi (Spotts *et al.*, 2009: Aguilar *et al*., 2018). To investigate why the current study's results differed from other published results, the susceptibility of *N. vagabunda* isolates was tested against a one- and two-times recommended concentration pyrimethanil dip treatment. The effect of incubation time of inoculated fruit prior to treatment with fludioxonil and pyrimethanil was also evaluated for its role in influencing fungicide efficacy. Of the isolates that were tested for sensitivity, none of the lesion diameters obtained in the 500 mg/L treatments were significantly different from the 1000 mg/L treatments, each isolate thus responded equally to both treatments. In terms of incubation time and pyrimethanil efficacy, there was a steady decrease in pyrimethanil efficacy in inhibiting bull's eye rot incidence the longer fruit was inoculated prior to treatment. Pyrimethanil was thus unable to successfully control disease incidence possibly because the inoculation infection was too established for the fungicide to completely inhibit further development. Fludioxonil was highly effective in reducing *N. vagabunda* disease incidence regardless of the incubation time.

The inoculation method applied in this study was an aggressive method compared to other spore suspension inoculations. The inoculation plug method was used because the *N. vagabunda* isolates used in this study did not sporulate in culture. Pilot trials were conducted according to other studies that successfully induced *N. vagabunda* isolates to produce conidia (Spotts *et al.*, 2009; Cameldi *et al.*, 2017; Everett *et al*., 2017). Unfortunately, consistent sporulation of isolates could not be obtained and when sporulation occurred, it was in extremely low levels (data not included). Inducing sporulation on infected apple fruit was also unsuccessful as no conidiomata was produced and fruit only decayed when kept for a prolonged period.

In general, the treatments responded better on 'Fuji' than 'Cripps Pink', even though higher fungicide residue levels were found on 'Cripps Pink'. The only exception was the fludioxonil dip treatment, where there was no difference in the disease incidence levels between the two cultivars. Fludioxonil had higher residual activity than pyrimethanil on both cultivars. For both fungicides, higher residues were recorded on 'Cripps Pink', although better control was obtained on 'Fuji'. Several studies found 'Cripps Pink' highly susceptible to bull's eye rot fungi even when fungicides were applied (Cameldi *et al*., 2016; Neri *et al.*, 2019). Therefore, in this study, the fungicides also struggled to control bull's eye rot on 'Cripps Pink' more than on 'Fuji' possibly due to host-pathogen interactions. The maximum residue limit (MRL) of fludioxonil allowed for the European Union (EU) is 5 mg/kg and 15 mg/kg for pyrimethanil (www.ec.europa.eu). Only the two-times recommended concentration (598 mg/L) fludioxonil dip treatment on 'Cripps Pink' exceeded the MRL limit with 7.1 mg/kg. Fludioxonil and pyrimethanil have high residue activity and competent residue levels 5 months after cold storage (Xiao and Boal, 2009). Xaio and Boal (2009) recommended using the fungicides either as a pre-storage treatment or as an in-line application before packing, as opposed to using one fungicide multiple times. This will improve efficacy of fungicide use against postharvest decay fungi due to the fact that fludioxonil and pyrimethanil belongs to two different FRAC codes, as well as to ensure the MRL's are not exceeded for the local and export markets (Xiao and Boal, 2009; Serfontein, 2018). However, excessive overuse of one fungicide in one season or repetitive use in consecutive seasons can lead to loss in efficacy (Serfontein, 2018).

The use of fungicides as postharvest treatments against bull's eye rot fungi on pome fruit to reduce incidence after storage has been previously reported (Lennox *et al.*, 2004; Cameldi *et al*., 2016; Lolas *et al.*, 2016; Wood and Fisher, 2017; Aguilar *et al*., 2018). However, it is apparent that the bull's eye rot pathogens are not affected equally by specific fungicides (Henriquez *et al.*, 2006; Spotts *et al.*, 2009; Aguilar *et al*., 2018). The current study showed that fludioxonil is effective against *N. vagabunda* on apple fruit in the Western Cape of South Africa, and successfully reduces disease incidence. Pyrimethanil had some success in reducing disease incidence when incubation time of mycelial plug inoculations was shortened.

Other studies that found pyrimethanil effective, inoculated fruit with conidial suspensions of *Neofabraea* either before or after harvest (Spotts *et al.*, 2009; Aguilar *et al*., 2018). Aguilar *et al*. (2018) performed a study where they inoculated apple fruit preharvest on the tree with conidial suspensions to mimic natural infection as closely as possible and they found postharvest application of pyrimethanil significantly reduced *N. perennans* and *N. kienholzii* disease incidence.

