Pathogenicity and Host Specificity of *Penicillium* spp. on Pome and Citrus Fruit

By

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DECLARATION

I, Johannes Petrus Louw, declare that the dissertation, which I hereby submit for the degree Magister Scientiae Agriculturae (Plant Pathology) at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institute.

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Johannes Petrus Louw

March 2014

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ABSTRACT

Title: The pathogenicity and host specificity of *Penicillium* spp. on pome and citrus fruit

Penicillium includes some of the most concerning postharvest pathogens of pome and citrus fruit. The pathogenicity and aggressiveness of selected *Penicillium* spp. previously isolated from South African and European Union fruit export chains were investigated on pome and citrus fruit. New insight and findings were documented in this study. *Penicillium digitatum*, the most aggressive pathogen on citrus, was also identified the most aggressive on 'Beurre Bosc', 'Beurre Hardy' and 'Sempre Rosemarie' pears. It was also the third most aggressive species on 'Granny Smith' and 'Cripps Pink' apples. To our knowledge this is the first report where *P. digitatum* has been described as an aggressive pathogen on certain pome fruit cultivars. The most concerning species in terms of decay on the evaluated apple cultivars ('Royal Gala', 'Granny Smith', 'Golden Delicious', 'Topred' and 'Cripps Pink') and two pear cultivars ('Packham's Triumph' and 'Forelle') were *P. expansum* and *P. crustosum* respectively. New reports concerning spoilage caused by these species were noted on citrus. *Penicillium expansum* decay and tissue-response lesions were noted on 'Nules Clementine', 'Owari Satsuma', 'Delta Valencia', 'Midknight Valencia' and 'Eureka' seeded. *Penicillium crustosum* caused decay and tissue-response lesions on 'Nules Clementine', 'Nova', 'Owari Satsuma', 'Delta Valencia', 'Cambria Navel', 'Eureka' seeded and 'Star Ruby'. In contrast to more aggressive infections and large surface lesions, some tissue-response lesions sporulated despite their small size, thus allowing the species to complete their life cycle. The second most aggressive species affecting citrus was *P. italicum*. Pathogenicity of *P. solitum* was also confirmed on some apple and pear cultivars, although a broader cultivar range and higher level of aggression was observed on pears. *Penicillium brevicompactum* was only found to be pathogenic on pears. New information regarding host-*Penicillium* interactions, the potential of cross-infection and the impact each species may have on fruit moving through the market chain was added. Future studies should examine the link between host susceptibility as influenced by maturity and the pathogenic potential of non-host pathogens. Further research is needed to elaborate on the pathogenicity of *P. digitatum* on pome fruit. Information on market-end losses, the causal agents involved, and inoculum levels and sources may prove beneficial in solving industry problems at the retail-end.

Supervisor: Prof. L. Korsten

Chapter 1

General Introduction

The South African citrus and deciduous fruit industries are considered major players in the world export markets (CGA, 2012; Hortgro, 2012). In combination, 1 151 490 tons of apples and pears were produced in 2011/12 of which 46.6% were traded as fresh produce, earning R3 543 335 147 in net export realisation (Hortgro, 2012). The citrus industry produced 2 230 887 tons of fruit of which 64% (1 424 715 tons) were exported fresh, earning an export gross total of R5 933 158 602 at the end of 2011 (CGA, 2012). Export volumes of this magnitude require well-organised fruit supply management systems, ensuring trade in blemish free, high quality fruit. Extended supply chains prolong exposure of fresh produce to postharvest losses at the market-end. In addition, climacteric fruit such as apples and pears (Kader, 2002; 2011) can undergo ripening under substandard storage and/or transport conditions (Lara and Vendrelle, 1998; Villalobos-Acuña and Mitcham, 2008), resulting in more susceptible fruit (Janisiewicz *et al*., 2008; Vilanova, *et al*., 2012a). Further understanding of postharvest losses throughout the supply chain will prove beneficial to the industries involved.

Previous studies have described citrus and pome fruit markets being under pressure and suffering severe economic losses due to *Penicillium* decay (Eckert and Ogawa, 1985; Rosenberger, 1990; Kinay *et al*., 2001; Reddy *et al*., 2010; Vico *et al*., 2010). Up to 25% of the total fruit crops can become infected with fungal pathogens in both developed and developing countries. Spoilage can in some instances result in postharvest losses of up to 50% (Eckert and Ogawa, 1985; FAO, 1989; Campbell and Reece, 2002; Spadaro and Gullino, 2004; Mahlo, 2009; Roslan *et al*., 2010; Marcet-Houben *et al*., 2012). Among the spoilage causing fungi, *Penicillium* spp. are one of the most important pathogens of apples and pears (Jones and Aldwinckle, 1990; Sanderson and Spotts, 1995; Spotts *et al*., 1998; Pianzzola *et al*., 2004; Kim *et al*., 2005), and are considered the main postharvest pathogen of citrus (Eckert and Ogawa, 1985; Kinay *et al*., 2001; Mahlo, 2009; Marcet-Houben *et al*., 2012). *Penicllium digitatum* (Pers. ex Fr.) Sacc is known as the most destructive postharvest pathogen of citrus causing up to 90% of total losses (Eckert, 1989, Marcet-Houben *et al*., 2012).

Penicillium belongs to the family Trichocomaceae and includes more than 250 accepted species (Houbraken and Samson, 2011). The genus is divided into four subgenera, if the formerly used teleomorph (*Eupenicillium*) is treated separate from *Penicillium*, according to Pitt (1980) (Pitt and Hocking, 2009; Houbraken and Samson, 2011). Of the subgenera, subgenus *Penicillium* includes the most concerning species in terms of food spoilage (Frisvad and Samson, 2004). In 2004 Frisvad and Samson (2004) described more species belonging to

the subgenus *Penicillium*, increasing the number from 23 (according to Pitt, 1979; 1980) to 58. These species all possesses terverticillate penicillin. Conidia can be spherical to ellipsoidal shaped and blue, green and/or grey coloured or ellipsoidal to fusiform/cylindriodal and blue, green or grey (Pitt and Hocking, 2009).

Pathogenic *Penicillium* spp. typically follow necrotrophic lifecycles (Marcet-Houben *et al*., 2012; Prusky *et al*., 2013; Vilanova *et al*., 2014). The genus produces copious amounts of air-borne asexual conidia, making most species ubiquitous (Frisvad and Samson, 2004). *Penicillium* spp. mainly rely on wounding for host entry, but some can also infect via natural openings (i.e. lenticels) (Rosenberger, 1990; Lahlali *et al*., 2005; Marcet-Houben *et al*., 2012; Vilanova *et al*., 2014). Numerous species are also quiescent, able to enter their host, lie dormant and develop symptoms further down the supply chain when conditions become more favourable (Prusky *et al*., 2013). The release of toxins, enzymes and other secondary metabolites produced by the species cause necrosis of living host tissue, which can then be invaded and colonised (Frisvad and Samson, 2004; Carris *et al*., 2012). The ability of some *Penicllium* spp. to dominate under certain conditions is mainly due to their capability to survive and/or grow at low temperatures and cover broad host ranges. *Penicillium digitatum* and *P.italicum* Wehmer for instance, cover small host ranges, where *P. expansum* Link ex Gray covers a broad host range (Frisvad and Samson, 2004; Pitt and Hocking, 2009), becoming a concern for numerous industries (Pitt and Hocking, 2009).

Penicillium spp. pathogenicity on pome fruit have extensively been reported (Frisvad and Samson, 2004; Pitt and Hocking, 2009). The concerning *Penicillium* spp. on pome fruit in terms of aggression include *P. expansum*, *P. crustosum* Thom and *P. solitum* Westling (Kim *et al*., 2005, Sanderson and Spotts 1995, Spotts *et al*., 1998). Other pathogenic species include *P. aurantiogriseum* Dierckx., *P. brevicompactum* Dierckx., *P. carneum* Frisvad , *P. commune* Thom, *P. griseofulvum* Dierckx. and *P. verrucosum* Dierckx. (Amiri and Bompeix, 2005; Jones and Aldwinckle, 1990; Moslem *et al*., 2010; Peter *et al*., 2012; Sholberg and Haag, 1996). Additional *Penicillium* spp. have been isolated from pome fruit environments, some of which are capable of causing decay on other fruit crops (Sanderson and Spotts, 1995). Examples include *P. digitatum* on citrus (Holmes and Eckert, 1999) and *P. glabrum* (Wehmer) Westling on pomegranates (Bardas *et al*., 2009). Importantly, *P. digitatum* was also isolated from lesions on market pears. However, the isolate used was not found pathogenic on fresh “d’Anjou” pears. Recently, Vilanova *et al*. (2012a) indicated *P.*

digitatum infection reactions on "Golden Smoothee" apples, also implying the pathogenic potential of *P. digitatum* on pome fruit.

The recognised pathogenic *Penicillium* spp. of citrus are *P. digitatum*, *P. italicum* and *P. ulaiense* Hsieh, Su & Tzean (Frisvad and Samson, 2004; Pitt and Hocking, 2009). The pathogenic ability of *P. crustosum* on citrus has been described in two papers (Garcha and Singh in 1976; Arrebolla *et al*., 2010). Pathogenic reactions caused by *P. expansum* on citrus have also been reported by other researchers (Macarisin *et al*. 2007; Vilanova *et al*., 2012b). *Penicillium fellutanum* Biourage was reported pathogenic on citrus in 1946 (Sinha, 1946), but confirmation is still lacking. Papers demonstrating *P. expansum* and *P. crustosum* pathogenicity on citrus seems uncommon, even absent when considering pathogenicity on all the economic important *Citrus* spp. Additionally, a need exist to report the aggressiveness of each species on a large citrus host range (different *Citrus* spp. and cultivars). This will indicate the decay pressure that different *Penicillium* spp. may have on *Citrus* spp. cultivars. Expressing decay prominence and *Penicillium* spp. dominance in the chain will provide more assertive direction into solving *Penicillium* related industry problems.

Motivation to do this project has been highlighted in the above mentioned paragraphs, attributing economic importance, lack of host range and specificity, and isolate aggressiveness. The project aims to evaluate *Penicillium* spp. pathogenicity on pome and citrus fruit cultivars. The most effective inoculation method and the effect of cold storage conditions on *Penicillium* spp. disease interactions will also be assessed in this study. The findings will shed more light on the nature of *Penicillium* spp. prominent in the pome and citrus fruit supply chains, demonstrate aggressiveness of isolates on a broad spectrum of cultivars and verify temperature based symptom expression and disease sensitivity of pathogenic *Penicillium* spp. Information provided in this dissertation will be beneficial in solving industry concerns related to postharvest *Penicillium* decay of pome and citrus fruit.

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Chapter 2

Literature Review

Pathogenic *Penicillium* spp. of Economic Important Deciduous and Citrus Fruit

1. INTRODUCTION

The genus *Penicillium* is very broad, including over 250 accepted species (excluding synonyms) (Houbraken and Samson, 2011) of which the major food spoilage species belong to *Penicillium* subgenus *Penicillium* (Frisvad and Samson, 2004; Pitt and Hocking, 2009). The pathogenic *Penicillium* spp. have necrotrophic lifecycles (Marcet-Houben *et al*., 2012; Prusky *et al*., 2013; Vilanova *et al*., 2014), relying on toxins and enzymes to cause necrosis of living host tissue, which will be colonised (Frisvad and Samson, 2004; Carris *et al*., 2012; Sanzani, 2012). Some of the mycotoxins produced by *Penicillium* spp. are of food safety concern to animals and humans when affected products are consumed (Serra *et al*., 2006; Frisvad and Samson, 2004; Pitt and Hocking, 2009). *Penicillium* spp. are wound pathogens, but some can enter through lenticels (Rosenberger, 1990; Lahlali *et al*., 2005; Marcet-Houben *et al*., 2012; Vilanova *et al*., 2014) and may possess an inactive infection stage (quiescent) prior to causing decay (Dijksterhuis, 2007; Prusky *et al*., 2013). Their ability to build up resistance against chemical control measures is of concern to fresh produce industries (Boubaker *et al*., 2009; Pitt and Hocking, 2009; Malandrakis *et al*., 2013).

Penicillium is a problem for numerous fruit, grain and vegetable products (fresh and processed) (Pitt and Hocking, 2009). However, their role in causing fresh fruit losses is of pronounced concern, especially on the export markets. *Penicillium* spp. greatly contribute to postharvest fruit losses and lower fruit quality in the pome fruit (Sanderson and Spotts, 1995; Pianzzola, *et al*., 2004; Kim, *et al*., 2005), stone fruit (Ceponis and Friedman, 1957; LaRue and Johnson, 1989; Molinu *et al*., 2012), table grape (Barkai-Golan, 2008) and citrus (Eckert and Eaks, 1989; Marcet-Houben *et al*., 2012) industries. *Penicillium* can also become a serious problem in the litchi industry (Jacobs and Korsten, 2004; Lichter *et al*., 2004). Numerous other fruit crops are also affected by *Penicillium* decay, including among others soft fruit (i.e. strawberries and raspberries) and other tropical (i.e. mango, pineapples) and subtropical fruit (i.e. avocado, kiwifruit, melon and pomegranate) (Snowdon, 1990; Pitt and Hocking, 2009). This literature study will primarily focus on *Penicillium* decay of pome fruit, stone fruit, table grapes and citrus, since these fruit crops are the most important in South African terms of production and exports (Faostat, 2014a, 2014b), and are seriously affected by postharvest *Penicillium* decay.

Well-documented pathogenic *Penicillium* spp. of pome fruit include *P. expansum* Link, *P. crustosum* Thom and *P. solitum* Westling (Sanderson and Spotts 1995; Spotts *et al*., 1998; Kim *et al*., 2005). Other reported pathogenic species include *P. digitatum* (Pers.:Fr.) Sacc. (Vilanova *et al*., 2012a; Louw and Korsten, 2014; Vilanova *et al*., 2014), *P. aurantiogriseum* Dierckx., *P. brevicompactum* Dierckx., *P. carneum* (Frisvad) Frisvad, *P. commune* Thom, *P. griseofulvum* Dierckx. and *P. verrucosum* Dierckx. (Jones and Aldwinckle, 1990; Sholberg and Haag, 1996; Amiri and Bompeix, 2005; Moslem *et al*., 2010; Peter *et al*., 2012). The pathogenic ability of *P. digitatum* has only recently been described on pome fruit (Louw and Korsten, 2014; Vilanova *et al*., 2014), although Sanderson and Spotts (1995) isolated the species from lesions on market pears in 1991-2. However, they failed to find the species pathogenic on fresh 'd'Anjou' pears. They theorised that the species is able to colonise over-mature pears (Sanderson and Spotts, 1995). Vilanova *et al*. (2012a) later showed *P. digitatum* causing infections and/or hypersensitive responses (HR), but not decay on 'Golden Smoothee' apples. Louw and Korsten (2014) were the first to fully describe *P. digitatum* as a pathogen on apples and pears, showing cultivar differences. A more recent paper confirms findings on apples related to this study (Vilanova *et al*., 2014).

The only confirmed pathogenic *Penicillium* sp. of nectarines and plums is *P. expansum* (Ceponis and Friedman, 1957; LaRue and Johnson, 1989; Snowdon, 1990; Barkai-Golan, 2001; Molinu *et al*., 2012). *Penicillium chrysogenum* Thom has been reported pathogenic on black plums (*Vitex doniana* Nielson) (Eseigbe and Bankole, 1996) and a single report demonstrated *P. crustosum* decay on peaches (Restuccia *et al*., 2006), but the host species differ from the more common, commercial nectarines and plums. *Penicillium digitatum* was isolated from plums in Parlier (Fresno Co., California) in 1996, but no further studies were conducted to conclude that the species is pathogenic on plums (Ma *et al*., 2003). Reports demonstrating a larger *Penicillium* spp. pathogen range on nectarines and plums are lacking.

Over 30 *Penicillium* spp. have been isolated from grapes (Crous and Phillips, 2000; Magnoli *et al*., 2003; Franck *et al*., 2005; USDA, 2005; Donoso and Latorre, 2006; Kim *et al*., 2007; Patiño *et al*., 2007; Serra and Peterson, 2007; Serra, 2007; Barkai-Golan, 2008; Nonaka *et al*., 2011; Deng *et al*., 2012; Sang *et al*., 2013). The majority of these species have unfortunately not been proven pathogenic on grapes. Pathogenic species according to Barkai-Golan (2008) include *P. aurantiogriseum*, *P. brevicompactum*, *P. chrysogenum*, *P. citrinum* Thom, *P. crustosum*, *P. cyaneofulvum* Biourge, *P. cyclopium* Westling, *P. decumbens* Thom, *P. frequentans* Westling, *P. glabrum* Westling (Wehmer), *P. puberulum* Bain., *P.*

stoloniferum Thom and *P. viridicatum* Westling. The information used by Barkai-Golan (2008) to mention the *Penicillium* spp. pathogenic on grapes was actually more illustrative of the species colonising/growing on the berry surface and not infecting the fruit (Benkhemmar *et al*., 1993). There is thus reason to question the pathogenicity of the above mentioned species on grapes. A *Penicillium* sp. that has been confimed a grape pathogen is *P. expansum* (Sanzani *et al*., 2013). Further research is needed to prove that the other species are pathogenic on grapes.

The major pathogenic *Penicillium* spp. of citrus in terms of decay are *P. digitatum*, *P. italicum* Wehmer and *P. ulaiense* Hsieh, Su & Tzean (Frisvad and Samson, 2004; Pitt and Hocking, 2009). There are reports demonstrating the pathogenic ability of *P. crustosum* (Garcha and Singh in 1976; Arrebolla *et al*., 2010) and *P. expansum* (Macarisin *et al*. 2007; Vilanova *et al*., 2012b) on citrus. Some of these reports are old and lack clarity, but the more recent papers have shown repeatability (i.e. Vilanova *et al*. (2012b)). One paper reported *P. fellutanum* Biourage pathogenic on citrus, but the article is old and lacks positive confirmation (Sinha, 1946). There thus remains a need to report on the pathogenic ability of *P. expansum* and *P. crustosum* on various citrus species.

South Africa is one of the world's leading fruit exporting countries (CGA, 2012; Hortgro, 2012; SATI, 2012). The combined production in tonnage of pome fruit (apples and pears), stone fruit (apricots, nectarines, peaches and plums) and table grapes equals 1 762 322 tons. More than 48% of these crops are exported fresh, with table grapes being the most popular for exporting at 86% of the total production. These deciduous crops earned South Africa over R7.9 billion in net export realisation for the 2011/2012 season (Hortgro, 2012; SATI, 2012). Concerning the citrus industry, 1 424 715 tons (64% of total production) freshly exported fruit grossed just under R6 billion for 2011 (CGA, 2012). These fruit industries are therefore of economic importance to the country and thus require effective management and distribution systems to maintain and advance further. The success of these industries do not only rely on their productivity, but also on their ability to maintain fruit quality and limit postharvest losses while fruit are distributed through the world and prior to consumption.

The shortcomings regarding host-*Penicillium* interactions discussed in preceding paragraphs support the need for further *Penicillium* research on both deciduous and citrus fruit. The review therefore proivdes a background to better understand the challenges associated with a wide spectrum of *Penicillium* spp. encountered in the deciduous and citrus

fruit industries and their possible role in terms of fruit spoilage. Further information regarding the suspected *Penicillium* pathogens (not confirmed) on the selected fruit hosts will be included, since a number of the identified shortcomings deal with these knowledge gaps. To provide context to the *Penicillium* challenges, an industry overview (South African) will also be given linked to host range and susceptibility.