Neofabraea pathogens survive during the latent period in the fruit lenticel as short hyphae that slightly penetrate the fruit flesh (Edney, 1958; Neri *et al.*, 2019). It is thus proposed, that pyrimethanil inefficacy in this study could be due to *N. vagabunda* infection being too advanced for the pyrimethanil to inhibit further development and lesion expression of the pathogen. However, this does not disprove pyrimethanil's ability to control bull's eye rot fungi as artificial inoculation does not adequately mimic natural infection of fruit. The hole puncture mycelial plug inoculation method used in this study ensured severe infection conditions and high disease incidence. Thus, this method tested the fungicides against severe *N. vagabunda* infected apple fruit by incubating fruit for 24 h before treating. Pyrimethanil completely lost its efficacy in reducing *N. vagabunda* incidence when fruit was treated 24 h after inoculation, but still significantly reduced lesion diameter. Thus, even though pyrimethanil could not reduce incidence, it had an effect on the pathogen and could significantly reduce *N. vagabunda* when the incubation times were shortened. With natural infections, *Neofabraea* sp. pathogens remain dormant in the infected lenticel cavities or just beneath the fruit surface when fruit get to the postharvest fungicide treatment stage (Henriquez *et al*., 2004; Neri *et al*., 2019). Pyrimethanil can therefore not be classified as ineffective against *N. vagabunda* from the Western Cape province of South Africa, because the inoculation method applied in this study completely breached the host's resistance by wounding the fruit.

This study found that submerging pome fruit in a fungicide suspension achieved better efficacy in reducing *N. vagabunda* incidence than drenching fruit with a recirculating fungicide suspension. Dipping fruit allows for uniform coverage of each individual fruit, which ensures improved fungicide contact time, as well as better managed application time. With the increase in concern for the use of pesticides and the risk to human consumption as well as high cost of waste disposal, use of alternative methods for pest management are encouraged. Fogging of fungicide active ingredients on fruit in the cold storage room is one such method. Less chemical waste is produced through fogging and significantly less amounts of water are required for treatment of fruit (Delele *et al.*, 2012). However, thermo-fogging is not always as effective as conventional methods due to the non-uniform distribution of fungicide related to various factors like air circulation, temperature and size of the cold storage room (Bertolini *et al.*, 1995; Delele *et al.*, 2012; Aguilar *et al*., 2018). Adjusting these factors can optimise for a specific fog application and increase fungicide efficacy (Delele *et al.*, 2012). Nonetheless,

fludioxonil and pyrimethanil as a fog treatment has been shown, in more than just this study, to significantly reduce bull's eye rot on apple (Aguilar *et al*., 2018). The pome fruit industry would benefit from a commercial scale trial with thermo-fogging of apple fruit with fludioxonil or pyrimethanil.

In conclusion, fludioxonil has the ability to reduce bull's eye rot incidence by *N. vagabunda* when applied as a postharvest fungicide application as either as a dip, drench or thermo-fog treatment. Pyrimethanil can reduce bull's eye rot incidence as a thermo-fog application and as a drench on 'Fuji' apples only. However, pyrimethanil as a dip or drench treatment cannot be classified as ineffective against *N. vagabunda* infection. As a recommendation, further investigation on the ability of the fungicides to reduce *N. vagabunda* as a postharvest application should be conducted on preharvest naturally infected or conidial suspension inoculated fruit.

REFERENCES

Aguilar, C.G., Mazzola, M. and Xiao, C.L. 2018. Control of bull's eye rot of apple caused by *Neofabraea perennans* and *Neofabraea kienholzii* using pre- and postharvest fungicides. Plant Disease 102: 905-910.

Bertolini, P., Guarnieri, A. and Venturi, P. 1995. Postharvest fog treatment of apples: Deposition patterns and control of *Phlyctema vagabunda* and superficial scald. Crop Protection 14: 345–348.

Bompeix, G. 1978. The comparative development of *Pezicula alba* and *P. malicorticis* on apples and in vitro (air and controlled atmosphere). Journal of Phytopathology 91: 97-109.

Cameldi, I., Neri, F., Ventrucci, D., Ceredi, G., Muzzi, E. and Mari, M. 2016. Influence of harvest date on bull's eye rot of 'Cripps Pink' apple and control chemical strategies. Plant Disease 100: 2287-2293.

Cameldi, I., Neri, F., Menghini, M., Pirondi, A., Nanni, I.M., Collina, M. and Mari, M. 2017. Characterization of *Neofabraea vagabunda* isolates causing apple bull's eye rot in Italy (Emilia-Romagna region). Plant Pathology 66: 1432-1444.

Coates, L. and Johnson, G. 1997. Postharvest diseases of fruit and vegetables. Pages 533-547 in: Plant Pathogens and Plant Diseases. Rockvale Publications, Burlington, USA.

Creemers, P. 1989. Chemical control of parasitic storage diseases on apple and pear. Acta Horticulturae 258: 645-653.

Delele, M.A., Vorstermans, B., Creemers, P., Tsige, A.A., Tijskens, E., Schenk, A., Opara, U.L., Nicolaï, B.M. and Verboven, P. 2012. Investigating the performance of thermonebulisation fungicide fogging system for loaded fruit storage room using CFD model. Journal of Food Engineering 109: 87-97.

Edney, K.L. 1958. Observations on the infection of Cox's Orange Pippin apples by *Gloeosporium perennans* Zeller and Childs. Annals of Applied Biology. 46: 622-629.

Edney, K.L. 1964. A comparison of the production of extracellular enzymes and rotting of apples by *Pezicula alba* and *P. malicorticis*. Transactions of the British Mycological Society 47: 215-225.

Everett, K.R., Pushparajah, I.P.S., Fisher, B.M. and Wood, P.N. 2017. A simple method for conidial production and establishing latent infections of apples by *Phlyctema vagabunda* (syn: *Neofabraea alba*). New Zealand Plant Protection 70: 106-111.

Grove, G.G. 1990. Anthracnose and perennial canker. Pages 36-38 in: Compendium of Apple and Pear Diseases. (A. L. Jones and H. S. Aldwinckle, eds.) St. Paul, Minnesota: The American Phytopathological Society.

Grove, G.G., Dugan, F. M. and Boal, R. J. 1992. Perennial canker of apple: seasonal host susceptibility, spore production, and perennation of *Cryptosporiopsis perennans* in infected fruit in eastern Washington. Plant Disease 76: 1109-1114.

Henriquez, J.L., Sugar, D. and Spotts, R.A. 2004. Etiology of bull's eye rot of pear caused by *Neofabraea* spp. in Oregon, Washington, and California. Plant Disease 88: 1134-1138.

Henriquez, J.L., Sugar, D. and Spotts, R.A. 2006. Induction of cankers on pear tree branches by *Neofabraea alba* and *N. perennans*, and fungicide effects on conidial production on cankers. Plant Disease 90: 481-486.

Henriquez, J.L., Sugar, D. and Spotts, R.A. 2008. Effects of environmental factors and cultural practices on bull's eye rot of pear. Plant Disease 92: 421-424.

Ivic, D. 2010. Curative and eradicative effects of fungicides. Fungicides. IntechOpen. www.intechopen.com.

Köhl, J., Wenneker, M., Groenenboom-de Haas, B.H., Anbergen, R., Goossen-van de Geijn, H.M., Lombaers-van der Plas, C.H., Pinto, F.A.M.F. and Kastelein, P. 2018. Dynamics of postharvest pathogens *Neofabraea* spp. and *Cadophora* spp. in plant residues in Dutch apple and pear orchards. Plant Pathology 67: 1264-1277.

Lattanzio, V., Di Venere, D., Linsalata, V., Bertolini, P., Ippolito, A. and Salerno, M. 2001. Low temperature metabolism of apple phenolics and quiescence of *Phlyctema vagabunda*. Journal of Agricultural and Food Chemistry 49: 5817-5821.

Lennox, C.L., Spotts, R.A. and Booyse, M. 2004. Incidence of postharvest decay of 'd'Anjou' pear and control with a thiabendazole drench. Plant Disease 88: 474-478.

Lolas, M., Díaz, G., Mendez, R., Cáceres, M. and Neubauer, L. 2016. Evaluation of the efficacy of fungicide fludioxonil in the postharvest control of bull's eye rot (*Neofabraea alba*) in Chile. Acta Horticulturae 1144: 461-464.

Moggia, C., Yuri, J.A., Lolas, M. and Pereira, M. 2003. Use of thermo-fogging for DPA and fungicides applications in Chile. Proceedings of Washington tree fruits postharvest conference, Wenatchee, USA.

Neri, F., Cappellin, L., Aprea, E., Biasioli, F., Gasperi, F., Spadoni, A. and Cameldi, I. 2019. Interplay of apple volatile organic compounds with *Neofabraea vagabunda* and other postharvest pathogens. Plant Pathology 68: 1033-1232.

Rosslenbroich, H.J. and Stuebler, D. 2000. *Botrytis cinerea* - history of chemical control and novel fungicides for its management. Crop Protection 19: 557-561.

Serfontein, K. 2018. Resistance management for the postharvest actives fludioxonil and pyrimethanil in pome fruit packhouses. South African Fruit Journal 17: 45-46.

Spotts, R.A., Seifert, K.A., Wallis, K.M., Sugar, D., Xiao, C.L., Serdani, M. and Henriquez, J.L. 2009. Description of *Cryptosporiopsis kienholzii* and species profiles of *Neofabraea* in major pome fruit growing districts in the Pacific Northwest USA. Mycological Research 113: 1301-1311.