2. *PENCILLIUM* CLASSIFICATION AS POSTHARVEST PATHOGENS

2.1 HISTORY AND CLASSIFICATION OF *PENICILLIUM*

The name *Penicillium* was introduced by Link (1809) and derives from penicillus, meaning little brush in Latin (Houbraken and Samson, 2011). The nomenclature of *Penicillium* was typified by *P. expansum*. The species with their terverticillate smooth-walled penicillin and synnemata was one of the first described *Penicillium* spp. (1809). Later studies have shown that some phenotypic features of *P. expansum* may vary, demonstrating the taxonomic instability associated with terverticillate penicilli species (Raper and Thom, 1949; Frisvad and Samson, 2004). Features such as micromorphology previously used in taxonomy became insufficient and techniques such as DNA sequencing became necessary to improve accuracy and ensure taxonomic stability with the identification and discovery of new species (Frisvad and Samson, 2004). Numerous new species were identified in the 19th century. Important classification work started by Dierckx (1901) brought the genus a long way to the four subgenera (excluding *Eupenicillium*), 10 sections, 21 series and more than 250 accepted species described today (Pitt, 1979b; Houbraken and Samson, 2011). Full organism classification up to genus level: Eukaryota, Fungi, Dikarya, Ascomycota, Pezizomycotina, Eurotiomycetes, Eurotiomycetidae, Eurotiales, Trichocomaceae, mitosporic and *Penicillium* (NCBI, 2013).

Penicillium spp. were in the past classified based on colony characteristics and conidiophore branching (Thom, 1930), conidiophore characters, phialide shapes and growth characteristics (Pitt, 1979b) or phialide shape and conidiophore branching (Stolk and Samson, 1985). Although the classification and taxonomy of *Penicillium* today require more advanced methods (DNA sequencing and production of extrolites), it does not eliminate micromorphology from the processes (Frisvad and Samson, 2004; Houbraken and Samson, 2011).

Penicillium mycelia compose of narrow septate hyaline hyphae that can be pale or brightly coloured. The conidiophores start as relatively narrow stalks (stipe) with a conidial apparatus (Penicillus) at the apex (Fig. 2.1). Some stipes can be hyaline, swollen at the apex and brown. Penicilli are made up out of conidiogenous cells (usually flask-shaped) called phialides directly situated on the stipe apex or on verticils of metulae attached to branches (one or more) called rami or ramulae. Branching of conidiophores can be mono- (one branch point; no branching), bi- (two branch points; one branch stage), ter- (three branch points; two branch stages) or quarter-verticillate (four branch points; three branch stages), or divaricate (branch in a spreading, divergent and irregular patter). Biverticillate can be symmetrical or asymmetrical. More complex branching systems have also been described. Single cell conidia are basipetally produced in unbranched chains from the phialides. Conidia can be aseptate, hyaline, smooth to rough walls with varying shapes from globose to cylindrical. Conidia colour can range from shades of green to olive or brown (rarely white) (Onions and Brady, 1987; Frisvad and Samson, 2004; Pitt and Hocking, 2009; Houbraken and Samson, 2011).

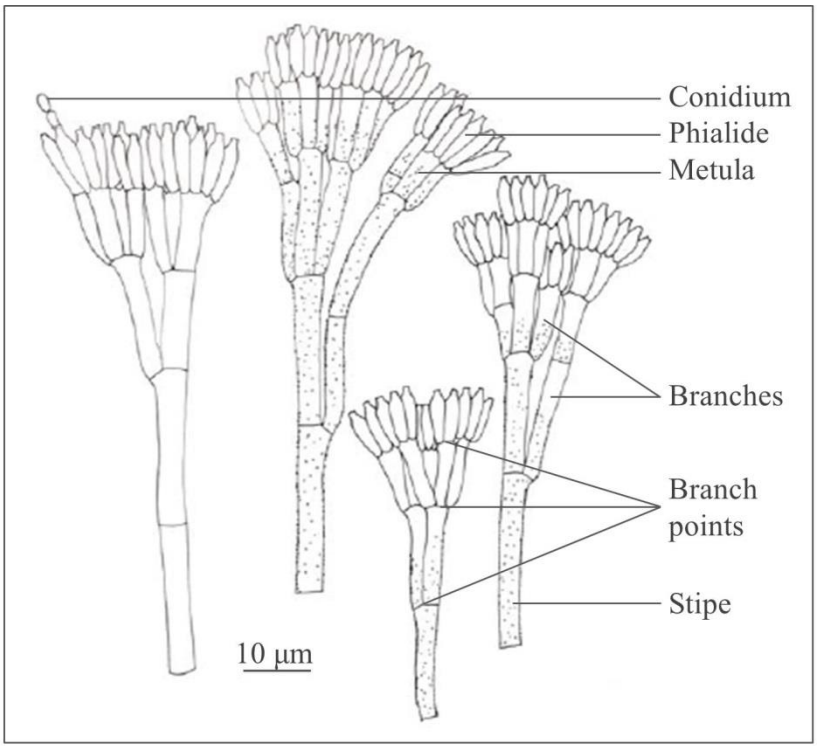

Fig. 2.1. Conidiophores with branching patterns (terverticillate and quarterverticillate) typically associated with *Penicillium* subgenus *Penicillium* (Adapted from Frisvad and Samson, 2004).

Mycelial masses, sclerotia or cleistothecia are produced by some species. Sclerotia are hard, thick-walled cells. Cleistothecia are hard, globose to subglobse shaped, sclerochymatous or pseudoparenchymatous and can come in various colours (white, pale, black, yellow, orange, brown or red). Species reproducing sexually can develop soft non-ostiolate (*Talaromyces*) or hard non-ostiolate sclerotiumlile ascomata (*Eupenicillium*). Asci are ellipsoidal to globose with usually eight lenticular ascospores typically with equatorially orientated ridges (Onions and Brady, 1987; Frisvad and Samson, 2004; Pitt and Hocking, 2009; Houbraken and Samson, 2011). The sexual stage of *Penicillium* will not be discussed further based on the one-fungus-one-name concept (the teleomorphs, *Talaromyces* and *Eupenicillium*, are no longer grouped separate from the anamorph, *Penicillium*) and the lack of economic significance associated with the sexual state of the genus (Onions and Brady, 1987).

Penicillium have four subgenera (*Eupenicillium* excluded); *Aspergilloides* Dierckx, *Furcatum* Pitt, *Penicillium*, *Biverticillium* Dierckx (Pitt, 1979b; Pitt and Hocking, 2009). *Penicillium* subgenus *Penicillium* is the most important subgenus of *Penicillium* in terms of food spoilage and nearly all species discussed in this document derive from this subgenus. Exceptions are *P. citrinum* which belongs to subgenus *Furcatum* and *P. glabrum* belonging to subgenus *Aspergilloides*. Subgenus *Penicillium* unfortunately also has the most difficult taxonomy among the subgenera (Frisvad and Samson, 2004; Pitt and Hocking, 2009). The subgenus mainly includes species with terminal terverticillate penicilli, but some are able to produce biverticillate and quaterverticillate penicilli. Phialides are dominantly flask-shaped (ampulliform), although few species produce needle shaped (acerose) phialides (Pitt and Hocking, 2009). Frisvad and Samson (2004) published a comprehensive revision of the subgenus *Penicillium*, bringing more certainty to the taxonomic framework of the species in the subgenus based on a polyphasic study using numerous isolates. They accepted 58 species, ordered in six sections and 17 series (Table 2.1).

Table 2.1. Section, series and species overview of *Penicillium* subgenus *Penicillium* as revised by Frisvad and Samson (2004)

Section	Series[a]	Species[b]
Coronata Pitt	***Olsonii*** Pitt	***P. olsonii*** Bain. & Sartory; ***P. bialowiezense*** K. Zalesski: ***P.***

		brevicompactum Dierckx
Chrysogena Frisvad & Samson, **sect. nov.**	***Chrysogena*** Raper & Thom ex Stolk & Samson	***P. chrysogenum*** Thom; ***P. dipodomyis*** (Frisvad, Filt. & Wicklow) Banke, Frisvad & S. Rosendahl; ***P. flavigenum*** Frisvad & Samson; ***P. nalgiovense*** Laxa
	Aethiopica Frisvad & Samson, **ser. nov.**	***P. aethiopicum*** Frisvad
	Mononematosa Frisvad	***P. mononematosum*** (Frisvad, Filt. & Wicklow) Frisvad; ***P. confertum*** (Frisvad, Filt. & Wicklow) Frisvad
	Persicina Frisvad and Samson, **ser. nov.**	***P. persicinum*** L. Wang, H. Zhou, Frisvad & Samson.
Digitata Frisvad & Samson, **sect. nov.**	***Digitata*** Raper & Thom ex Stolk & Samson	***P. digitatum*** (Pers.:Fr.) Sacc.
Penicillium	***Claviformia*** Raper & Thom ex Stolk, Samson & Frisvad	***P. vulpinum*** (Cooke & Massee) Seifert & Samson; ***P. clavigerum*** Demelius; ***P. concentricum*** Samson, Stolk & Hadlok; ***P. coprobium*** Frisvad; ***P. coprophilum*** (Berk. & Curt.) Seifert & Samson; ***P. formosanum*** Hsieh, Su & Tzean; ***P. glandicola*** (Oud.) Seifert & Samson
	Expansa Raper & Thom ex Fassatiová	***P. expansum*** Link; ***P. marinum*** Frisvad & Samson, **sp. nov.**; ***P. sclerotigenum*** Yamamoto
	Gladioli Raper & Thom ex Stolk & Samson	***P. gladioli*** McCulloch & Thom
	Italica Raper & Thom ex Pitt	***P. italicum*** Wehmer; ***P. ulaiense*** Hsieh, Su & Tzean
	Urticicolae Fassatiová	***P. griseofulvum*** Dierckx; ***P. dipodomyicola*** (Frisvad, Filt. & Wicklow) Frisvad
Roqueforti Frisvad & Samson **sect.**	***Roqueforti*** Raper & Thom ex Frisvad	***P. roqueforti*** Thom; ***P. carneum*** (Frisvad) Frisvad; ***P. paneum*** Frisvad

nov.		
Viridicata Frisvad & Samson, **sect. nov.**	***Viridicata*** Raper & Thom ex Pitt	***P. viridicatum*** Westling; ***P. aurantiogriseum*** Dierckx; ***P. cyclopium*** Westling; ***P. freii*** Frisvad & Samson, **sp. nov.**; ***P. melanoconidium*** (Frisvad) Frisvad & Samson, **comb. nov.**; ***P. neoechinulatum*** (Frisvad, Filt. & Wicklow) Frisvad & Samson, **comb. nov.**; ***P. polonicum*** K. Zaleski; ***P. tricolor*** Frisvad, Seifert, Samson & Mills
	Camemberti Raper & Thom ex Pitt	***P. camemberti*** Thom; ***P. atramentosum*** Thom; ***P. caseifulvum*** Lund, Filt. & Frisvad; ***P. commune*** Thom; ***P. crustosum*** Thom; ***P. palitans*** Westling
	Corymbifera Frisvad	***P. hirsutum*** Dierckx; ***P. albocoremium*** (Frisvad) Frisvad; ***P. allii*** Vincent & Pitt; ***P. hordei*** Stolk; ***P. radicicola*** Overy & Frisvad; ***P. tulipae*** Overy & Frisvad; ***P. venetum*** (Frisvad) Frisvad
	Solita Frisvad	***P. solitum*** Westling; ***P. cavernicola*** Frisvad & Samson, **sp. nov.**; ***P. discolor*** Frisvad & Samson; ***P. echinulatum*** Fassatiová
	Verrucosa Frisvad	***P. verrucosum*** Dierckx; ***P. nordicum*** Dragoni & Cantoni ex Ramírez; ***P. thymicola*** Frisvad & Samson, **sp.nov**.

a, Series order: arranged to give typus species first (if assigned), series thereafter follow in an alphabetical order; **b,** Species order: species listed first are the type species, species thereafter include accepted species in an alphabetical order.

The sections are phenotypically distinct (Frisvad and Samson, 2004).

2.2 THE LIFECYCLE OF *PENICILLIUM* SPP.

Penicillium spp. are characterised as necrotrophic filamentous fungal pathogens (Marcet-Houben *et al*., 2012; Prusky *et al*., 2013; Vilanova *et al*., 2014) that illustrates a global presence (ubiquitous) due to their rapid production of numerous asexual conidia and effective dissemination characteristics (Adaskaveg *et al.*, 2002; Amiri and Bompeix, 2005; Sonjak *et al*., 2005; Hart, 2006). They infect and colonise their hosts by secreting various secondary metabolites, including mycotoxins and lytic enzymes capable of causing necrosis of living host tissue (Frisvad and Samson, 2004; Carris *et al*., 2012). The role of all mycotoxins is not clear, but they can play a role in pathogenicity, virulence (Sanzani, 2012) and/or fungal defence (against antagonistic microorganisms) (Gloer, 1995). The toxic effect of some mycotoxins affecting animals and humans has been well-documented (Frisvad and Samson, 2004). These characteristics and their ability to grow and/or survive harsh environmental conditions (Fresca and Sampson, 2004; Sonjak *et al.*, 2005; Dijksterhuis, 2007), allow *Penicillium* spp. to infect, invade and/or contaminate diverse sets of hosts, substrates and environments. This allows *Penicillium* to contaminate any food product and be of concern to numerous industries if not properly controlled (Eckert and Ogawa, 1985; LaRue and Johnson, 1989; Kinay *et al*., 2001; Aziz and Moussa, 2002; Engelbrecht *et al*., 2004; Frisvad and Sampson, 2004; Franck *et al*., 2005; Reddy *et al*., 2010; Vico *et al*., 2010).

Necrotrophs are characterised by their destructive lifestyle which leads to rapid host degradation, cellular collapse, tissue maceration and rotting (Laluk and Mengiste, 2010). They are prone to infect opportunistically through wounds, but can penetrate the host through natural openings (Rosenberger, 1990; Alfano and Collmer, 1996; Lucas and Dickinson, 1998; Lahlali *et al*., 2005; Marcet-Houben *et al*., 2012; Vilanova *et al*., 2014). Fungi can also infect via penetration pegs produced from appressoria (Prins *et al*., 2000; Łaźniewska *et al*., 2010). Necrotrophs do not occupy living plant cells. They grow intercellularly within host tissue (among the cells) from where they will secrete lytic enzymes and toxins to kill and/or degrade host cells (Stone, 2001; Diéguez-Uribeondo *et al*., 2005; Shlezinger *et al*., 2011). Nutrients released from dead and dying tissue will be absorbed, supporting invasion, colonisation and growth (Stone, 2001). This type of lifestyle is associated with a broad host range and does not limit the pathogen to the host, but allows saprophytic growth. Pathogens with such lifestyles are also known as non-obligative (Lucas and Dickinson, 1998; Oliver and Ipcho, 2004).

Penicillium spp. primarily reproduce asexually via conidia. Their lifecycle is relatively short and fast (Fig. 2.2). Conidia are primarily disseminated via water and air (Hart, 2006; Frisvad and Samson, 2004; Dijksterhuis, 2007). Once environmental conditions are favourable and conidia are present in or attach to suitable substrates or hosts, they germinate. A substrate with adequate nutrients will support further mycelial growth and colonisation. Epiphytic growth on non-host surfaces is also possible (Korsten, 2006; Johnston, 2008). A recognised host will be infected, invaded and colonised, if not resisted or halted by host defence or control practices. Some *Penicillium* spp. are known to cause quiescent infections; a period in which the pathogen appears to be inactive and almost no growth occurs. Infections transude from the quiescent infection stage to necrotrophic attack once the quiescence state is lifted (Prusky *et al*., 2013). Reproduction, if allowed (i.e. anti-sporulant activity of imazalil against *P. digitatum* (Brown and Dezman, 1990; Brown *et al*., 1983)), will follow colonisation. Conidiophores (Fig. 2.1) will develop from mycelia, producing conidia which can function as primary or secondary inoculum to repeat the lifecycle. Mycelail fragments can also function as inoculum. The sexual stage of *Penicillium* will not be discussed in this document.

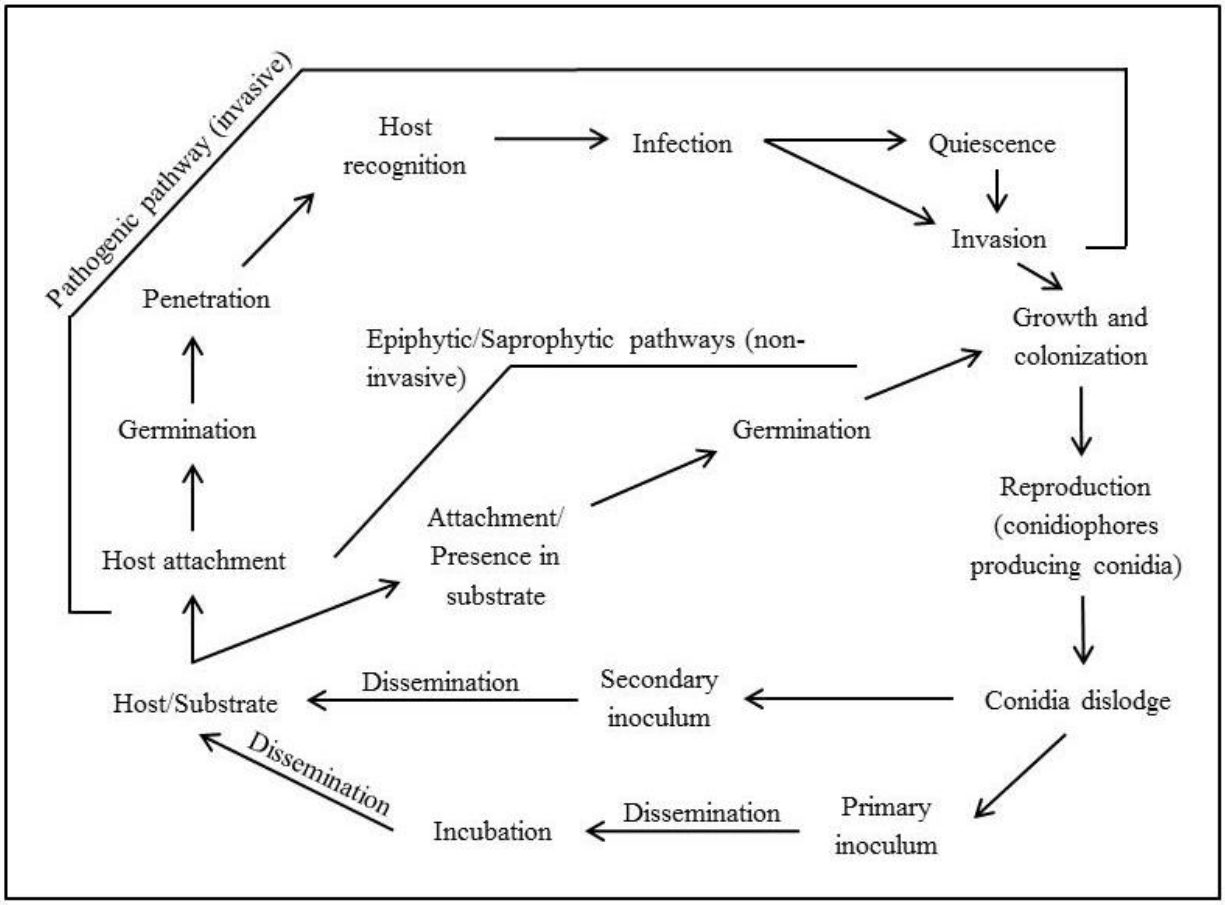

Fig. 2.2. Asexual lifecycle (conidia) of *Penicillium* spp. (Adapted from Agrios, 2005 and Johnston, 2008).