Wood, P.N. and Fisher, B.M. 2017. The effect of fungicides on spore germination, mycelial growth and lesion development of *Phlyctema vagabunda* (syn: *Neofabraea alba*) (bull's eye rot of apples). New Zealand Plant Protection 70: 112-119.

Xiao, C.L. and Boal, R.J. 2009. Residual activity of fludioxonil and pyrimethanil against *Penicillium expansum* on apple fruit. The American Phytopathological Society 93: 1003-1008.

TABLES AND FIGURES

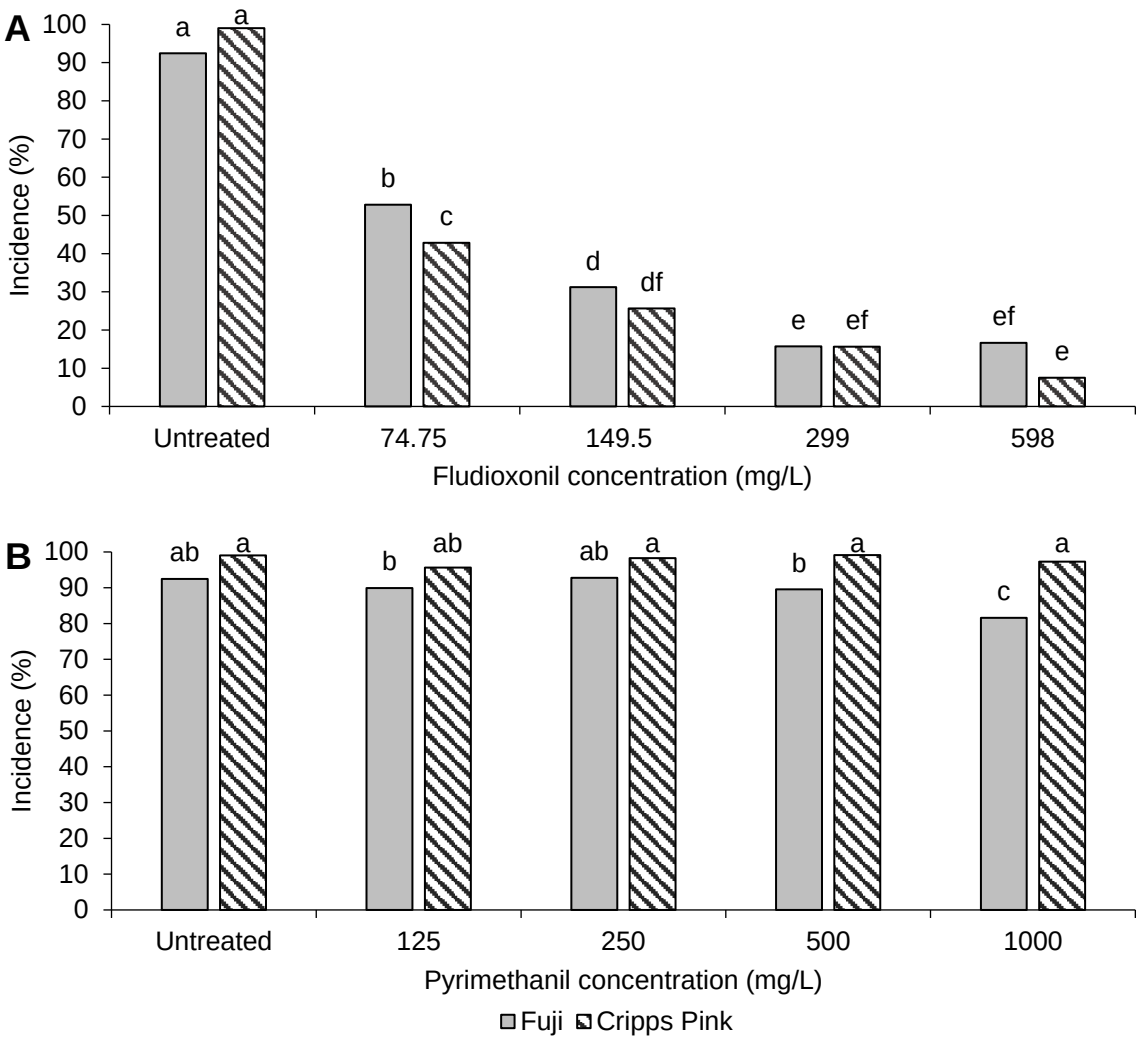

Figure 1. Effect of the fungicides fludioxonil (**A**) and pyrimethanil (**B**) as a postharvest curative dip application on *Neofabraea vagabunda* incidence of 'Fuji' and 'Cripps Pink' apples. Fruit were inoculated 24 hours before treatment. Fungicides were applied at four different concentrations. Recommended application concentration postharvest on pome fruit for fludioxonil is 299 mg/L and 500 mg/L for pyrimethanil. The Duncan's Multiple Range Test was used to identify significant differences between fungicide concentrations. Means with the same letter are not significantly different (P<0.05).