3. PATHOGENIC *PENICILLIUM* SPP. ON DECIDUOUS AND CITRUS FRUIT

Pathogenic *Penicillium* spp. reported on pome fruit include *P. aurantiogriseum*, *P. brevicompactum*, *P. carneum*, *P. commune*, *P. crustosum*, *P. digitatum*, *P. expansum*, *P. griseofulvum*, *P. solitum* and *P. verrucosum* (Jones and Aldwinckle, 1990; Sanderson and Spotts 1995, Sholberg and Haag, 1996; Spotts *et al*., 1998; Amiri and Bompeix, 2005; Kim *et al*., 2005; Moslem *et al*., 2010; Peter *et al*., 2012; Vilanova *et al*., 2012a; Louw and Korsten, 2014; Vilanova *et al*., 2014). Pathogenic *Penicillium* spp. reported on stone fruit comprise of *P. chrysogenum*, *P. crustosum* and *P. expansum* (Ceponis and Friedman, 1957; LaRue and Johnson, 1989; Snowdon, 1990; Eseigbe and Bankole, 1996; Barkai-Golan, 2001; Restuccia *et al*., 2006; Molinu *et al*., 2012). Although not all *Penicillium* spp. have been reported pathogenic on commercial stone fruit (*Prunus* spp.), they will be included in the research study.

Numerous *Penicillium* spp. have been isolated from grapes (>30 species). These species include *P. adametzioides* Abe ex Smith, *P. astrolabium* Serra & Peterson, *P. aurantiogriseum*, *P. bialowiezense* K. Zaleski, *P. brevicompactum*, *P. chrysogenum*, *P. citrinum*, *P. crustosum*, *P. cyclopium*, *P. daejeonium* sp. nov., *P. decumbens*, *P. echinulatum* Raper & Thom ex Fassat, *P. elongatum* Dierckx, *P. expansum*, *P. funiculosum* Thom, *P. cyaneofulvum*, *P. expansum*, *P. glabrum* (including *P. frequentans*), *P. griseofulvum*, *P. neocrassum* Serra & Peterson, *P. puberulum*, *P. purpurogenum* Stoll, *P. spinulosum* Thom, *P. solitum*, *P. stoloniferum*, *P. thomii*, Maire, *P. viridicatum*, *P. viticola* Nonaka & Masuma, sp. nov. and an unidentified *Penicillium* sp. (Crous and Phillips, 2000; Magnoli *et al*., 2003; Franck *et al*., 2005; USDA, 2005; Donoso and Latorre, 2006; Kim *et al*., 2007; Patiño *et al*., 2007; Serra and Peterson, 2007; Serra, 2007; Barkai-Golan, 2008; Nonaka *et al*., 2011; Deng *et al*., 2012; Sang *et al*., 2013). Very few of these species have been proven to be grape pathogens. Barkai-Golan (2008) regarded roughly 14 of the mentioned species as pathogenic (according to literature research). These species include *P. aurantiogriseum*, *P. brevicompactum*, *P. chrysogenum*, *P. citrinum*, *P. crustosum*, *P. cyaneofulvum*, *P. cyclopium*, *P. decumbens*, *P. expansum*, *P. frequentans*, *P. glabrum*, *P. puberulum*, *P. stoloniferum*, *P. viridicatum* and others (Donoso and Latorre, 2006; Barkai-Golan, 2008). Unfortunately, some literature sited by Barkai-Golan (2008) did not contain results confirming the species pathogenic on grapes (Benkhemmar *et al*., 1993). Considering the large number of *Penicillium* spp. isolated from grapes and the lack of information proving them pathogenic, makes it difficult to identify all the species of concern to the grape industry.

Penicillium expansum can be regarded the most concerning *Penicillium* pathogen of grapes in terms of spoilage and toxicity (Sanzani *et al*., 2013). *Penicillium viridicatum* and *P. brevicompactum* are also probable grape pathogens (Pitt and Hocking, 2009). *Penicillium decumbens* (subgenus Aspergilloides, section *Exilicaulis*) was only isolated from grapes, but spoilage was not recorded. It is actually unusual to observe food spoilage from *P. decumbens* (Valero *et al*., 2007a, 2007b; Pitt and Hocking, 2009). Species mentioned that are synonyms of accepted species include *P. cyaneofulvum* (synonym of *P. chrysogenum*), *P. frequentans* (synonym of *P. glabrum*), *P. puberulum* (presumed synonym of *P. cyclopium*) and *P. stoloniferum* (synonym of *P. brevicompcatum*) (Frisvad and Samson, 2004; Pitt and Hocking, 2009). The pathogenic *Penicillium* spp. mentioned by Barkai-Golan (2008) that will be discussed in this document include *P. brevicompactum*, *P. citrinum*, *P. cyclopium*, *P. expansum*, *P. glabrum* and *P. viridicatum*. *Penicillium cyclopium* and *P. viridicatum* are not related, but will be discussed together due to close similarities (Onions and Brady, 1987; Frisvad and Samson, 2004).

Pathogenic *Penicillium* spp. of citrus according to the literature include *P. crustosum*, *P. digitatum*, *P. expansum*, *P. fellutanum*, *P. italicum* and *P. ulaiense* (Sinha, 1946; Garcha and Singh, 1976; Frisvad and Samson, 2004; Macarisin *et al*., 2007; Pitt and Hocking, 2009; Arrebolla *et al*., 2010; Vilanova *et al*., 2012b). *Penicillium fellutanum* will not be included in the review due to a lack of papers confirming the species pathogenic on citrus. The paper by Sinha (1946) was the only report illustrating *P. fellutanum* pathogenic on citrus.

3.1 *PENICILLIUM AURANTIOGRISEUM*

Penicillium aurantiogriseum produces granular, sometimes floccose shaped colonies with a bright greyish-green colour. Conidiophores have a bi-, ter-, or quarterverticillate morphology and stipes are roughened or smooth. Conidia are smooth, sub-spherical to ellipsoidal shaped (Pitt, 1979a; Frisvad and Samson, 2004).

The species is, among others, one of the most commonly encountered fungi. It has consistently been isolated from crops (maturing and drying), specifically in cereals and products derived thereof. Spoilage has been reported on apples, pears, grapes, melons, strawberries, tomatoes, cassava and potatoes. However, there is reason to question spoilage on some fruit and vegetables, since not all the reports have been authenticated (Pitt and Hocking, 2009). It has also been isolated from dried beans and peas, dried fruit, eggs (cold stored), frozen pastries (fruit), health foods, nuts, olives, spices, soybeans (Pitt and Hocking,

1997; Crous and Phillips, 2000; Pitt and Hocking, 2009) and luncheon meats (Ismail and Zaky, 1999).

The species can grow between 1°C and 30°C (optimally at 23°C) (Armolik and Dickson, 1956; Mislivec and Tuite, 1970b; Pitt and Hocking, 2009) and at a minimum water activity (a_w) of 0.81 (Mislivec and Tuite, 1970b). They also have a high lipolytic activity (production of lipase in oily substrates) (Magan *et al*., 1993). They can contribute to food contamination by producing a cocktail of mycotoxins; some neprotoxic glycopeptides (partially characterised), penicillic acid (neurotoxin), terrestic acid (cardiotoxin) and verrucosidin (neurotoxin) (Pitt, 1988; Frisvad and Samson, 2004; Khaddor *et al*., 2007; Pitt and Hocking, 2009). Other extrolites include auranthine, aurantiamine, anacine and pseurotin (Frisvad and Samson, 2004).

3.2 *PENICILLIUM BREVICOMPACTUM*

The colonies produced by *P. brevicompactum* appear velvet blue to grey-green that can crinkle with a thin white margin (Onions and Brady, 1987). It has been shown that the species can express two morphological branching systems; bi- or terverticillate. Biverticillate is however believed to be the predominant and representative branch stage of the species (Seifert and Frisvad, 2000; Frisvad and Samson, 2004). Conidiophores are further granular or rough shaped and conidia can be sub-globose, pear-shaped to broadly ellipsoidal with finely roughened or smooth walls (Onions and Brady, 1987; Frisvad and Samson, 2004). Recently, tree linages were noticed from the species depending on its phylogeny, but further clarification is required (Scott *et al*., 2008).

The species has a widespread occurrence (various soils, including rainforests) and a xerophilic nature (Frisvad and Samson, 2004; Pitt and Hocking, 2009). It has been isolated from numerous dried foodstuffs; beans, health foods, soybeans, various nuts (including peanuts) and pepper (including peppercorns) (Pitt and Hocking, 1997; Freire *et al*., 1999). Other products include bakery products, bottled water, curry paste, dairy products, fruit purée, margarine, sumac (Pitt and Hocking, 2009), biltong, ham (Pitt and Hocking, 1997), cheese (Hayaloglu and Kirbag, 2007) and salami (Cantoni *et al*., 2007). It has also exhibited weak pathogenic behaviour towards fresh produce; apples, cassava, mushrooms, potatoes (Pitt and Hocking, 1997), ginger (Overy and Frisvad, 2005), grapes (Patiño *et al*., 2007), litchi (Pitt and Hocking, 2009) and yams (Aboagye-Nuamah *et al*., 2005).

Growth is possible between -2°C and 30°C (Mislivec and Tuite, 1970b), although growth at 30°C is poor (Frisvad and Samson, 2004). The pathogen can germinate and grow at a a_w as low as 0.78 if temperatures are around 25°C (Hocking and Pitt, 1979) and grow better in media with 5% NaCl (Frisvad and Samson, 2004). A concerning mycotoxin produced by the species is botryodiploidin, but patulin (highly toxic broad/general toxin) has also been reported (Frisvad and Samson, 2004; Rand, *et al*., 2005; Barkai-Golan, 2008). Other extrolites include asperphenamate, botryodiploidin, brevianamide A, pebrolides, raistrick phenols and silvatin derivatives. Pharmaceuticals that can be produced are compactin and mycophenolic acid (Frisvad and Samson, 2004). *Penicillium brevicompactum* was the first reported species to produce the anticholerolemic agent; compactin (Brown *et al*., 1976). Compactin can be used to lower cholesterol and has antifungal properties (Frisvad and Sampson, 2004).

3.3 *PENICILLIUM CARNEUM*

The species produces di- or terverticillate conidiophores with tuberculate stipes and short, wide necked phialides. Conidia are large, smooth, globose to sub-globose and dark blue-green in colour (Frisvad and Samson, 2004). *Penicillium carneum* is associated with the spoilage of meat products, although it has been isolated from beer, beverages, cheese, cork, mouldy bakers yeast, rye bread, silage, water and yoghurt (Boysen *et al*., 1996; Lund *et al*., 1996; Frisvad and Samson, 2004; Pitt and Hocking 2009). Fruit spoilage is not common, but a recent paper from Peter *et al*. (2012) reported blue mould on apples caused by *P. carneum*. They isolated *P. carneum* from decayed 'Golden Delicious' apples in controlled atmosphere storage at a commercial Pennsylvania apple packinghouse. Morphological and molecular techniques identified the species as *P. carneum* and Koch's postulates were confirmed by inoculating apples with 50 µl of a 10^6 conidia/ml conidial suspension (Peter *et al*., 2012).

A close interaction between lactic acid bacteria and *P. carneum* exists, since *P. carneum* has only been isolated from substrates where lactic acid bacteria grow or the substrates resembles similar characteristics caused by the bacteria. Similar characteristics may be caused by organic acids as preservative (i.e. sorbic acid, benzoic acid, propionic acid and acetic acid), alcohol and/or high CO_2 or low O_2 levels (Boysen *et al*., 1996; Lund *et al*., 1996). The species has thus been described growing at 30°C and tolerating low pH levels, high concentrations of alcohol and high CO_2 levels (Frisvad and Samson, 2004; Peter *et al*., 2012). Mycotoxins produced by the species include isofumigaclavine A and B, patulin,

penitrem A (highly toxic tremorgen) and roquefortine C (neurotoxin) (Frisvad and Samson, 2004). The species can also produce a pharmaceutical; mycophenolic acid (Bentley, 2000; Frisvad and Samson, 2004; Nielsen *et al*., 2006). Other extrolites include cyclopaldic acid, penicillic acid and geosmin (Frisvad and Samson, 2004).

3.4 *PENICILLIUM CHRYSOGENUM*

The conidiophores produced by *P. chrysogenum* are mononematous, can be di-, ter-, quarterverticillate or even more-stage branched (morphologic similarities with *P. griseofulvum*). Stipes are predominantly smooth and long. Conidia are dull green, smooth-walled or finely roughened and ellipsoidal to globose or sub-globose shaped (Frisvad and Samson, 2004). The species is ubiquitious and typically found in indoor air, common in dry habitats and prefers/tolerates high salt levels and low a_w (Samson *et al*., 2004a, 2004b).

It is a more common food contaminant than *P. aurantiogriseum* and has been reported pathogenic on black plums (Eseigbe and Bankole, 1996), carrots (Snowdon, 1991), cantaloupe (Raper and Thom, 1949), grapes (Barkai-Golan, 1974) and able to cause spoilage of margarine (Hocking, 1994). The species is common in cereals (rice, wheat, barely, maize, flour), maize-based snacks (Pitt and Hocking, 1997; Lugauskas *et al*., 2006), dry-cured ham (Rodríquez *et al*., 1998), fish (dried), nuts, spices (Pitt and Hocking, 1997), luncheon meats (Mohamed and Hussein, 2004), cheese and other dairy products, lactose powder, pharmaceutical products, spoiled bakery products and timber (causes tainting in shipping containers used for food transport) (Pitt and Hocking, 2009). Mycotoxins produced are PR-toxin and roquefortine C (Frisvad and Samson, 2004). Other extrolites and/or toxins are chrysogine, meleagrin, riboflavin, secalonic acids, xanthocillin X (Frisvad and Filtenborg, 1989; Frisvad and Samson, 2004) and kojic acid (Frisvad and Filtenborg, 1983; Hohn *et al*., 1991; Dai *et al*., 1993; Möller *et al*., 1997). *Penicillium chrysogenum* also produces the well-known pharmaceutical; penicillin (Andersen and Frisvad, 1994).

3.5 *PENICILLIUM CITRINUM*

The species belongs in subgenus *Furcatum*, section *Furcatum*, series *Citrina* (Pit, 1979b). There are seven other accepted species in this series; *P. gorlenkoanum* Baghdadi, *P. hetheringtonii* Houbraken, Frisvad and Samson, sp. nov., *P. sizovae* Baghdadi, *P. tropicum* Houbraken, Frisvad and Samson, *P. tropicoides* Houbraken, Frisvad and *P. steckii* K.M. Zalessky, and Samson, sp. nov. *Penicillium hetheringtonii* is very closely related to *P.*

citrinum with few differing features. Conidiophores have smooth stipes and shared symmetric biverticillate (infrequently an additional branch, more often associtated with fresh isolates). Some isolates have shown monoverticillate or variously branched conidiophores. Conidia are small, globose, smooth-walled and blueish grey-green coloured. The colour of conidia is however not an exclusive characteristics (Houbraken *et al*., 2010).

The species has a world-wide distribution (Pit, 1979b) and has been isolated from nearly all food types surveyed for fungi, the most common being cereals (Pitt and Hocking, 2009). Isolates were made from wheat, rice, ray, corn, barley, oats, peanuts, soybean and amaranth (Scott *et al*., 1972; Bonera *et al*., 1982; Nelson *et al*., 1985; Pohland and Wood, 1987; Bresler *et al*., 1995; Reddy *et al*., 2010). Some other products or environments it has been isolated from are indoor environments, soil, spices (Samson *et al*. 2004), leaves, stems and roots of coffee plants (endophyte) (Posada *et al*., 2007), roots of *Ixeris repens* (Khan *et al*. 2008), coconut milk, coffee beans, compost, peanuts (Houbraken *et al*., 2010), cheese, curd (Kumaresan *et al*., 2003), dried vine fruits (Romero *et al*., 2005), grapes (Bau *et al*., 2005; Kim *et al*., 2007), cashews, copra, sorghum, soybeans (Pitt *et al*., 1993, 1994), dried fish and numerous other food products (Pitt and Hocking, 2009). Reports, including others not referenced, fail to test *P. citrinum* pathogenicity on grapes (Koch's postulates were not performed).

Some *P. citrinum* strains can grow at 37°C (Houbraken *et al*., 2010). *Penicillium citrinum* produces citreoviridin and is the main producer of the citrinin, hence honouring the toxin with its name (Hetherington and Raistrick 1931; Barkai-Golan, 2008; Pitt and Hocking, 2009; Houbraken *et al*., 2010). Citrinin has strong anti-bacterial properties, but can cause nephropathy in consumers (Timonin, 1942; Phillips *et al*., 1980; Voss *et al*., 2001; Stoev, 2008a, 2008b; Stoev, 2010). Other extrolites and toxins include anthraquinone with emodin chromophore, asteric acid, citrinadins, compactin, tanzowaic acid A, quinolactacins and quinocitrinines (Turner and Aldridge 1983; Malmstrøm *et al*., 2000; Kim *et al*., 2001; Kozlovskiĭ *et al*., 2003a, 2003b; Houbraken *et al*., 2010).

3.6 *PENICILLIUM COMMUNE*

Penicillium commune can produce slightly roughened ter- and quarterverticillate conidiophores with short and wide necked phialides. Conidia are large, smooth and spherical (sometimes sub-spherical) shaped. Colonies can be greyish turquoise to dull green in colour. The species is strongly associated with food rich in lipids and protein (i.e. dairy) (Frisvad and

Samson, 2004) and is well-known for causing cheese spoilage (Lund *et al*., 1995; Frisvad *et al*., 1997; Frisvad and Samson, 2004). Spoilage of apples and pears (blue mould) has also been reported (Jones and Aldwinckle, 1990; Sanderson and Spotts, 1995; Sholberg and Haag, 1996). It has been isolated from various products including cakes, lactose powder, margarine, nuts, sour cream, yoghurt and to a lesser extent dried meat products (Frisvad and Samson, 2004; Pitt and Hocking, 2009). *Penicillium camemberti* Thom originates from *P. commune* and is used in cheese production, distinctly known as the white mould on Camembert and Brie (Lund *et al*., 1996). *Penicillium commune* is very similar to *P. palitans* Westling, yet distinct (Polonelli *et al*., 1987).

Indoor air is a common environment for *P. commune* and physiologically it can grow between 5°C and 35°C, require a minimum a_w of 0.85 and show improved growth on media with 5% NaCl (Taniwaki, 1995; Frisvad and Samson, 2004; Pitt and Hocking, 2009). It is able to grow on creatine and use nitrite as sole N-source. Mycotoxins produced by *P. commune* include rugulovasins (ergot-like alkaloid) and cyclopiazonic acid and pharmaceuticals include 3-methoxyviridicatin (active against HIV) (Frisvad and Samson, 2004). Other extrolites produced: cyclopenin and cyclopaldic acid, cyclopolic acid, cyclopiamine, palitantin and viridicatin (Frisvad and Filtenborg 1989; Lund, 1995; Larsen *et al*., 2002a; Frisvad and Samson, 2004).

3.7 *PENICILLIUM CRUSTOSUM*

Penicillium crustosum has terverticillate conidiophores, produces darkish green colonies (can be dull green grey depending on age) and sporulates to such an extent that the surface of colonies appears smooth. Conidia are sub-globose, small and roughened (Pitt, 1979a; Frisvad and Samson, 2004). The species is ubiquitous, common in soil, has been associated with pomaceous fruit and can infect and/or cause spoilage in a range of foods. It has been described as a weak pathogen of citrus and melons (Snowdon, 1990), although two papers demonstrated it differently on citrus (Arrebolla *et al*., 2010; Garcha and Singh in 1976). Pathogenicity and decay of other crops has been reported on apples and pears (blue mould) (Sanderson and Spotts, 1995; Pitt and Hocking, 2009), onions (Pitt and Hocking, 2009) and peaches (Restuccia *et al*., 2006). *Penicillium crustosum* can also infect maize, pine peanuts, mung beans, pepper, rice, sorghum and soybeans (Pitt *et al*., 1993, 1994 and 1998). Spoilage has been reported in biscuits, cakes, cheese, fruit juice, maize and processed meats (Pitt and Hocking, 2009). It has additionally been isolated from animal feeds, bread, cereal, jellies

(dessert), rice products, spoiled dairy, soup, various nuts (including peanuts and fresh pistachios) (Pitt and Hocking, 1997; Frisvad and Samson, 2004; Lewis *et al*., 2005), amaranth grain, cabbage (fresh), dried peas (Pitt and Hocking, 1997), grapes (Benkhemmar *et al*., 1993), thyme (Sonjak *et al*, 2005) and vegetation in temperate regions (Pitt and Hocking, 1997).

The species grows in temperate or subtropical environments (>30°C; optimal at 25°C) (Pitt and Hocking, 2009), over a pH range of 2.2 - 10.0 pH (optimum 4.5 - 9.0 pH) (Wheeler *et al*., 1991) and can survive extreme cold conditions (Arctic) (Sonjak *et al*, 2005). Mycotoxins and other extrolites that can be produced include penitrem A, roquefortine C and terrestric acids (Frisvad and Samson, 2004), cyclopenol, cyclopenin, cyclopiazonic acid, festuclavine, geosmin, viridicatin (Pitt, 1979a, Mantle, 1987), andrastin A, viridicatol (Sonjak *et al*, 2005) and patulin (Hye-Jeong, *et al*. 2006). *Penicillium crustosum* can produce the pharmaceutical called 3-methoxyviridicatin (Frisvad and Samson, 2004) and the important pectic and cellulolytic enzymes involved in pathogenesis and food spoilage (Mantle, 1987).

3.8 *PENICILLIUM CYCLOPIUM* AND *PENICILLIUM VIRIDICATUM*

Penicillium cyclopium is very similar to *P. viridicatum* Raper & Thom and *P. verrucosum* (Onions and Brady, 1987), but has distinctively been separated (Frisvad and Samson, 2004). *Penicillium cyclopium* and *P. viridicatum* belong within the series *Viridicata*, which now covers nine other closely associated *Penicillium* spp. *Penicillium verrucosum* belongs within series *Verrucosa*. The three mentioned species are difficult to distinguish from each other because they produce varying restricted fasciculate colonies that may appear morphologically similar. *Penicillium cyclopium* and *P. viridicatum* have di- or terverticillate conidiophores and conidia are globose to sub-globose, smooth to very finely roughened and blue-green (*P. cyclopium*) or pure green (*P. viridicatum*) coloured.

Penicillium cyclopium and *P. viridicatum* are very common in cereals, resulting in spoilage and toxicity problems (mycotoxins). They are also found in soil. *Penicillium viridicatum* has been reported as a weak pathogen of grapes (Pitt and Hocking, 1997; USDA, 2005). *Penicillium cyclopium* may tend to prefer colder climates than *P. viridicatum*, but both species grow poorly when using creatine as sole N-source (Frisvad and Samson, 2004; Pitt and Hocking, 2009).

Mycotoxins commonly associated with *P. cyclopium* and *P. viridicatum* include the hepatotoxic and nephrotoxic extrolites; xanthomegnin, viomellein, vioxanthin (Lund and Frisvad, 1994; Frisvad, 1995; Frisvad and Samson, 2004) and penicillic acid (Mislivec *et al*., 1975; Frisvad and Samson, 2004). *Penicillium cyclopium* exhibits beneficial properties in rehabilitating heavy metal (copper) polluted water (Ianis *et al*., 2006) and producing pharmaceuticals (3-methoxyviridicatin and benzodiazepine-like alkaloids; cyclopenin, cyclopenol, cyclopeptin and dehydrocyclopeptin) (Heguy *et al*., 1998; Lund, 1995; Frisvad and Samson, 2004). Other extrolites produced by *P. cyclopium* are pseurotin, puberulonic acid and verrucofortine (Frisvad and Samson, 2004). *Penicillium viridicatum* can additionally produce viridic acid (tripeptide mycotoxin) (Holzapfel *et al*., 1986) and other extrolites which include brevianamide A, viridamine, viridicatum and xanthomegnin (Frisvad and Samson, 2004). Other mycotoxins that have been reported are roquefortine, patulin and ochratoxin A (Braselton and Rumler, 1996; Barkai-Golan, 2008). Although ochratoxin A was mentioned, it has been shown that it is actually only associated with *P. verrucosum* and *P. nordicum* Dragoni & Cantoni ex Ramírez (Frisvad, 1985; Land and Hult, 1987; Pitt, 1987; Larsen *et al*., 2002a, 2002b; Frisvad and Samson, 2004).

3.9 *PENICILLIUM DIGITATUM*

This is the only species in the section and series *Digitata* (Frisvad and Sampson, 2004). *Penicillium digitatum* produces velvety, fading yellow green, greyish, or blue-green colonies on MEA (Pitt, 1979a). The species is characterised by only producing biverticillate, frequently irregular and smooth-walled conidiophores. Phialides are long and cylindrical in shape. Conidia are large, smooth, ellipsoidal to cylindrical and olive coloured (Frisvad and Samson, 2004), although not necessarily on all media. *Penicillium digitatum* prefers warmer climates, although it is universally distributed together with its hosts (Pitt and Hocking 2009).

It is a destructive pathogen of citrus (closely associated) causing green mould rot of the fruit (Stange *et al*., 2002; Frisvad and Samson, 2004). A study has illustrated the importance of citrus peel volatiles (α-pinene, limonene and myrcene) associated with the germination of *P. digitatum* conidia (Droby *et al*., 2008). Evidence associating the species with other produce is few or lacking (Frisvad and Samson, 2004). Recent papers, including one from this study, have demonstrated *P. digitatum* able to infect apples and pears (Vilanova *et al*., 2012a; Louw and Korsten, 2014; Vilanova *et al*., 2014). Decay was recorded on some cultivars (Louw and Korsten, 2014; Vilanova *et al*., 2014). The species has also been isolated

from black olives (Heperkan *et al*., 2006), nuts (including peanuts) (Pitt *et al*., 1993, 1994; Pitt and Hocking, 1997), maize, meats, rice, sorghum and soybeans (Pitt *et al*., 1993, 1994).

Penicillium digitatum can grow at 5°C and as high as 37°C (Domsch *et al*., 1980; Plaza *et al*., 2003), although Frisvad and Samson (2004) reported no growth at 30°C. The species requires a high a_w for growth: a minimum a_w of 0.9 when incubated at 25°C and 0.99 when incubated at 5°C (Plaza *et al*., 2003). It is strongly inhibited by 5% NaCl and acidic preservatives (acetic acid, benzoic acid, propionicacid and sorbic acid). It is uniquely the only species in the subgenus *Penicillium* that cannot use nitrate as N-source (Frisvad and Sampson, 2004). A harmful mycotoxin produced by *P. digitatum* is tryptoquialanins (chemically similar to toxic tryptoquivalins) (Cole and Cox, 1981; Ariza *et al*., 2002), although the natural occurrence of it in infected citrus is unknown (Frisvad and Samson, 2004). The species makes use of extracellular polysaccharides called pectic enzymes to macerate citrus peel tissue (Mantle, 1987).

3.10 *PENICILLIUM EXPANSUM*

Penicillium expansum conidiophores have smooth stipes, are mononematous (less common synnematous) and can have two- to three-staged branches. Conidia are dull green, ellipsoidal to sub-globose in shape and smooth-walled (Frisvad and Samson, 2004; Pitt and Hoking, 2009). It is a broad spectrum pathogen (Pitt and Hoking, 2009), but well-known for causing decay (blue mould) of apples and pears (Frisvad and Samson, 2004; Pitt and Hoking, 2009). It is the only *Penicillium* sp. that can express pathogenicity over all fruit types discussed in this document (pome fruit, stone fruit, grapes and citrus). Decay can be caused on apricots, crabapples, persimmons (Sommer *et al*., 1974), grapes, mangoes, strawberries, tomatoes (Snowdon, 1990; Pitt and Hocking, 1997), nectarines, plums (Ceponis and Friedman, 1957; LaRue and Johnson, 1989; Snowdon, 1990; Barkai-Golan, 2001; Batta, 2006; Molinu *et al*., 2012), kiwifruit (Batta, 2007) and even citrus (Vilanova *et al*., 2012b). Although uncommon, it has been isolated from vegetables (cabbages, carrots and onions) (Lugauskas *et al*., 2005) and cereals (maize, rice, wheat, barley) (Aziz *et al*., 2006). It has also been isolated from avocadoes (Snowdon, 1990; Pitt and Hocking, 1997), dried fish, health foods, frozen pastries (fruit), meat products, rapeseed (Pitt and Hocking 1997), soil (Onions and Brady, 1987), cheese (Hayaloglu and Kirbag, 2007), margarine (Hocking, 1994), nuts (including peanuts) (Pitt and Hocking 1997; Aziz *et al*., 2006), olives (Kivanç and

Akguel, 1990), desserts (jelly with fruit), fruit yoghurt, and juice and sauce of apples (Pitt *et al*., 1993, 1994).

The species has been reported growing between -2°C (Mislivec and Tuite, 1970b) and 35°C, poorly at 30°C (Frisvad and Samson, 2004) and optimally at 25°C (Panasenko, 1967). It can grow at low O_2 levels (Golding, 1945) and requires a minimum a_w of 0.82-0.83 (Mislivec and Tuite, 1970b). *Penicillium expansum* is one of the concerning producers of mycotoxins; chaetoglobosins, citrinin (nephrotoxin), communesin B (cytotoxic), roquefortine C and patulin, (Cole and Cox, 1981; Frisvad and Samson, 2004). Other important extrolite are expansolide, geosmin, penicillic acid and viridicatum toxin (Mantle, 1987; Sommer *et al*., 1974; Frisvad and Samson, 2004). The species also produces a large number of enzymes ranging from extracellular polysaccharides such as pectic enzymes, amylase and polygalacturonases (Mantle, 1987).

3.11 *PENICILLIUM GLABRUM* (*PENICILLIUM FREQUENTANS*)

Penicillium glabrum belongs to subgenus and section *Aspergilloides*, and series *Glabra* (Barreto *et al*., 2011; Houbraken and Samson, 2011). Seven other species belong to the series; *P. chermesinum* Biourge, *P. decumbens*, *P. donkii* Stolk, *P. purpurescens* (Sopp) Biourge, *P. spinulosum*, *P. sclerotiorum* Wang, Zhang & Zhuang and *P. thomii*, (Pitt *et al*., 1990). *Penicillium glabrum* was previously also classified as *P. frequentans*, thus forming a cross reference between the two species names (Onions and Brady, 1987; Pitt and Hocking, 2009). *Penicillium glabrum* is the referred name today (Barreto *et al*., 2011; Houbraken and Samson, 2011). It is difficult to distinguish the species from *P. spinulosum* and *P. subericola* sp. nov. using microscopy. All three species primarily have monoverticillate conidiophores with vesiculate and phialides are the same size and shape (ampulliform). Differences were mainly noted at the conidia; smooth to slightly rough in both *P. glabrum* and *P. subericola* sp. nov. (more likely roughened), but distinctly rough in *P. spinulosum*. In addition, *P. subericola* sp. nov. occasionally produces branched conidiophores (not observed with the other species). Conidia produced by *P. glabrum* are globose and grey-green coloured (Barreto *et al*., 2011).

Penicillium glabrum (including reports with *P. frequentans*) is ubiquitous and has been isolated from various environments and products; caves, mines (uranium), quarries (chalk), synthetic materials, water (from estuaries, salt water to sewage) (Smith, 1969; Domsch *et al*., 1993; Pitt and Hocking, 1997), paper (paper, paper pulp), paintings (Inoue and Koyano,

1991), cork (oak trees) (Barreto *et al*., 2011), various soils (importantly deciduous tree and vegetable soils) (Domsch *et al*., 1993; Pitt and Hocking, 1997) and numerous food products. It has been reported causing spoilage of cheese (Northolt *et al*., 1980; Hocking and Faedo, 1992; Pitt and Hocking, 1997), margarine (Pitt and Hocking, 1997), onions (Pitt and Hocking, 2009) and pomegranates (Spadaro *et al*., 2010). It has additionally been isolated from chestnuts (Overy *et al*., 2003), grapes (Mikusová *et al*., 2010), jams (Udagawa *et al*.,1977), maize (Mislivec and Tuite, 1970a), rice (Kurata *et al*.,1968), beverages, cabbage, meet products, peanuts, yams, wheat (Pitt and Hocking, 1997; Pitt and Hocking, 2009) and soybeans (Pitt *et al*., 1998). Like most *Penicillium* spp., *P. glabrum* grows optimally around 25ºC, but can grow between 0°C to near 30°C and minimum a_w of 0.8 (Pitt and Hocking, 1997; Pitt and Hocking, 2009). Extrolites that can be produced are asterric acid, bisdechlorogeodin, citromycetin, PI-3, PI-4, questin, questinol and sulochrin (Barreto *et al*., 2011).

3.12 *PENICILLIUM GRISEOFULVUM*

Penicillium griseofulvum has complex conidiophores. They are smooth, long, variably patterned, can have between three to five, or even more branch stages. Phialides are typical flask shape, but very small. Conidia are ellipsoidal, smooth-walled and grey-green coloured (Frisvad and Samson, 2004). The species occur commonly on dry cereals (barley maize, rice and wheat), grasses, grass seeds and nuts (Frisvad and Samson, 2004; Aziz *et al*., 2006; Pitt and Hocking, 2009). It has also been found in bakery products, dried peas and beans, flour, frozen pastries (fruit), health foods, meats, rapeseed (Pitt and Hocking, 1997) and pasta (Frisvad and Samson, 2004). Moslem *et al*. (2010) was the first to associate blue mould of apples with *P. griseofulvum*. Despite the production of lesions on apples and pears, the species is not commonly associated with spoilage of pome fruit (Sanderson and Spotts, 1995; Pitt and Hocking, 2009). It is present in soil, able to grow between 4°C and 35°C (optimally at 23°C), and 0.81 a_w (Mislivec and Tuite, 1970b; Frisvad and Samson, 2004). Mycotoxins produced by the species include cyclopiamine, cyclopiamide, cyclopiazonic acid, patulin and roquefortine C. Other extrolites are fulvic acid, mycelianamide, griseofulvin (antifungal pharmaceutical) (Frisvad and Samson, 2004) and penicillin (mycotoxin) (Laich *et al*., 2002).

3.13 *PENICILLIUM ITALICUM*

Smooth di- or terverticillate mononematous to definitely synnematous conidiophores are produced by *P. italicum*. Stipes can be long and phialides cylindrical and long. Conidia are smooth-walled, can be cylindrical to ellipsoidal and greyish blue-green coloured (infrequently avellaneous or white). Sclerotia with asci have been reported (Frisvad and Samson, 2004; Pitt and Hocking, 2009).

Penicillium italicum is strongly associated with citrus (Frisvad and Samson, 2004; Droby *et al*., 2008; Hernández-Montiel *et al*., 2010), although it has rarely been isolated from avocados, rice, sapodillas, tomatoes (Pitt and Hocking, 1997), fruit juice (Wyatt *et al*., 1995), cheese, sausages, salami (Guillet *et al*., 2003) and other meat (Papagianni *et al*., 2007). The importance of citrus peel volatiles (limonene, myrcene and α-pinene) related to *P. italicum* conidia germination was also demonstrated by Droby *et al*. (2008).

A report has shown *P. italicum* growing between -3°C and 32-34°C (Panasenko, 1967), although recent studies have shown a minimum of 0°C (Wyatt *et al*., 1995) and very poor growth at 30ºC (Frisvad and Samson, 2004). The minimum required a_w for growth is 0.87 (Panasenko, 1967; Plaza *et al*., 2003) and the pH range is between 1.6 and 9.8 (Panasenko, 1967). Toxicity realated to the species has been reported (Faid and Tantaoui-Elaraki, 1989), but no mycotoxins are associated with the species. Extrolites produced are deoxybrevianamide E, formylxanthocillin X, italinic acid and PI-3 (Frisvad and Samson, 2004). Extracellular enzymes produced by *P. italicum* to degrade host tissue include amylase, β- D-1,2-glucanase and pectic enzymes (Mantle, 1987).

3.14 *PENICILLIUM SOLITUM*

Conidiophores produced by *P. solitum* have rough walled stipes and are predominant terverticillate but can also be di- or quaterverticillate (Pitt and Hocking, 2009; Frisvad and Samson, 2004). Conidia are smooth to finely roughen, relatively large, dark bluish-green or dark green coloured and spherical to sub-spheroidal (uncommonly broad ellipdoidal) (Pitt and Hocking, 2009). The species was ignored for almost 40 years (Pitt and Hocking, 2009) as a synonym of *P. verrucosum* var. *cyclopium* strain ananas-olens Ramírez (Samson *et al*., 1976) or *P. aurantiogriseum* (Pitt, 1979a). Limited background information on the species is thus available (Pitt and Hocking, 2009). First signs of it being classed as distinctive species

was in 1987 (Cruickshank and Pitt, 1987), but proper descriptions only started in 1991 (Pitt *et al*. 1991).

It does not have an extremely broad host range, but has been reported causing spoilage problems on apples and pears (blue mould). Although *P. expansum* is the dominant pome fruit pathogen (Sanderson and Spotts, 1995; Amiri and Bompeix, 2005), *P. solitum* may gain dominance when fungicides are used (Pitt *et al*., 1991). The species can also cause spoilage of cheese (Lund *et al*.,1995) and has been isolated from cashews (Pitt *et al*., 1993), cured meat products, vegetables, indoor air (Frisvad and Samson, 2004), maize, mung beans and peanuts (Pitt and Hocking 2009).

Penicillium solitum is able to grow at low a_w and temperatures, but not at 30°C and is not known to produce mycotoxins (Frisvad and Samson, 2004; Pitt and Hocking, 2009). It is one of the species that can grow faster when 5% NaCl is added to media and can use creatine as sole N-source (Frisvad and Samson, 2004). Extrolites produced include compactin, cyclopenin, cyclopenol, palitantin and viridicatin (Lund, 1995; Frisvad and Sampson, 2004). Important enzymes produced by the species are lipase (Bogdanova *et al*., 1978), pectinases and polygalacturonases (McEvoy *et al*., 2006).

3.15 *PENICILLIUM ULAIENSE*

Penicillium ulaiense is genetically closely related to *P. italicum*, able to produce very similar colonies with similar morphology (Holmes *et al*., 1994; Frisvad and Samson, 2004). Both species cause blue mould on citrus and are very closely associated with the crop. Excluding genetic differences, a few distinctive characteristics also exist; *P. ulaiense* grows much slower, exhibits a higher fungicide resistance (imazalil, thiabendazole and *o*-phenylphenol), is less aggressive and is able to produce 1 to 7 millimetre tall white stalked synnemata. The tall white stalked synnemata have lead to colonies or symptoms being described as whiskery or whisker mould (Holmes *et al.*, 1993; Holmes *et al.*, 1994; Ligorio *et al*., 2009; Pitt and Hocking, 2009; Youssef *et al*., 2010).

The species is an example of extreme conditions forcing change in a species. *Penicillium ulaiense* is rare, but extensive use of imazalil, the choice fungicide for citrus, may select for *P. ulaiense* in packinghouses (Holmes *et al*., 1994). The species is yet to be found outside the boundaries of citrus or citrus packinghouses, but has already been reported in Australasia, Denmark, Egypt, Israel, North America (Arizona, Texas, California, Florida), South America,

South Africa, Southern Europe, Taiwan and Turkey (Holmes *et al.*, 1993; Holmes *et al.*, 1994; Carrillo, 1995; Samson *et al*., 2004a, 2004b; Youssef *et al*., 2010). It exhibits strong growth at 5°C and weak growth at 30°C (optimal at 25°C) (Holmes *et al*., 1994). No growth was described at 30°C by Frisvad and Samson (2004), where Holmes *et al*. (1994) found no growth at 33°C. *Penicillium ulaiense* does not produce mycotoxins, but is biphenyl resistant and can produce deoxybrevianamide E (Frisvad and Samson, 2004). Enzymes produced are carboxymethylcellulase, naringinase, pectinase and protease (Rajal *et al*., 2002; Rajal and Cuevas, 2008).