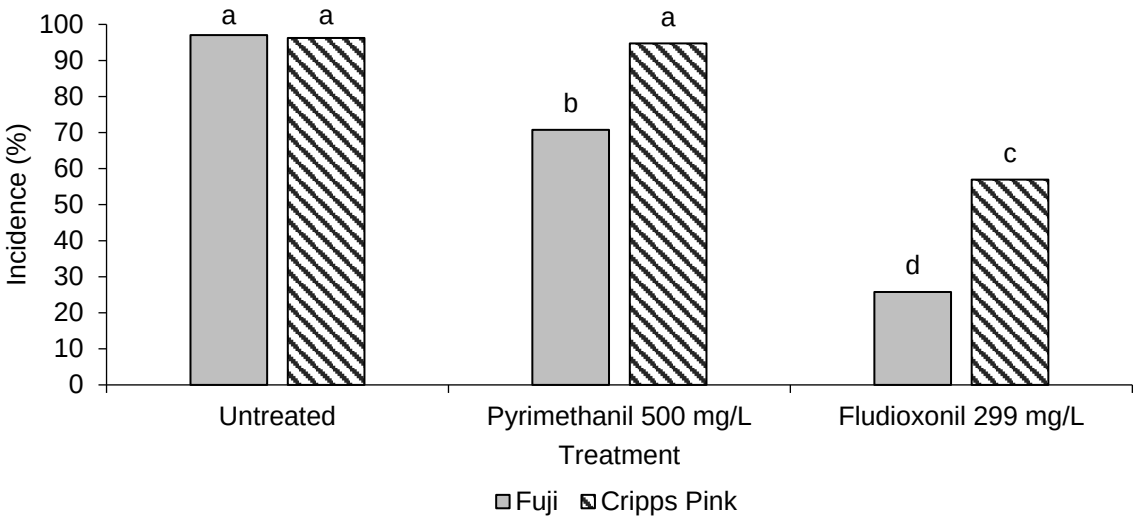

Figure 2. Effect of fludioxonil and pyrimethanil applied at recommended concentrations as a postharvest curative drench treatment on *Neofabraea vagabunda* incidence of 'Fuji' and 'Cripps Pink' apples. Fruit were inoculated 24 hours before treatment The Duncan's Multiple Range Test was used to identify significant differences between fungicide treatments. Means with the same letter are not significantly different (P<0.05).

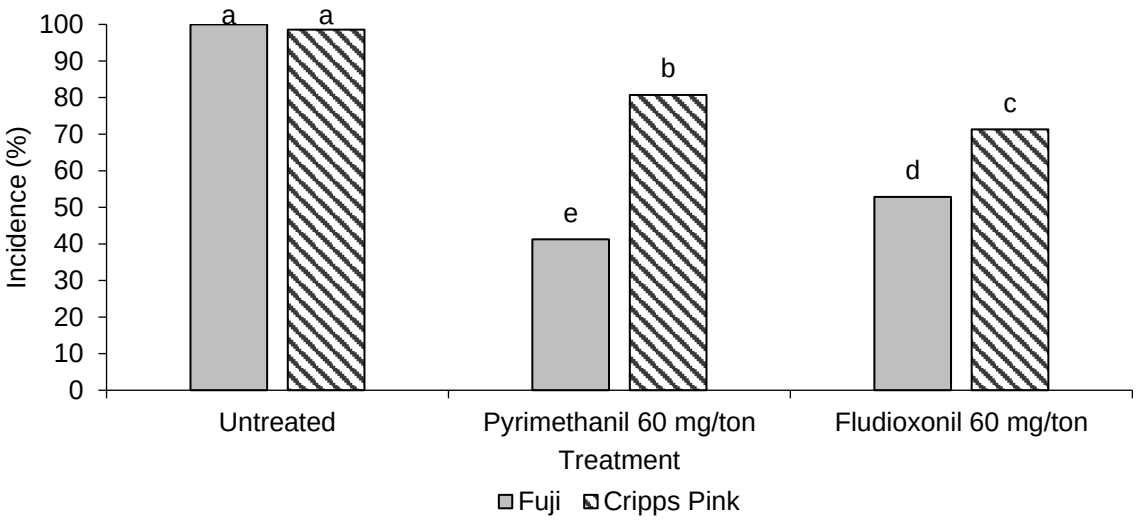

Figure 3. Effect of fludioxonil and pyrimethanil applied at recommended concentrations as a postharvest curative thermo-fog treatment on *Neofabraea vagabunda* incidence of 'Fuji' and 'Cripps Pink' apples. Fruit were inoculated 24 hours before treatment. The Duncan's Multiple Range Test was used to identify significant differences between fungicide treatments. Means with the same letter are not significantly different ($P<0.05$).