3.16 *PENICILLIUM VERRUCOSUM*

Conidiophores produced by *P. verrucosum* are rough with various branching patterns ranging from di-, ter- and/or quaterverticillate which can be compact or irregularly disposed. Conidia are small, smooth, spherical (uncommonly sub-sperical or ellipsoidal) and bright green (Frisvad and Sampson, 2004; Pitt and Hocking, 2009). Species from *Viridicata* have often been linked to *P. verrucosum*, although Frisvad and Sampson (2004) have distinctly differentiated between the species using various features.

Penicillium verrucosum is common in stored cereals, although it has been found in animal feed and tissue, and even isolated from salami (Frisvad and Sampson, 2004; Pitt and Hocking, 2009). A report has demonstrated *P. verrucosum* causing decay on apples (Penrose *et al*., 1984). Amiri and Bompeix (2004) investigated the study and reported the species able to infect apples through their lenticels.

The species has never been found in warm habitats (Frisvad and Samson, 2004) and associated with cool temperate cereals, however it is able to grow between 0°C and 31°C (optimally at 20ºC) (Pitt and Hocking, 2009). It can grow over a wide pH range (2.1-10.0) (Wheeler *et al*., 1991), at 0.8 a_w (Northolt *et al*., 1979; Cairns-Fuller *et al*., 2005; Pardo *et al*., 2006) and has improved growth on media with 5% NaCl. The species can use nitrite as its sole N-source, but grow weakly with creatine as sole N-source (Frisvad and Samson, 2004). *Penicillium verrucosum* is a major contributor of the mycotoxin ochratoxin A when growing in related foods, but can also produce citrinin (Frisvad, 1985; Land and Hult, 1987; Pitt, 1987; Larsen *et al*., 2002b; Frisvad and Sampson, 2004). Other extrolites include verruculone and verrucin. 2-methylisobirneol can also be produced (Larsen *et al*., 2002a; Frisvad and Sampson, 2004).

4. THE ORIGIN, PHYSIOLOGY AND ECONOMIC SIGNIFICANCE OF SOME DECIDUOUS AND CITRUS FRUIT

4.1 CLASSIFICATION AND ORIGIN OF SOME DECIDUOUS AND CITRUS FRUIT

Apples and pears belong to the subfamily *Pomoideae* and are among the oldest and most important deciduous fruit crops. They can grow in a relatively broad spectrum of environments, ranging from temperate to tropical regions (Jackson, 2003). The species name of apples date back to 1753 as *Pyrus malus* L. to the now preferred classification of *Malus* x *domestica* Borkh. *Malus* x *domestica* refers to cultivated apples as interspecific hybrids. European pears are called *Pyrus communis* L. and Asian pears are called *Pyrus pyrifolia* Burm. f. Nak. or *Pyrus serotina* Rehd. *Pyrus communis* possibly derives from *Pyrus caucasia* Fed. and snow pears (*Pyrus nivalis* Jacq.) (Rieger, 2006).

Stone fruit comprise of plums (including prunes), peaches, nectarines, cherries and apricots. All of these fruit fall within the genus *Prunus*, which is thought to originate from Central Asia. Nectarines (*Prunus persica* (L.) Batsch var. *nectarina* (Aiton) Maxim or *P. persica* (L.) Batsch var. *nucipersica* (Suckow) C. Schneider) arise from peaches (*Prunus persica* (L.) Batsch var. *persica*), but their geographical origin is unknown. Commercial plums primarily include Chinese or Japanese plums (*Prunus salicina* Lindl.) and European plums (*Prunus* x *domestics*, L.). European plums are also characterised as prunes (Crisosto and Day, 2012).

Grapes are globally one of the most valuable cultivated fruit crops. *Vitis vinifera* L. is the dominant grape species used for fresh consumption, drying (raisins) and wine production. The genus *Vitis* belongs to the family Vitaceae and includes approximately 60 species with around 5000 cultivars (Alleweldt and Dettweiler, 1994; This *et al*., 2006; Owens, 2008). Grape cultivars are subdivided into groups; occidentalis (western European cultivars), orientalis (Central Asia) and pontica (Eastern Europe and the Black Sea Basin cultivars), although the precise domestication and occurrence of cultivar groups are debatable (Owens, 2008).

Citrus predominantly originates from southern Asia (Scott, 1995). The taxonomy of the genus is confusing since a wide range of closely related varieties were originally (1918 and 1937) classified as different species according to Tanaka. Swingle (1943) divided *Citrus* into

subgenus groups with the help of genetic and chemical techniques. Mabberley more recently (1998) approved of this, but further shortened the species range using gene sequencing to form hybrid groups with the ancestral species; *Citrus maxima* Burm., *Citrus medica* L. and *Citrus reticulate* Blanco. This was not accepted as there was low similarity between the main ancestral species (*C. medica*) and the other species (De Araújo *et al*., 2003). De Araújo *et al*. (2003) also stated that Swingle's two subgenera were very closely related, making it unnecessary. Although still with some uncertainty, Swingle's classification is today the more preferred and widely used classification (Page, 2008).

Primary citrus fruit crops in the world, also grown in South Africa, include mandarin/soft citrus (*C. reticulate*), sweet oranges (*Citrus* x *sinensis* (L.) Osbeck (pro sp.)), grapefruit (*Citrus* x *paradisi* Macfad.), lemons (*Citrus* x *limon* (L.) Osbeck, (pro sp.)) and limes (*Citrus* x *aurantiifolia* (Christm) Swingle (pro sp.)). Other economic important groups in South Africa are pummel/shaddock (*Citrus grandis* (L.) Osb./*Citrus maxima* (L.) Osb.) and kumquats (*Citrus* japonica Thunb**.**/*Fortunella margarita* Lour./*Fortunella spp.*). However, calamondins (*Citrus mitis* Blanco), citrons (*C. medica*), sour orange (*Citrus* x *aurantium* L., pro sp.), trifoliata orange (*Poncirus trifoliate* **(**L.) Raf.) and other hybrid species also form part of the citrus family (Hepburn and Mitchell, 1981; Scott, 1995; Rieger, 2004; Seidemann, 2005; Ladanyia, 2008; Page, 2008; Boning, 2010).

4.2 FRUIT PHYSIOLOGY AND MATURITY

Managing and/or controlling fruit maturity and ripeness is fundamental in the fresh produce industry. Importantly, it will impact on fruit quality (internal and external), susceptibility (mechanical injury and pathogens), shelf life, handling, storage and distribution practices, as well as marketability. Deterioration rate or perishability of postharvest produce is relative to their respiration rate (the conversion of O_2 and starch and/or sugar to energy, CO_2 and water). There is also a relationship between ethylene and perishability. Ethylene plays a regulatory role in growth, development, senescence and abscission. Synthesis of the hormone is dependent on or influenced by genetics and environmental conditions (importantly temperature, O_2 levels and CO_2 levels). Ethylene production can also, in general, increase with disease, injuries, maturity at harvest, temperature (up to 30°C) and water stress. Respiration rate together with ethylene (C_2H_4) production during maturation and ripening allows the grouping of fruit into climacteric (CO_2 and C_2H_4 production rate increases

significantly as fruit ripen) or non-climacteric (CO_2 and C_2H_4 production rate are generally low and does not change as fruit ripen) fruit (Kader, 2011).

Respiration and ethylene production can be reduced by controlling temperature, relative humidity (RH), O_2 levels and CO_2 levels. Low temperatures, O_2 levels below 8% and CO_2 levels above 2% will drastically impact on ethylene production and fruit maturity. The loss in firmness, change in internal acidity, and decrease of starch and increase of sugars levels (starch-to-sugar conversion) are important physiological factors associated with maturing and ripening. Soft fruit are very vulnerable to spoilage as they are more susceptible to mechanical injury and infections. Wounded fruit will exhibit an increased water loss, have wound openings ideal for fungal infections and have stimulated CO_2 and C_2H_4 production, all contributing to increased disease incidence and/or severity. Colour changes linked to maturity; loss of chlorophyll (de-greening), carotenoid production (yellow or orange pigments), development of anthocyanins (red and blue pigments), change in anthocyanins and other phenolics (browning; undesirable although increases antioxidant capacity). Other physiological changes include sugar-to-starch conversion (related to vegetables), change in organic acid, protein, amino acid and lipid composition (flavour and nutritional impact), and water loss (including transpiration) (Kader, 2011). Refer to Table 2.2 for perishability information of apples, pears, nectarines, plums, grapes and citrus.

4.3 MARKET SIGNIFICANCE FROM A SOUTH AFRICAN PERSPECTIVE

The South African pome fruit industry produced less than 1.2 million tons of apples and pears of which just under 47% (536 305 tons) were exported (fresh), earning a net export realisation above R3.5 billion for the 2011/2012 season. The country's competitive ranking for apples moved from 12th (2011) to 10th (2012), but pears remained at ninth (2012). South Africa is the seventh largest apple and sixth largest pear exporter (metric tons) in the world (preliminary 2011). The top five apple cultivars planted descend from 'Golden Delicious', 'Granny Smith', 'Royal Gala', 'Topred' to 'Cripps Pink'. These cultivars also form the top five exported cultivars. The top five planted pear cultivars are 'Packham's Triumph', 'Forelle', 'Williams Bon Chretien', 'Abate Fetel' and 'Rosemarie'. These cultivars are also among the top five export cultivars (descending order): 'Packham's Triumph', 'Forelle', 'Williams Bon Chretien', 'Vermont Beauty' and 'Abate Fetel' (Hortgro, 2012). Please refer to Table 6.1 and Table 6.2 in Appendix A for cultivar harvest seasons in South Africa.

Table 2.2. Perishability and storage of some deciduous and citrus fruit crops

	Apples	Pears	Nectarines and plums	Grapes	Citrus
Climacteric	Yes	Yes	Yes	No	No
Respiration rate (mg CO_2/kg.hr at 5°C)	2-10 (low)	10-20 (moderate)	10-20 (moderate)	2-10 (low)	2-10 (low)
Ethylene production rate (µL C_2H_2/kg.hr at 20°C)	10.0-100.0	10.0-100.0	10.0-100.0	<0.1	<0.1
High-temp injury	>38-40°C	>38-40°C	>38-40°C	>38-40°C	>38-40°C
Optimum ripening temp	20-25°C	20-25°C	20-25°C	20-25°C	20-25°C
Ideal transit and storage temp	0-2°C	0-2°C	0-2°C	0-2°C	9-14°C
Chilling injury	No	No	Some	Some	0-9°C
Freezing injury	-5°C	-5°C	-5°C	-5°C	<0°C°C
Relative perishability	Low to moderate[a]	Low to moderate[a]	High	Moderate to high[b]	Low-high[d]
Potential storage life (weeks)	8-16 or 4-8[a]	8-16 or 4-8[a]	2-4	4-8 or 2-4[b]	8-16, 4-8 or 2-16[d]
Cold storage conditions	0-1°C to 3.5-4.5°C; RH 90-95%[a]	European pears: -1 to 0°C for 2-3 to 6-7 months; 95%[a]	Below 0°C for overseas shipment	-1°C to 0°C; RH 90-95%	Very broad, depending on *Citrus* spp. and cultivar[e]
Controlled atmosphere (CA) storage (months)	4-6 and 7-11[a]	3-5 to 7-9[a]	Yes[c]	Yes[c]	Yes[c]

a, depending on cultivar; **b,** depending on SO_2 treatment; **c,** less applicable; **d,** lemon: low (8-16 weeks), orange, grapefruit, lime and pomelo: moderate (4-8 weeks), mandarin and loquat: high (2-4 weeks); **e,** mandarins: 2-3 to 6-7°C, 80-85 to 90-95% RH and 2-5 to 12-18 weeks storage, oranges: 1-2 to 3-9°C, 85-90 to 90-95% RH and 4-8 to 12 weeks storage, grapefruit: 9-10 to 15-15°C, 90-92 to 90% and 4 to 16-20 weeks storage, lemons: 7-8 to 13-14°C, 85-90% RH and 3-4 to 16-24 weeks storage, lime: 8-9 to 9-10°C, 85-90% RH and 6-8 to 10-12 weeks storage, pomelo: 8-9°C; 85-90% and 10-12 weeks storage.
References used: Hardenburg *et al*., 1986; Richardson and Kupferman, 1997; Kupferman, 1997; literature research by Ladanyia, 2008; Kader, 2011; Mitcham and Mitchell, 2011; Crisosto and Mitchell, 2011a, 2011b.
Variables can also be influenced by various other, unmentioned, factors such as waxing, chemical treatments, packaging and palletising, and more.

The South African stone fruit industry yielded under 260 thousand tons of nectarines, peaches and plums, fresh exported 24% (61 847 tons) thereof and earned just over R0.7 billion for 2011/2012. Plums are the primary contributors with 74% of the net export realisation coming from the crop. The top five most popular nectarine cultivars per area planted (descending order): 'Alpine', 'Experimental', 'August Red', 'May Glo' and 'Ruby Sweet'. The top exported cultivars are 'Alpine', 'August Red', 'May Glo', 'Ruby Sweet' and 'Summer Bright'. The top five plum cultivars produced in South Africa descend from

'Laetitia', 'Songold', 'Sapphire', 'African Delight' to 'Angeleno'. The top five exported cultivars are 'Laetitia', 'Songold', 'Fortune', 'Angeleno' and 'Pioneer' respectively (Hortgro, 2012). See Table 6.3 and Table 6.4 in the Appendix A for the South African nectarine and plum cultivar harvests seasons.

The South African table grape industry produced 285 810 tons of berries during the 2011/12 season. Grapes are high value crops. The majority of the berries were exported as fresh (245 797 tons; 86% of the total production), generating a net export realisation of just over R3.6 billion. Of the numerous table grape varieties produced in South Africa, 15 can be described as top varieties. The majority of these varieties fall within the white seeded variety group. The export popularity of variety groups decline from white seedless, red seedless, red seeded, black seeded, white seeded, black seeded to mixed grapes. The top five grape varieties per area planted are 'Crimson Seedless', 'Thompson Seedless', 'Prime', 'Flame Seedless' and 'Redglobe' respectively. The top export varieties showed a similar ranking: 'Crimson Seedless', 'Prime' and 'Thompson Seedless', 'Redglobe', 'Sugraone' and 'Flame Seedless' (SATI, 2012). Refer to Table 6.5 in Appendix A for the South African table grape harvest seasons.

The total production for citrus (mandarins, oranges, grapefruit, lemons and limes) is over 2.2 million tons for 2011. A large amount of the total production (1.4 million tons; 63%) was exported and grossed nearly R6 billion in net export realisation. Oranges are the main contibuters; alone bringing in nearly R3.8 billion (64%). The top five export cultivars for mandarins decline from 'Clementines', 'Satsuma', 'Nova', 'Nules' to 'Nadorcott'. Top five oranges exports are 'Valencia' (general/unclassed), 'Navel' (general/unclassed), 'Midknight Valencia', 'Delta Valentia' (seedles) and 'Navelate Navel'. Top five grapefruit exports include 'Star Ruby', 'Marsh', 'Rose', 'Ruby Red' and 'Oroblanco'. Lemon cultivar classing is less specific in the market and is dominated by 'Eureka' seeded. The top five categories are lemons (general/unclassed), 'Eureka', 'Eureka Seedless', seedless lemon (general/unclassed) and'Genoa'. Other dominating cultivars in their groups are 'Valencia' (nearly double that of 'Navel') and 'Star Ruby' (more than six times the closest competitor) (CIGR, 2012). Please see Table 6.6 in Appendix A for the South African citrus harvest seasons.

Deciduous and citrus industries continually need to apply proper control practices and chain management to preserve fruit quantity and quality. Pathogens add significantly to postharvest losses of fresh fruit and vegetables (Snowdon, 1990; Kader, 2011). Total fresh

produce losses can range between 5 to 25% in developed countries and 20 to 50% in developing countries (Eckert and Ogawa, 1985; Spadaro and Gullino, 2004; Mahlo, 2009; Kader, 2011; Marcet-Houben *et al*., 2012). Fruit are primarily infected with fungi, mainly contributing between 10 to 50% of total fresh fruit losses, but can be much higher (Eckert and Eaks, 1989; Campbell and Reece, 2002; Roslan *et al*., 2010; Marcet-Houben *et al*., 2012). Among all the fungal pathogens contributing to postharvest losses, *Penicillium* spp. include some of the most important in the deciduous and citrus fruit industries (Eckert and Eaks, 1989; Jones and Aldwinckle, 1990; Sanderson and Spotts, 1995; Spotts *et al*., 1998; Pianzzola *et al*., 2004; Kim *et al*., 2005; Pitt and Hocking, 2009; Kader, 2011; Marcet-Houben *et al*., 2012). Two of the *Penicillium* spp. play a substantial role in their respective industries: *P. expansum* (deciduous) and *P. digitatum* (citrus) are considered the most aggressive. Total citrus losses of up to 90% can be due to *P. digitatum* (Eckert and Eaks, 1989, Marcet-Houben *et al*., 2012). Additionally, a paper published form this work (Louw and Korsten, 2014) has shown *P. digitatum* to be more aggressive than *P. expansum* on some pear cultivars.

5. CONCLUSION

Numerous species are included in *Penicillium*, although only a small group is of importance in terms of food spoilage and contamination. The effectiveness of *Penicillium* spp. to survive and spread is primarily due to their ability to produce numerous, resilient asexual conidia, easily dislodged and disseminated via air or water flow. Fungicide resistance is also easily expressed in *Penicillium* populations. The necrotrophic lifestyles of *Penicillium* spp. do not limit the species to their host/s but allow non-obligative saprophytic growth on some substrates or surfaces. The production of harmful and highly toxic mycotoxins and lytic enzymes allow species to infect broader host ranges and/or cause spoilage of a wide spectrum of food types. The production of copious amounts of conidia, easily disseminated via wind makes these species ubiquitous, allowing them to be isolated from various associated or un-associated environments, fresh produce and food products. Some *Penicillium* spp. may thus be able to occupy and survive (mainly as conidia) in diverse environments and products, but lack the ability to cause decay or spoilage of those products.

Pathogenic *Penicillium* spp. of apples and pears are accepted as *P. aurantiogriseum*, *P. brevicompactum*, *P. carneum*, *P. commune*, *P. crustosum*, *P. digitatum*, *P. expansum*, *P.*

griseofulvum, *P. solitum* and *P. verrucosum*. The pathogenic *Penicillium* spp. of commercial nectarines and plums can be regarded as *P. expansum* and possibly *P. crustosum*. The dominant pathogenic *Penicillium* sp. on grapes is *P. expansum*. Other reported pathogenic species include *P. aurantiogriseum*, *P. brevicompactum*, *P. chrysogenum* and *P. viridicatum*. Pathogenic *Penicillium* spp. of citrus can be grouped as *P. crustosum*, *P. digitatum*, *P. expansum*, *P. italicum* and *P. ulaiense*. *Penicillium digitatum* and *P. italicum* are found to be the most aggressive species.

Some confusion related to pathogenicity exists in the literature. *Penicillium* spp. isolated from fresh produce does not prove they are pathogens of the produce they were isolated from. The capability of a species to cause disease on a susceptible host (according to the definition of pathogenicity by Agrios (2005)) needs to be validated (confirmed by Koch's postulates) before it can be described as a pathogen. The pathogenic *Penicillium* spp. on pome and citrus fruit are well-documented, but storage and transport of fruit under varying conditions over extended periods of time will cause fruit to mature and ripen as it moves through the supply chain. Over-mature or riper fruit will be more susceptible to pathogens. Reports demonstrating the pathogenicity of various *Penicillium* spp. on stone fruit and grapes remain insufficient and additional research is required on these crops.

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Chapter 3

Pathogenic *Penicillium* spp. on Apples and Pears

ABSTRACT

Numerous *Penicillium* spp. have been associated with postharvest fruit spoilage. This study investigates pathogenicity and aggressiveness of selected *Penicillium* spp. previously isolated from South African and European Union fruit export chains. *Penicillium expansum* was the most- and *P. crustosum* the second most aggressive on all apple ('Royal Gala', 'Granny Smith', 'Golden Delicious', 'Topred' and 'Cripps Pink') and two pear ('Packham's Triumph' and 'Forelle') cultivars tested. *Penicillium digitatum* was the most aggressive on 'Beurre Bosc', 'Beurre Hardy' and 'Sempre' ('Rosemarie') pears and the third most aggressive on 'Granny Smith' and 'Cripps Pink' apples. To our knowledge this is the first report where *P. digitatum* has been described as aggressive on certain pome fruit cultivars. These cultivars are also the most commonly associated with decay in the export markets resulting in considerable market-end losses. *Penicillium brevicompactum* was shown pathogenic on pears, but was not further evaluated in this study. *Penicillium solitum* covered a broader cultivar range, expressed higher disease incidence and was more aggressive (larger lesions) on pear cultivars than on apple cultivars. This study provides new information on host specificity and the importance of pathogenic *Penicillium* spp. isolated from various environments in the shipping and marketing channels.

1. INTRODUCTION

The South African pome fruit industry is economically important in terms of global trade volumes and foreign exchange. The industry produces over 1.1 million tons of fruit of which 46.6% are exported fresh, earning over R3.5 million in net export realisation in 2012 (Hortgro, 2012). Although specific data is not available for pome fruit, postharvest losses can be as high as 50% (Campbell and Reece, 2002; Spadaro and Gullino, 2004; Roslan *et al*., 2010). These losses can partly be attributed to decay caused by *Penicillium* spp. (Jones and Aldwinckle, 1990; Sanderson and Spotts, 1995; Spotts *et al*., 1998; Pianzzola *et al*, 2004; Kim *et al*., 2005; Moslem *et al*., 2010).

A report by Sanderson and Spotts (1995) indicates that numerous *Penicillium* spp. naturally inhabit pome fruit environments (packinghouses and storage facilities) and are prominent on fruit bins. Of these species, *P. expansum* Link ex Gray, *P. crustosum* Thom and *P. solitum* Westling have been described as the most important decay causers of apples (*Malus domestica* L. Borkh.) and pears (*Pyrus communis* L.) (Sanderson and Spotts, 1995; Spotts *et al*., 1998; Kim *et al*., 2005). Other pathogenic species reported include *P. aurantiogriseum* Dierckx., *P. brevicompactum* Dierckx., *P.commune* Thom, *P. griseofulvum* Dierckx., *P. verrucosum*, Dierckx. and *P. carneum* (Frisvad) Frisvad (Jones and Aldwinckle, 1990; Sholberg and Haag, 1996; Amiri and Bompeix, 2005; Moslem *et al*., 2010; Peter *et al*., 2012). However, these species have less often been associated with decay of pome fruit.

Several other *Penicillium* spp. isolated from pome fruit environments (Sanderson and Spotts, 1995; Amiri and Bompeix, 2005) are best known as pathogens on other fruit crops such as *P. digitatum* (Pers.:Fr.) Sacc. on citrus (Holmes and Eckert, 1999) and *P. glabrum* (Wehmer) Westling on pomegranates (Bardas *et al*., 2009). Complex fruit trade networks result in a large variety of fruit from different countries being retained together in storage or holding facilities (Vermeulen *et al*., 2006). In addition, citrus and pome fruit are sometimes re-packed in the same facility and the infrequent removal of decaying fruit may constitute an inoculum source and point of potential cross-contamination. This practice can potentially introduce a wider range of pathogens to the hosts that would not occur under initial packhouse conditions. Recently, Vilanova *et al*. (2012) reported *P. digitatum* able to infect 'Golden Smoothee' apples but with lesions not developing beyond the initial infection site. Infectivity was described as limited to specific fruit maturity conditions (commercial and over-mature) and inoculum load, resulting in peel reactions of up to 6 mm in diameter (including wound site).

The lack of transparency, more specifically towards the end of the pome fruit supply chain, makes it difficult to assess the impact of pathogen presence and the inoculum loads present. Costs associated with market-end losses are often passed back to the farmer without verification of the point of contamination or cause of losses. End-of-the-season fruit, long-term storage and extended transit periods can result in physiologically older fruit that may be more susceptible towards decay (Lara and Vendrell, 1998; Bower *et al*., 2003; Janisiewicz *et al*., 2008; Villalobos-Acuña and Mitcham, 2008). In this context, opportunistic pathogens encountered further down the supply chain may infect fruit previously considered non-hosts for those pathogens. Effective cold chain management principles are often not maintained after the point of distribution, making fruit more prone to decay.

The aim of this study was to determine the pathogenicity, aggressiveness and host specificity of representative dominant *Penicillium* spp. isolated from various environments in the citrus and pome fruit supply chains on apple and pear cultivars. This knowledge will provide an improved understanding of the causal agents involved, inoculum build-up and sources, and fruit decay potential at the market-end of the supply chain.

2. MATERIALS AND METHODS

Fungal cultures. The *Penicillium* spp. isolates were obtained from the fungal culture collection at the University of Pretoria. Isolates originated from pear (2010/2011) and citrus (2009/2010) export chain studies (unpublished data) (Table 3.1). The criterion for initial selection of species from the culture collection for the present study was based on the identified species being well-known pathogens or the species representing dominance in the supply chain environment. Cultures of the isolates for experimental trials were prepared by single spore isolation. Cultures were grown for up to three weeks on malt extract agar (MEA) (Merck, Biolab Diagnostics (Pty) Ltd, Johannesburg, South Africa) plates in darkness at 25°C. Original isolates were previously purified, morphologically grouped, molecularly identified and preserved in water as working cultures and in 10% glycerol for long-term storage. Water preservations were made by placing five to six pieces of the fungal mycelial growth culture on MEA into double-sterilised distilled water in McCartney bottles, sealed with a strip of Parafilm and stored at room temperature. Cryo-preservation was made by placing five to six pieces of MEA grown cultures into 10% glycerol (autoclaved five times) in cryotubes and stored at -72°C in a freezer.

Table 3.1. *Penicillium* isolates used in the pome fruit trials

Isolate code	*Penicillium* spp.	Fruit chain	Year	Country of origin	Source (location of isolation)
P.eC	*P. expansum*	Citrus	2009 /2010	Germany	Distributer/repack facility-wall. Koch postulates confirmed on apples (2011).
P.eP	*P. expansum*	Pear	2011	United Kingdom	Cold storage facility-air.
P.cC	*P. crustosum*	Citrus	2009/ 2010	Germany	Air, walls or floors of packhouse.
P.cP	*P. crustosum*	Pear	2011	South Africa	Packinghouse holding area-wall.
P.sC	*P. solitum*	Citrus	2009/ 2010	Germany	Distributer/repack facility-wall.
P.sP	*P. solitum*	Pear	2011	United Kingdom	Retail storage facility-wall.
P.dC	*P. digitatum*	Citrus	2009/ 2010	Netherlands	Distributer/repack facility-floor. Koch postulates confirmed on plums (2011).
P.dP	*P. digitatum*	Pear	2011	United Kingdom	Repack facility area small waste bins.
P.bP	*P. brevicompactum*	Pear	2010	United Kingdom	Distribution centre-air.

The isolates were identified by PCR-RFLP (polymerase chain reaction-restriction fragment length polymorphism) method and identity was confirmed by sequencing. DNA was extracted using Neuclospin® Plant II kit (Macherey-Nagel GmbH and Co. KG, Düren, Germany), PCR amplified, restriction digested (Pianzzola *et al*., 2004; Oliveri *et al*., 2007; Johnston, 2008) and submitted for sequencing to conduct final identification. The PCR reactions were performed in a 2700 Prekin-Elmer PCR thermocycler using beta-tubulin (β-tubulin) primers (Bt2a and Bt2b) (Glass and Donaldson, 1995). The cycle conditions included a preliminary three minute denaturation step at 95ºC, 35 cycles of denaturation (94ºC for 30 sec), annealing (57ºC for 45 sec) and elongation (72ºC for two min), followed with a final elongation step (72ºC for seven min) and samples held at 4 ºC upon completion. The yield, purity and quality of PCR amplicons were verified on an agarose gel with a 1kb ladder/marker.

A restriction enzymes BfaI (isochitzomer - FspBI) (Inqaba, Pretoria, South Africa) was used to restriction digest the PCR amplicons. The fragments were separated by electrophoresis on 3% agarose gel run at 75 V for three to five hours (minimum and maximum run time) with a 100bp ladder/marker to separate the fragment and determine

polymorphisms. For sequencing, the PCR amplicons were purified according to MSB® Varo CleanUp (Invitek GmbH, D13125 Berlin, Germany). The sequencing of PCR samples was conducted with the BigDye® Terminator V3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, USA) including the forward primer (Bt2a). Sequence cycle profiles followed in a 2700 PrekinElmer PCR thermocycler. The cycle conditions were 96ºC for one min, trailed by 25 cycles (96ºC for 10 sec, 50ºC for five sec and 60ºC for four min) and samples held at 4ºC. The sequenced PCR products were analysed with an ABI3500 Genetic Analyzer (Applied Biosystems, Foster City, USA).

Confirming pathogenicity and comparing citrus isolates to pear isolates. The *Penicillium* spp. selected for pathogenicity trials included isolates from the citrus environment (*P. expansum*, *P. crustosum*, *P. solitum* and *P. digitatum*) and pear environment (*P. brevicompactum*). Conidial suspensions were prepared in sterilised Ringers (Merck) solution containing 0.05% Tween 80 (Associated Chemical Enterprises, Johannesburg). Concentration of the conidial suspensions were determined with a haemocytometer and the suspensions were diluted to the final concentrations of 6.3 x 10^4 conidia/ml. Freshly-harvested, untreated and commercially graded mature apples (‘Golden Delicious’) and pears (‘Beurre Hardy’) from a packinghouse in the Western Cape Province, with different postharvest practices, were used in the pathogenicity trials (Table 3.2). Fruit were surface-sterilised by dipping into 0.002% sodium hypochlorite solution for approximately ten min and allowed to air dry. Ten apples and 10 pears were inoculated with each isolate of *Penicillium* spp. Each fruit was wounded on opposite sides (1.5 mm x 1.5 mm x 3 mm) by gently piercing the fruit surface with a sterile yellow micropipette tip (20-200 μ l). Inoculation was conducted by depositing 20 µl of conidial suspension into each wound using a pipette. Each fruit was treated as a replicate. Controls consisted of un-inoculated wounded fruit. All wounds (inoculated and un-inoculated) were taped with Parafilm to prevent cross-contamination during the experiment. Fruit were randomised on a disinfected table and incubated under ambient conditions (24.1±0.9°C; 48.8±5.8% RH) for seven days. The decay was assessed three, five and seven days post-inoculation by measuring the horizontal and vertical (stem-calyx axis vertical) diameters of lesions. Control wound sizes were also measured and the mean diameter of control wounds was subtracted from the measured lesion diameters. The experiment was repeated and arranged according to the complete randomised design.

Table 3.2. Pome fruit origin and handling practices

Cultivar	Time of harvest (season)[a]	Postharvest practices	Cold storage before inoculation (days)
Pathogenicity trials/Inoculation comparison method trial/Cold storage trial			
Golden Delicious	Mid	A	18/20
Beurre Hardy	Late	A	12/13
Isolate comparison trial			
Beurre Hardy	Late	A	32
Aggressiveness trials on different cultivars			
Golden Delicious	Mid	A	12/13
Granny Smith (2012)	Late	A	31
Granny Smith (2011)	Previous year (long-term storage)	B	12/13
Cripps Pink	Late	B	31
Topred	Late	C	1/2
Royal Gala	Mid/Late	D	8/12
Packham's Triumph (Region 1)	Early	D	8/12
Packham's Triumph (Region 2)	Mid	E	1/2
Forelle	Early/Mid	E	1/2
Beurre Hardy	Late	A	12/13
Beurre Bosc	Mid	D	8/12
Sempre (Rosemarie)	Mid	D	8/12

A, Standard packaging (8 1.5kg bags per box) and conventional transport (commercial transport via truck) to Tshwane Fresh Produce Market; **B,** Standard packaging (layering in box) and commercial transport to Tshwane Fresh Produce Market; **C,** Sent directly from the farm (loose packed in box) and commercial transport to Tshwane Fresh Produce Market; **D,** Standard packaging (layering in box), flown (cargo) to O.R. Tambo International airport; **E,** Standard packaging (layering in box) and commercial transport to Tshwane Fresh Produce Market; **a,** The period when the fruit was harvested within the season.

The citrus isolates that were confirmed pathogenic to apples and pears were further evaluated to observe if they can be considered pathogenic representatives of their species on pome fruit by comparing them with environmental isolates from a pear chain study (Table 3.1). The isolates included *P. expansum*, *P. crustosum*, *P. solitum* and *P. digitatum*. This comparative trial was conducted by inoculating five surface-sterilised pears ('Beurre Hardy') per isolate using conidial suspensions. Each fruit represented a replicate. Inoculation and incubation of the fruit and the measuring of lesion diameters were as previously described. The isolate comparison trial was repeated using a complete randomised design.

Comparison of methods used for fruit inoculation trials. Three unrelated methods were compared to select the most suitable approach for pome fruit inoculation trials. The methods included; inoculation via conidial suspensions, (as previously described), plugs (simulate direct contact inoculation of wounds) and aerial dispersed conidia (simulate inoculation of wounded fruit via aerial dispersed conidia). Only citrus environment isolates were used in subsequent trials based on the absence of isolate significant differences. Ten 'Golden Delicious' apples were wound inoculated with either *P. expansum*, *P. crustosum*, *P. solitum* and *P. digitatum* for each method. Each fruit represented a replicate. Wounding of fruit for inoculation via conidial suspensions were as previously described, but wounding via plugs and air were made with a 5 mm sterile cork borer (5 mm diameter; 2-3 mm deep). Mycelial plugs (5 mm), intended for inoculation via plugs, were cut from two to three week old cultures (MEA) and placed into the wound sites with a sterilised scalpel. Control fruit were wounded but received no *Penicillium* agar plugs. Wounds were covered with Parafilm, fruit were incubated and data was recorded as previously described.

Inoculation via air was conducted inside an inoculation chamber (Fig. 3.1). The chamber composed of a metal frame overlaid with a plastic bag (61cm x 102.5cm). The inside of the chamber was sterilised with 70% ethanol and allowed to air dry. Ten wounded fruit were placed in the chamber with a two to three week old *Penicillium* culture (MEA) plate. A disinfected fan (YJ 58-12C motor with a 15cm diameter double blade prop) was stationed inside, behind the open *Penicillium* plate. The chamber was closed to ensure air circulation across the plate and prevent air entering from outside while the fan was operational (10 min). The fruit were removed, covered with Parafilm, incubated under ambient conditions and data was recorded as in the pathogenicity trial. This was completed for all four *Penicillium* spp. Control fruit were treated similarly to control fruit intended for inoculation via plugs.

The trial was randomised according to a complete randomised block design (CRBD) with a factorial arrangement. The trial was repeated. Inoculation via plugs and air resulted in larger wounds (5 x 5 mm plugs) in comparison to wounds for inoculation via conidial suspensions (2 x 2 mm piercings). The difference was annulled by subtracting the mean diameter of the control from the mean of the lesions of the respective method.

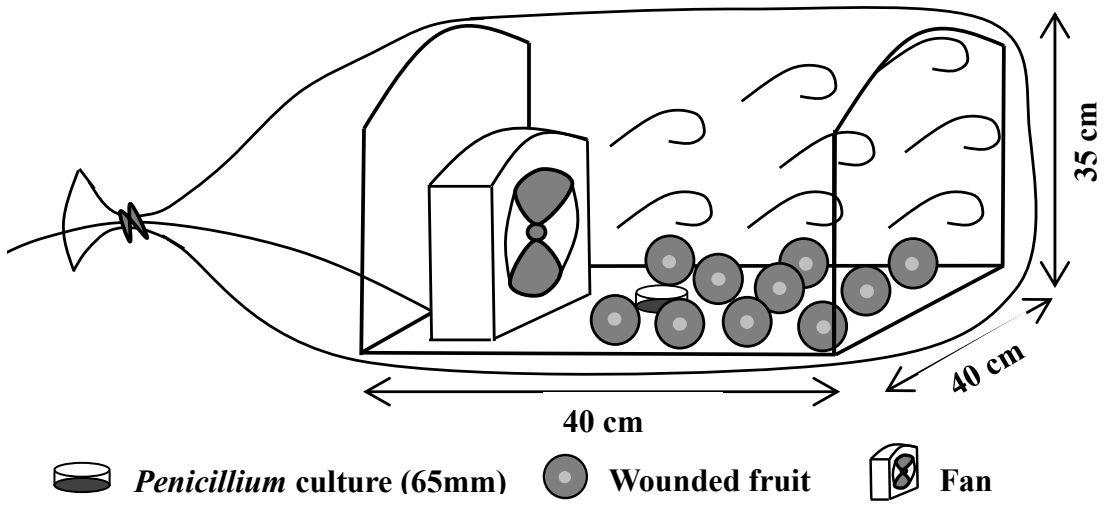

Fig. 3.1. Experimental setup for air inoculation studies using a mini-air chamber.

Decay caused by *Penicillium* spp. under cold storage conditions. The cold storage trial was performed to determine the effect of temperature on disease expression on 'Golden Delicious' apples (Table 3.2) inoculated with *P. expansum*, *P. crustosum*, *P. digitatum* and *P. solitum* (all isolated from the citrus environment). Each pathogen was inoculated into ten surface-sterilised apples (each a replicate) using the conidial suspension method. One set of inoculated apples was incubated under ambient conditions while the second group was incubated under refrigerated conditions (6.2±1.7°C; 63.3±3.0% RH). During the 43-day incubation period, the lesion diameters were recorded (as previously described) every second day starting from the third day after inoculation. Mean wound sizes for controls were also subtracted from the measured lesion diameter means to present decay lesion diameter alone. The trial included a repeat with factorial arrangements on a CRBD.

Aggressiveness of *Penicillium* spp. on apple and pear cultivars. Five different apple ('Golden Delicious', 'Granny Smith', 'Royal Gala', 'Topred' and 'Cripps Pink') and five different pear ('Beurre Hardy', 'Beurre Bosc', 'Forelle', 'Packham's Triumph' and 'Sempre' ('Rosemarie')) cultivars were inoculated with conidial suspensions of *P. expansum*, *P. crustosum*, *P. digitatum* and *P. solitum*. Isolates were from the citrus environment. Fruit for the test originated from two packinghouses in the Western Cape Province, but were collected from various sectors in the supply chain ranging from freshly-harvested (fully mature) to packed or commercially stored for the different trials (Table 3.2). Ten surface-sterilised fruit per *Penicillium* spp. per cultivar were inoculated with the pathogens, incubated under

ambient conditions and the lesion diameters were measured as previously described. Each inoculated fruit represented a replicate. Mean wound sizes for the controls were subtracted from the measured lesion diameter means in the results. Symptom expression (necrotic pattern, visual fungal growth and sporulation) and disease progression were recorded. The experiment was repeated and organised according to a factorial arrangement in a complete randomised design.

Isolations from lesions, culture preservation and *Penicillium* spp. identification. Two to three fruit from all experiments were selected for each cultivar or combination tested for re-isolation of the pathogen. Isolations were made onto MEA medium and incubated as previously described. Once sufficient growth occurred the cultures were observed for morphological similarity, purity and one culture for each *Penicillium* spp. from each cultivar or combination was preserved and identified using the genetic approach as previously described.