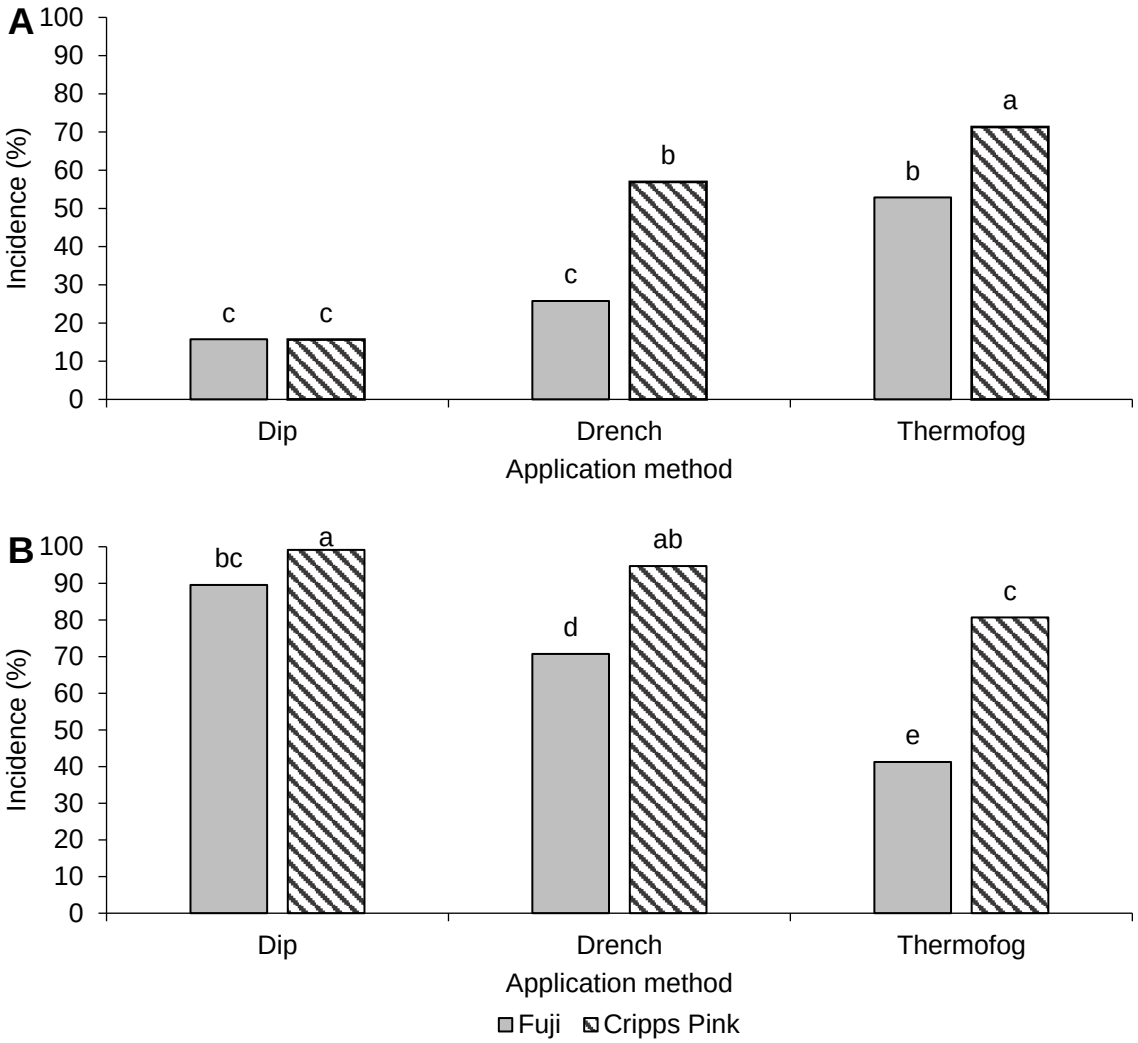

Figure 4. Influence of fludioxonil (**A**) and pyrimethanil (**B**) on *Neofabraea vagabunda* incidence on 'Fuji' and 'Cripps Pink' apple when applied as different postharvest treatment methods. Fruit were inoculated 24 hours prior to treatment. The fungicides were applied at recommended concentrations for each application method. The Duncan's Multiple Range Test was used to identify significant differences between fungicide treatments. Means with the same letter are not significantly different (P<0.05).