Statistical analysis. Statistical analysis was performed with SAS (version 9.2; SAS Institute Inc., Carry, NC, USA). Each fruit in every trial was treated as a replicate, with its mean derived from four measurements (two wounds with horizontal and vertical diameter measurements). Where means follow with ±*x*; *x* refers to standard deviation. Bars on graphs illustrate standard deviation. Least-square mean t-test was used to analyse similarity between repeated tests. The independent experiments for all the trials proved not significantly different and were thus pooled. Fisher's Least Significant Difference (LSD) was used to separate means.

Lesion diameter (mm) and disease incidence (%) of the inoculated wounds from the pathogenicity trials were recorded and used to calculate disease intensity, a measure that represents the combined effect of disease severity and disease incidence. Infected wounds may progress to decay or be resisted by the host (hypersensitive response (HR)). Tissue-response lesions (HR) may become large and have a negative impact on fruit quality. Decay lesions and tissue-response lesions significantly different from the control were regarded as concerning to industry and used in calculating disease incidence. Disease intensity can be used to compare the potential importance of each *Penicillium* sp. on the respective cultivar, indicating possible disease-associated concerns within the fresh produce chain. The disease intensity relation is as follows: Disease intensity = $[(d \times F)/(T_n \times D)] \times 100$; d represented degree of disease severity assessed or specific lesion size classified on the empirical scale, F

frequency, T_n total number of fruit examined and D highest numerical number value of the empirical scale used (Van Eeden and Korsten, 2013). The relation was applied with mean lesion diameter (d), number of reactions observed (F), total number of inoculated wounds examined (T_n) and maximum lesion diameter measurable (D).

3. RESULTS

Confirming pathogenicity and comparing citrus isolates to pear isolates. The independent pathogenicity experiments (trial and repeat) for both apples and pears did not differ significantly (P = 0.6 and P = 0.9). Different host-pathogen interactions did however differ significantly (Table 3.3). All the *Penicillium* spp. originating from the citrus environment were pathogenic on pears. Fewer *Penicillium* spp. were pathogenic on apples compared to pears. *Penicillium brevicompactum* was not pathogenic, whereas *P. solitum* and *P. digitatum* expressed low incidence and caused small lesions on apples within the seven day period assessed. Mean lesion diameters caused by *P. solitum* and *P. digitatum* on apples

Table 3.3. Apple and pear pathogenicity and disease intensity results

Cultivar	*Penicillium* spp.	Mean of all inoculated wounds[a] (mm)	Mean of significant lesions[a,b] (mm)	Incidence (% significant lesions)[b]	Disease intensity (%)
Beurre Hardy	*P. expansum*	34.1 ± 3.2 b	34.1 ± 3.2	100	36.3
	P. crustosum	29.5 ± 4.5 b	29.5 ± 4.5	100	31.1
	P. solitum	8.1 ± 3.3 dc	9.8±2.2	70	7.3
	P. digitatum	81.7 ± 11.1 a	81.7 ± 11.1	100	87.0
	P. brevicompactum	3.7 ± 2.7 de	8.4 ± 0.9	20	1.8
	Control	0.1 ± 0.2 e	-	-	-
Golden Delicious	*P. expansum*	27.3 ± 3.9 a	27.3 ± 3.9	100	27.1
	P. crustosum	12.5 ± 3.5 b	12.5 ± 3.5	100	12.4
	P. solitum	1.5 ± 1.1 c	2.6 ± 0.5	40	1.1
	P. digitatum	1.5 ± 1.4 c	4.0 ± 0.6	20	0.8
	P. brevicompactum	0.2 ± 0.1 c	-	0	-
	Control	0.0 ± 0.1 c	-	-	-

Disease intensity = [(d x F)/T_n x D] x 100: D pears = 93.95mm and apples = 100.48mm

a, Mean wound diameter for controls were already subtracted from measured lesion diameters; **b,** Only lesions significantly different from the control were used to calculate these means (non-significant lesions were excluded).

Means followed by ±*x*; *x* refers to standard deviation.

did not differ significantly when compared to the control, but some significantly different lesions were noted to calculate disease intensity (Table 3.3).

Lesion sizes were not significantly different in two independent experiments for isolate comparison on pears (P = 0.9). The pear and citrus isolates also did not differ significantly in terms of lesion sizes (Fig. 3.2).

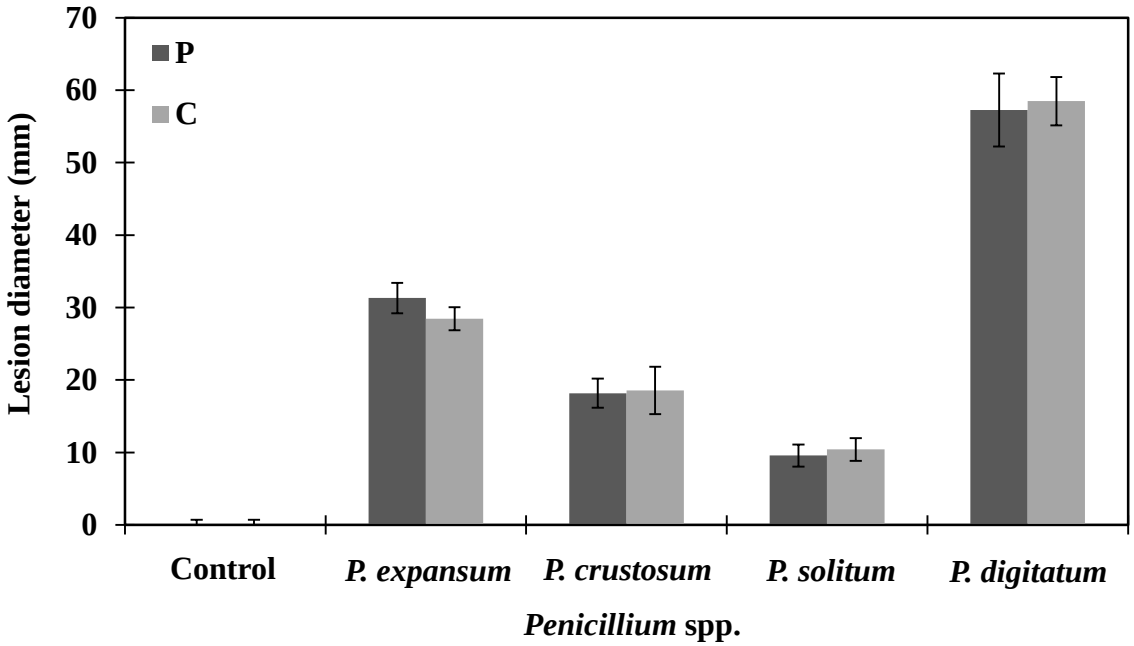

Fig. 3.2. Lesion diameters (seven days incubation at ambient conditions) caused by different *Penicillium* isolates on 'Beurre Hardy' pears; **P,** Pear supply chain isolate; **C,** Citrus supply chain isolate. Bars illustrate standard deviation.

Comparison of methods used for fruit inoculation trials. Independent experiments were pooled (P = 0.4). The inoculation methods differed significantly (P < 0.0001). Inoculation via plugs and conidial suspensions were grouped together where inoculation via air was grouped separately, although trends resembled strong similarity (Fig. 3.3). Inoculation via plugs resulted in highly specific symptoms, well-defined and easy to distinguish. Inoculation via air on the other hand; delivered the smallest overall mean lesion diameter, was the least convenient and more sensitive to air contamination in comparison to the other methods. Inoculation via conidial suspensions was the most convenient and least time consuming compared to the other methods.

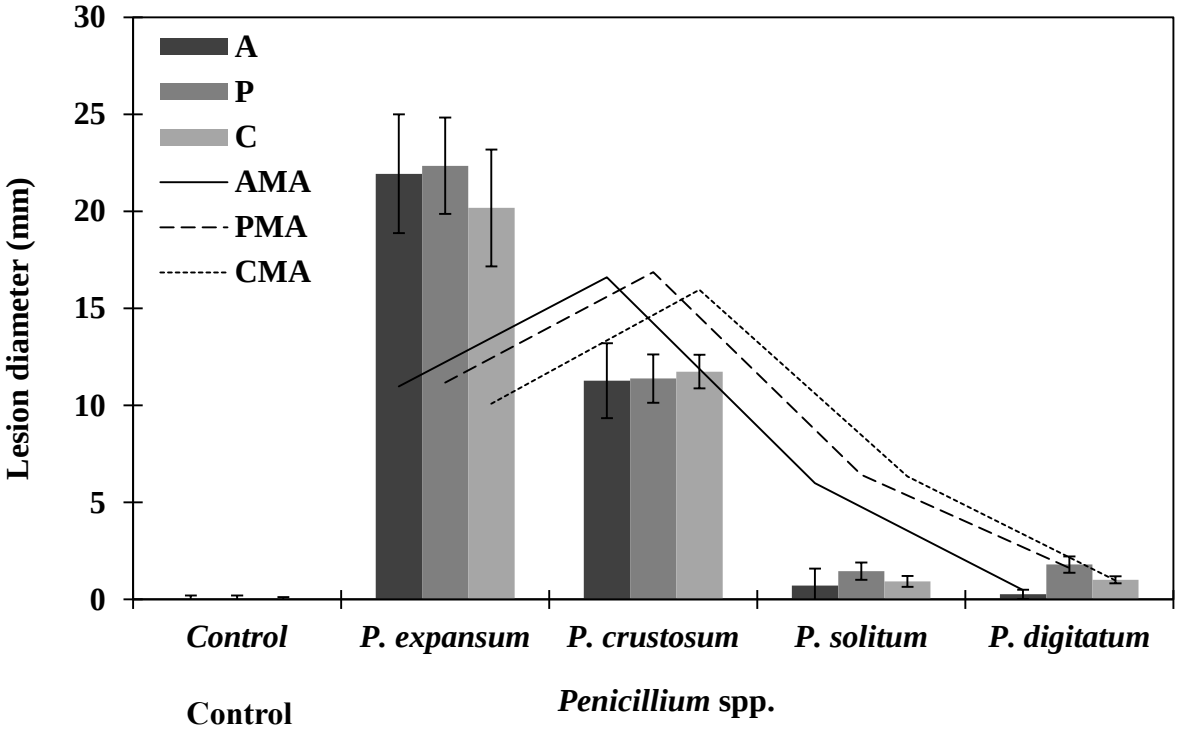

Fig. 3.3. Lesion diameters (seven days incubation at ambient conditions) produced by *Penicillium* spp. inoculated into 'Golden Delicious' apples via three different inoculation methods; **A,** Inoculation via air (**AMA,** Air inoculation moving average); **P,** Inoculation via plugs (**PMA,** Plug inoculation moving average); **C,** Inoculation via conidial suspensions (**CMA,** Conidial suspension inoculation moving average). Bars illustrate standard deviation.

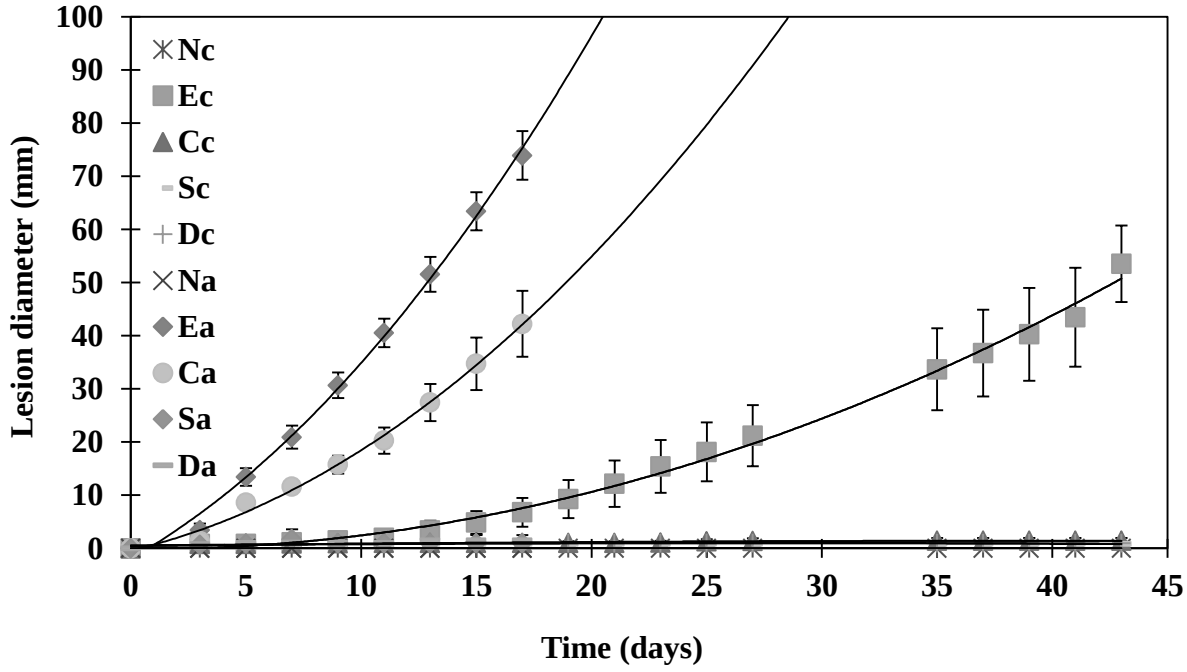

Fig. 3.4. Lesion diameters of *Penicillium* spp. on 'Golden Delicious' apples over 17 days ambient storage and 43 days cold storage; **N,** Control; **E,** *P. expansum*; **C,** *P. crustosum*; **S,** *P. solitum*; **D,** *P. digitatum*; **c,** cold storage (6.2±1.7°C); **a,** ambient storage (24.8±0.3°C). Bars illustrate standard deviation.

Decay caused by *Penicillium* spp. under cold storage conditions. The temperature had a significant impact on rate of lesion development ($P < 0.0001$). The cold storage trial and repeat did not differ significantly ($P = 0.8$). *Penicillium expansum* was the only species able to cause symptoms on 'Golden Delicious' under cold storage conditions over the 43 day storage period (lesion diameter (*ld*) = 49.5±17.3 mm) (Fig. 3.4). *Penicillium crustosum* did however produce small lesions of 4.0±0.5 mm (mean wound diameter of the control was already subtracted) at low incidence (10%) under cold storage conditions. Under ambient conditions (17 days) large lesions were caused by *P. expansum* (73.9±9.2 mm) and *P. crustosum* (42.2±12.4 mm). *Penicillium digitatum* and *P. solitum* again caused small tissue-response lesions at low incidence on the 'Golden Delicious' apples. The experiment was terminated on the 17th day due to total decay of fruit by *P. expansum* at ambient conditions. Under cold storage conditions lesions began to develop seven days post-inoculation while fruit kept under ambient conditions developed decay two days post-inoculation. No surface mycelium or sporulation was observed on inoculated fruit kept in cold storage. Fruit inoculated with *P. expansum* and stored under ambient conditions displayed mycelial growth on the fruit surface after six days (no sporulation noted).

Aggressiveness of *Penicillium* spp. on apple and pear cultivars. The *Penicillium*-cultivars interactions were significantly different ($P < 0.0001$). The repeated experiments were not significantly different, except *P. digitatum* on 'Beurre Bosc' pears where in the initial experiment small lesions (seven day *ld* = 7.6±3.7 mm) were observed compared to large lesions (fifth day *ld* = 84.0±5.2 mm) in the second experiment. A similar aggressiveness shift occurred between the two experiments for 'Beurre Bosc' inoculated with *P. crustosum*, but differences were not as prominent (25.0±7.7 mm vs. 40.3±3.0 mm) (Fig. 3.5). The same cultures were used for inoculation, but in the second experiment the fruit were presumed riper as they were kept in cold storage four days longer (Table 3.2). Due to differences in production practices, cultivar availability and/or seasonality, the trial experiments could not be completed simultaneously (Lara and Vendrell, 1998; Villalobos-Acuña and Mitcham, 2008).

Decay development by *P. expansum* and *P. crustosum* over the cultivar ranges was more consistent compared to *P. digitatum* and *P. solitum*. *Penicillium digitatum* and *P. solitum* did not cause decay on 'Royal Gala' apples (Fig. 3.6). The mean of lesions caused by *P. digitatum* on 'Packham's Triumph' (Region 1) were not significantly different from the control, but few independent tissue-response lesions were noted significantly different; 10.5%

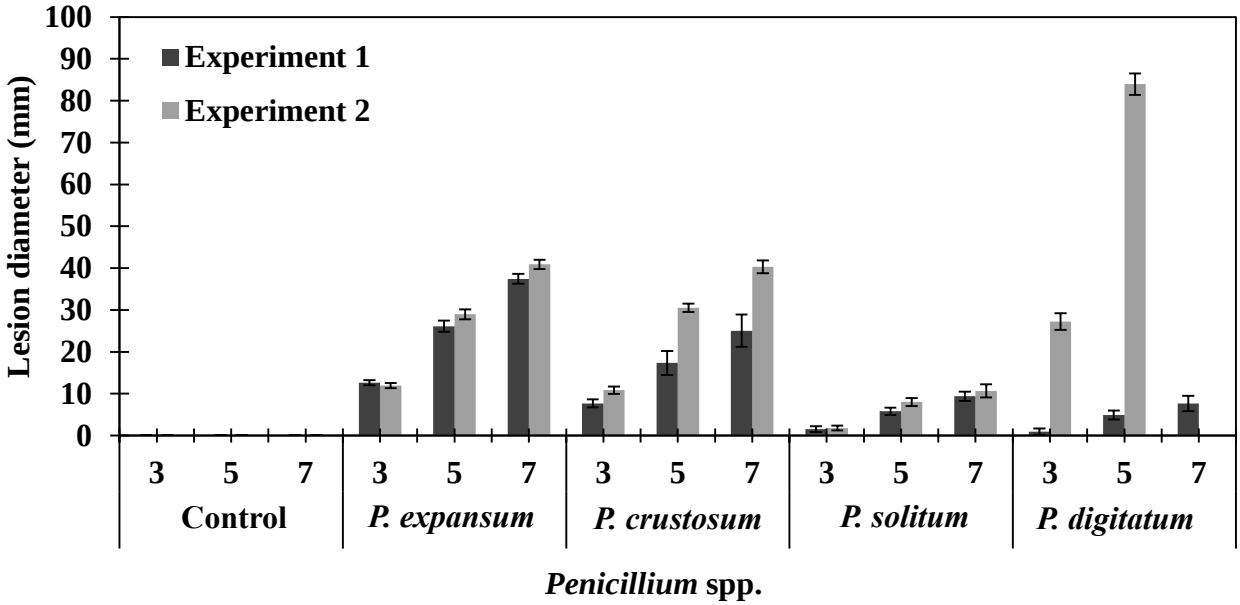

Fig. 3.5. *Penicillium* spp. lesion diameter growth on 'Beurre Bosc' pears over seven days incubation at ambient conditions in two individual experiments. Bars illustrate standard deviation.