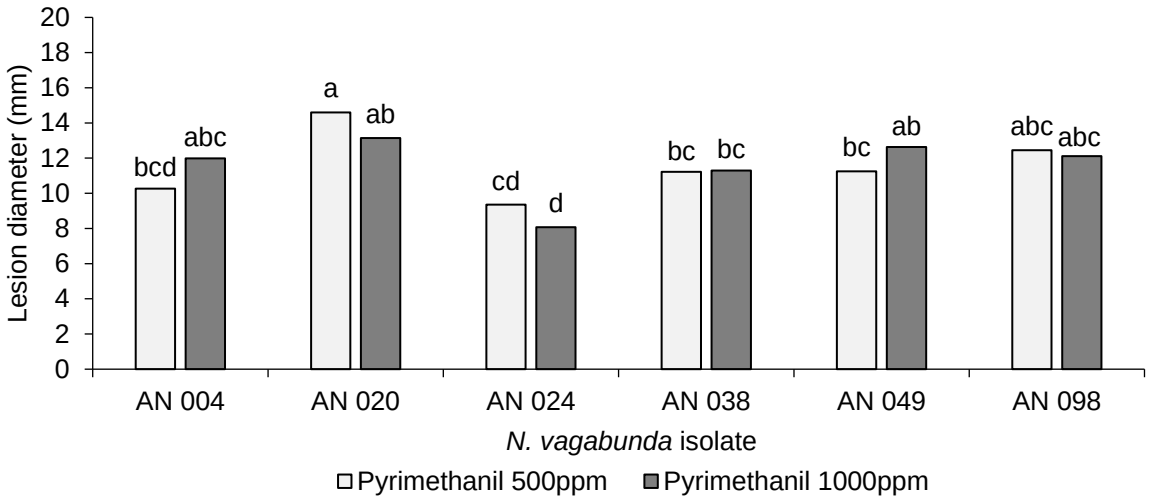

Figure 5. Influence of the fungicide pyrimethanil as a postharvest dip treatment at two different concentrations on the lesion diameter of *Neofabraea vagabunda* on 'Cripps Pink' apple. Fruit was inoculated with six different isolates and incubated for 24 hours before treatment. The Duncan's Multiple Range Test was used to identify significant differences between fungicide treatments. Means with the same letter are not significantly different (P<0.05).

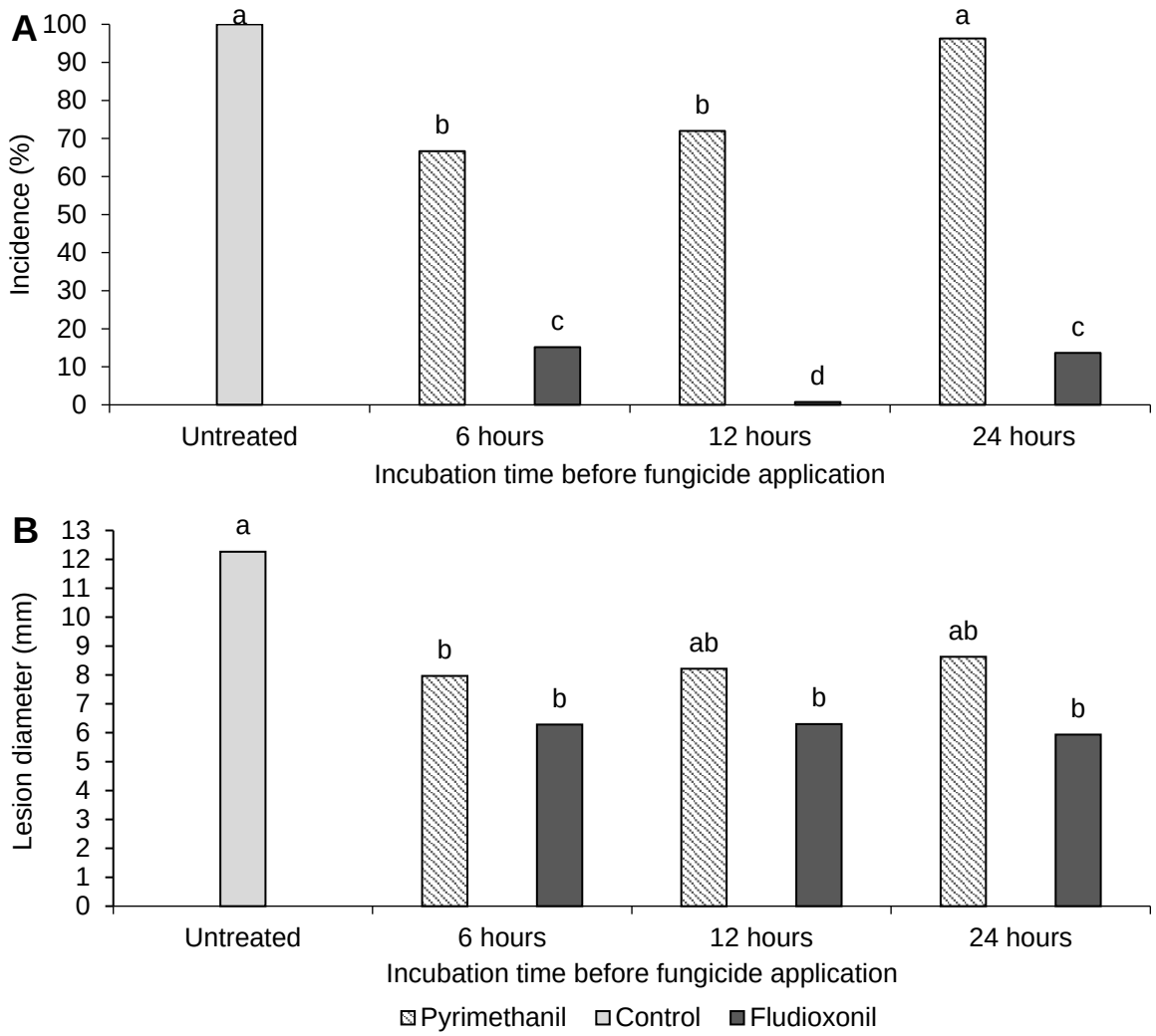

Figure 6. Influence of incubation time on the efficacy of pyrimethanil and fludioxonil against *Neofabraea vagabunda* disease incidence (**A**) and disease severity (**B**) on 'Cripps Pink' inoculated apple fruit. The Duncan's Multiple Range Test was used to identify significant differences between fungicide treatments. Means with the same letter are not significantly different (P<0.05).

APPENDIX

Table 1. Analysis of variance for the average bull's eye rot lesion diameter caused by *N. vagabunda* inoculated 'Cripps Pink', 'Fuji' and 'Golden Delicious' apple fruit. *Neofabraea vagabunda* isolates were obtained from different areas throughout the Western Cape.

Effects	DF[a]	Sum of Squares	F	Pr > F
Isolate	7	724,27	69,135	<0,0001
Cultivar	2	188,72	18,014	<0,0001
Isolate*Cultivar	14	18,19	1,736	0,045670

[a]Degrees of Freedom

Table 2. Analysis of variance for percentage incidence of bull's eye rot caused by *N. vagabunda* inoculated 'Cripps Pink' and 'Fuji' apple fruit dip treated with fludioxonil at 0, 74.75, 149.5, 299 and 598 mg/L 24 hours after inoculation.

Effects	DF[a]	Sum of Squares	F	Pr > F
Cultivar	1	0,4434	2,959	0,085609
Treatment	4	118,8111	198,229	<0,0001
Cultivar*Treatment	4	1,2240	2,042	0,086226

[a]Degrees of Freedom

Table 3. Analysis of variance for percentage incidence of bull's eye rot caused by *N. vagabunda* inoculated 'Cripps Pink' and 'Fuji' apple fruit dip treated with pyrimethanil at 0, 125, 250, 500 and 1000 mg/L 24 hours after inoculation.

Effects	DF[a]	Sum of Squares	F	Pr > F
Cultivar	1	2,130	36,43	<0,0001
Treatment	4	0,615	2,63	0,033131
Cultivar*Treatment	4	0,416	1,78	0,130924

[a]Degrees of Freedom

Table 4. Analysis of variance for percentage incidence of bull's eye rot caused by *N. vagabunda* inoculated 'Cripps Pink' and 'Fuji' apple fruit drench treated with fludioxonil and pyrimethanil at recommended concentrations 24 hours after inoculation.

Effects	DF[a]	Sum of Squares	F	Pr > F
Cultivar	1t	6,2538	48,035	<0,0001
Fungicide	2	42,2622	162,305	<0,0001
Cultivar*Fungicide	2	3,4688	13,322	<0,0001

[a]Degrees of Freedom

Table 5. Analysis of variance for percentage incidence of bull's eye rot caused by *N. vagabunda* inoculated 'Cripps Pink' and 'Fuji' apple fruit thermo-fog treated with fludioxonil and pyrimethanil at recommended concentrations 24 hours after inoculation.

Effects	DF[a]	Sum of Squares	F	Pr > F
Cultivar	1	7,5328	51,815	<0,0001
Fungicide	2	26,9109	92,556	<0,0001
Cultivar*Fungicide	2	5,9082	20,320	<0,0001

[a]Degrees of Freedom