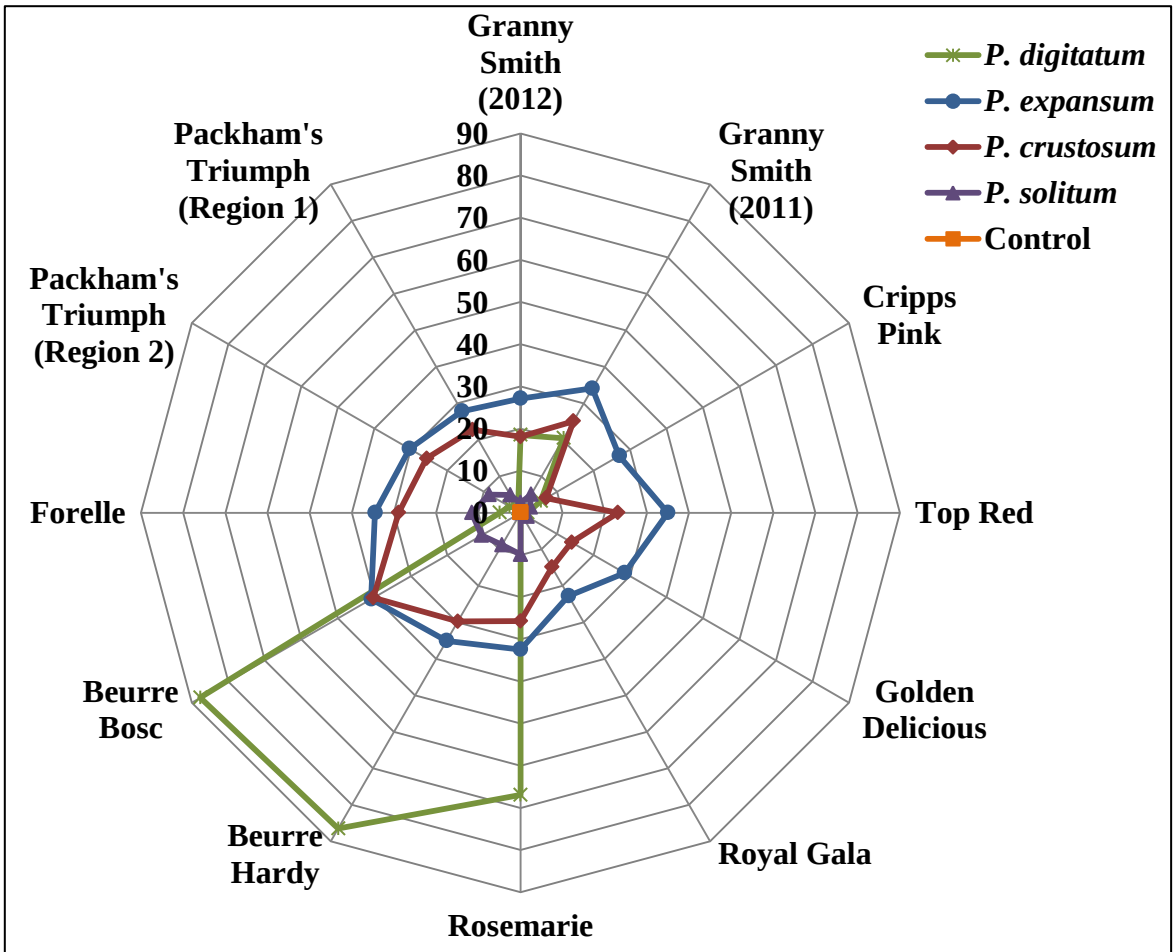

Fig. 3.6. Lesion diameter (mm) of *Penicillium* spp. inoculated apple and pear cultivars after seven days incubation at room conditions.

of the inoculated wounds had lesions 4.7±1.3 mm in diameter. Means of *P. digitatum* lesions on the remaining cultivars were significantly different from the control, however incidences (only lesions significantly different from the control were regarded as significant qualitative concerns) varied with some (Table 3.4). *Penicillium digitatum* was the most aggressive species on 'Beurre Bosc' (100.0% fifth day incidence with 83.9±5.2 mm *ld* for the second experiment), 'Beurre Hardy' (100.0% seventh day lesion incidence with 84.7±8.4 mm *ld*) and 'Rosemarie' (100.0% seventh day incidence with 73.6±11.5 mm *ld*). Means of *P. solitum* lesions on all the cultivars, except 'Royal Gala', were significantly different compared to the control, but incidence also varied (Table 3.4). In general, greater incidence and larger lesions were observed from the *Penicillium* spp. on pears than apples (Fig. 3.6).

Table 3.4. *Penicillium digitatum* and *P. solitum* disease interactions displaying incomplete incidence (<100%) after seven days incubation on tested apple and pear cultivars

***Penicillium* spp.**	**Cultivar**	**Mean of significant lesions[a]**	**Incidence[a] (%)**
P. digitatum	Topred	4.0 ± 2.3	27.8
	Golden Delicious	3.1 ± 1.1	27.5
	Cripps Pink	6.6 ± 3.9	81.8
	Granny Smith 2011	20.3 ± 12.7	97.3
	Granny Smith 2012	19.1 ± 8.8	96.8
	Packham's Triumph (Region 2)	7.0 ± 2.7	51.3
	Forelle	7.1 ± 3.1	64.5
P. solitum	Topred	3.4 ± 0.8	30.0
	Golden Delicious	3.0 ± 1.1	35.0
	Cripps Pink	4.5 ± 1.8	40.0
	Granny Smith 2011	5.0 ± 2.4	89.5
	Granny Smith 2012	4.3 ± 1.9	40.0
	Packham's Triumph (Region 1)	5.8 ± 2.6	70.0
	Packham's Triumph (Region 2)	6.9 ± 3.4	85.0
	Beurre Hardy	8.4 ± 3.4	90.0
	Rosemarie	8.4 ± 3.2	88.2

a, Only lesions significantly different from the control were used to calculate these means (non-significant lesions were excluded).
Means followed by ±*x*; *x* refers to standard deviation.

Symptom expression on apple and pear cultivars. Expressions of decay symptoms on various cultivars were compared to previously described symptoms caused by *P. expansum*, *P.crustosum*, *P. solitum* and *P. digitatum*. This study noted additional symptoms not

previously described from various *Penicillium* pathogens screened on different cultivars. Symptom expression was not uniform on different cultivars. The lesions produced on all apple and pear cultivars tested were brown, circular and slightly sunken. The shade of the brown lesions differed depending on the *Penicillium* spp. and the cultivar host they infect. Thus, lesion colouration cannot be associated with either a specific species or cultivar, but rather to an interaction between *Penicillium* sp. and fruit cultivar (Fig. 3.7 and Fig. 3.8). Additionally, *P. digitatum* produced lesions that were not distinctly defined with brown blotching, softening and swollenness of the tissue. The brown skin of lesions on sensitive hosts later exhibited a bulged appearance (Fig. 3.9). These lesions can be seen during development from a central infection point or as in the case of 'Granny Smith' appearing five to six days after inoculation, from below the skin surface. In the latter case the skin of the apple will appear healthy during the first few days, but the application of pressure reveals a hollowing or softening underneath the skin surface. *Penicillium expansum* and *P. crustosum* frequently produced bull's-eye rot type symptoms as the growth progressed on all cultivars (Fig. 3.7 and Fig. 3.8). *Penicillium solitum* frequently produced small lesions on apples. Some apple cultivars displayed typical HR reactions (as described by Agrios, (2005)) as skin and/or tissue darkening was restricted to the inoculated wound areas (Fig. 3.7: *P. solitum* reactions on 'Granny Smith', 'Golden Delicious' and 'Topred'; *P. digitatum* reactions on 'Golden Delicious' and 'Topred'). HR reactions were also observed on few independent 'Packham's Triumph' pears inoculated with *P. digitatum*.

Penicillium expansum and *P. crustosum* produced white mycelia on all apple cultivars, although the amount produced by *P. expansum* made it more apparent. *Penicillium solitum* and *P. digitatum* produced only small amounts of mycelia when infecting 'Granny Smith' and 'Cripps Pink'. No *Penicillium* conidia were observed on any apple cultivars during the duration of the experiment. Mycelial growth with limited sporulation was observed on all pear cultivars inoculated with *P. expansum* and *P. crustosum*. *Penicillium solitum* never sporulated, but mycelial growth was observed on all pear cultivars. *Penicillium digitatum* produced very limited mycelia and condia on the large lesions produced on 'Beurre Bosc'. More sporulation was noted on 'Beurre Hardy' with 'Rosemarie' supporting profuse sporulation after seven days incubation. No mycelial growth or conidia were on the remaining pear cultivars inoculated with *P. digitatum*. *Penicillium expansum* and *P. crustosum* typically produced blue conidia (blue mould) whereas *P. digitatum* produced lime green conidia (green mould) (Fig. 3.8).

Fig. 3.7. *Penicillium* spp. (columns left to right: *P. digitatum, P. crustosum, P. expensum, P. solitum*) symptom expression on apple cultivars (rows top to bottom: Granny Smith, Golden Delicious, Cripps Pink, Royal Gala), cultures (Malt Extract Agar) and PCR-RFLP (fragments of restriction digested DNA separated on 3% agarose gel).

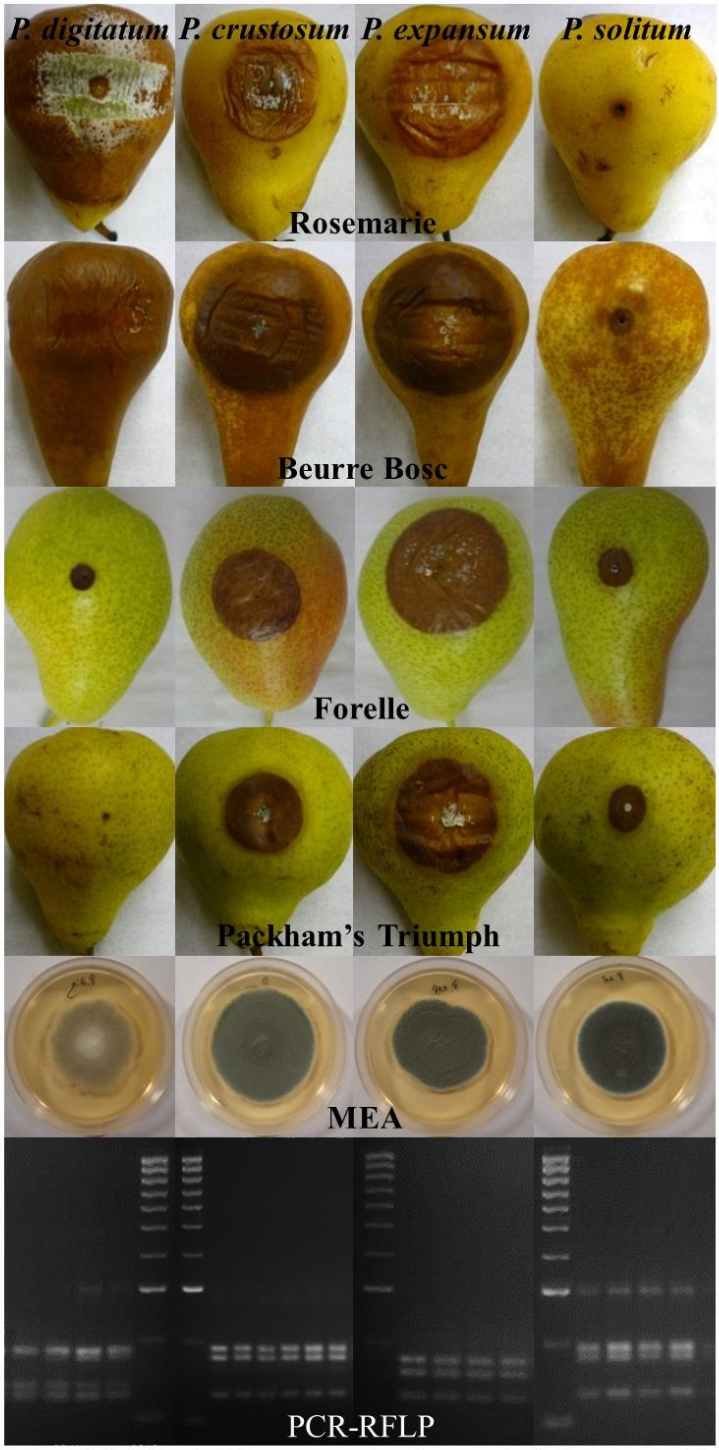

Fig. 3.8. *Penicillium* spp. (columns left to right: *P. digitatum, P. crustosum, P. expensum, P. solitum*) symptom expression on pear cultivars (rows top to bottom: Rosemarie, Beurre Bosc, Forrel, Packham's Triumph), cultures (Malt Extract Agar) and PCR-RFLP (fragments of restriction digested DNA separated on 3% agarose gel).

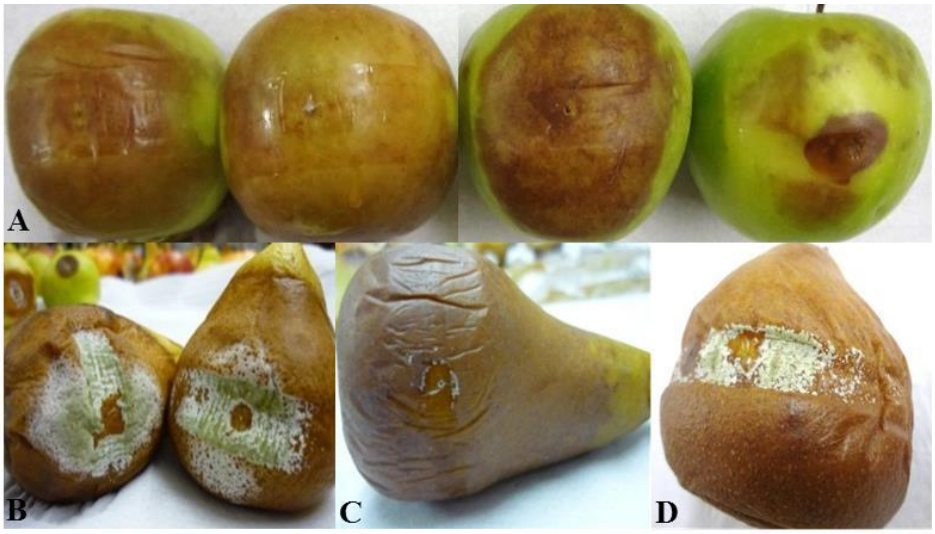

Fig. 3.9. *Penicillium digitatum* disease symptoms on pome fruit. **A,** 'Granny Smith' (11 days incubation); **B,** 'Rosemarie'; **C,** 'Beurre Bosc'; **D,** 'Beurre Hardy' (B-D: seven days incubation).

Confirmation of identity. Koch's postulates were confirmed on all species tested in the trails. The identity of the *Penicillium* spp. re-isolated from infected fruit were confirmed using PCR-RFLP and sequencing as the same species that were used for the inoculation of the fruit (Table 3.5).

Table 3.5. Accession numbers as allocated by GenBank for the β-tubulin gene sequences of *Penicillium* isolated from pome fruit

Isolate no.	Sequence identification	GenBank accession no.
8	*P. solitum*	KF303072
28	*P. expansum*	KF303073
32	*P. expansum*	KF303074
54	*P. digitatum*	KF303075
58	*P. digitatum*	KF303076
78	*P. crustosum*	KF303077
109	*P. brevicompactum*	KF303079
110	*P. brevicompactum*	KF303080
111	*P. brevicompactum*	KF303081

4. DISCUSSION

The most important pathogenic *Penicillium* spp. in terms of decay on pome fruit identified in this study were *P. expansum*, *P. crustosum*, *P. digitatum* and *P. solitum*. *Penicillium brevicompactum* was found to be non-pathogenic on apples and was not tested further in this study. The first mentioned species have formerly been described in pome fruit environments (Sanderson and Spotts, 1995; Amiri and Bompeix, 2005) and pathogenic (excluding *P. digitatum*) on pome fruit (Sanderson and Spotts, 1995; Spotts *et al*., 1998; Kim *et al*., 2005). Sanderson and Spotts (1995) previously reported isolating *P. digitatum* with low incidence (1.8% of 57 pears) from decay lesions on winter pears ('d'Anjou', 'Bosc', 'Comice' and 'Red Anjou'). In their study, decaying apples and pears were sampled from both packinghouses and local markets. The sampling from the packinghouses was regarded as biased because samples were selected based on a specific type of decay. Nonetheless, *P. digitatum* was only isolated from the market winter pears and was not pathogenic when inoculated into fresh 'd'Anjou' pears. The authors never demonstrated, nor explicitly stated that *P. digitatum* was pathogenic on pears, but theorised that the species may be able to colonise "over-mature" fruit (market fruit). The authors did not screen a spectrum of different cultivars or test physiologically older fruit obtained from the market (Sanderson and Spotts, 1995).

Penicillium digitatum produced the largest lesions on three pear cultivars ('Rosemarie', 'Beurre Bosc' and 'Beurre Hardy') indicating its importance as a postharvest pathogen on pears. These cultivars were also described by the industry as being the most prone to decay on export markets. To our knowledge, this is the first report where *P. digitatum* was described as a highly aggressive pathogen on selected pear cultivars. *Penicillium digitatum* infections are more prominently associated with over-mature fruit (Sanderson and Spotts, 1995; Vilanova *et al*., 2012), possibly causing problems much later in the market chain, similar to our results in the second experiment on 'Beurre Bosc'. Although fruit for both trials were selected at commercial harvest maturity stage, fruit for the second experiment was refrigerated (6.2±1.7°C) four days longer (total of 12 days) before inoculation trials. Storage at 5-10°C for three, five or seven days enhances pear ripening (Villalobos-Acuña and Mitcham, 2008), thus influencing susceptibility towards decay (Janisiewicz *et al*., 2008; Vilanova *et al*., 2012). The prolonged storage at the specified temperature is a potential explanation for the fruit being more mature and thus more susceptible to decay. *Penicillium expansum* lesion development was less affected by the prolonged stored fruit, although *P. crustosum* was influenced, causing larger lesions. This raises the question if *P. crustosum*

should be regarded more problematic on pears after suboptimum storage or transport conditions? It also illustrates that stored or transported fruit may become more susceptible towards certain pathogenic *Penicillium* spp., reflecting a shift in prevalence towards the market-end. Effective cold chain management and reduction of inoculum loads still remain important for disease control.

Although fruit maturity indices for each cultivar were not tested in the laboratory prior to inoculation in this study, the fruit were harvested at the commercial mature stage and officially inspected for compliance with national quality standards according to the Agricultural Products Standards Act No 119 of 1990 (South African Department of Agriculture, Forestry and Fishery, 2009a; 2009b). Through this study we have come to understand the importance of taking fruit seasonality and postharvest changes into account in pathogenicity/aggressiveness studies. Some reports dealing with pathogenicity or aggressiveness do not test or report on the physiological status of the host (Spotts *et al*., 1999; Amiri and Bompeix, 2005; Moslem *et al*., 2010). However, this aspect should be included to more effectively assess host-pathogen interactions in the future.

It is important to consider further steps in the supply chain when fruit is stored for months prior to packaging and marketing. Fruit designated for export markets with long distribution systems will be more prone to decay, because it will be more mature after a long sea shipment (16 days or longer) and may be exposed to pathogens from other fruit species (e.g. citrus and apples when seasons overlap) that may be handled in the same central facility. In addition, during re-packing the cold chain is broken, fruit handling can cause wounding (Vermeulen *et al*., 2006) and exposure to high *Penicillium* inoculum levels can result in decay at the consumer-end of the chain. *Penicillium digitatum* in this study has been identified as a pathogen that has the potential to cause rapid decay at the market-end (total fruit decay within five days), especially when fruit are riper. Pathogenicity trials on market aged fruit are therefore required to identify the pathogens that can cause rapid decay and extensive losses at the end of the market chain.

Sanderson and Spotts (1995) noted that *P. digitatum* probably colonise over-mature fruit because they isolated the species from market pears, but could not reproduce infections on newly harvested pears. Unfortunately no symptom descriptions were given of the isolated lesions from the market fruit. Vilanova *et al*. (2012) described reactions of *P. digitatum* infections on ‘Golden Smoothee’ apples, but restricted the descriptions to “no decay

development" with limited disease symptoms and or a HR. The host resistance responses and reactions of *P. digitatum* described on 'Golden Smoothee' were very similar to symptoms produced by *P. digitatum* on 'Golden Delicious' apples in our study. *Penicillium digitatum* was not isolated from the described infection sites on 'Golden Smoothee' to confirm the identity of the organism causing the reaction. Books or publications with pome fruit *Penicillium* pathogen symptom descriptions are usually limited to explanations or illustrations of one or two cultivars (Snowdon, 1990; Kader, 2002; Vilanova *et al*., 2012). A compendium describing symptoms associated with different *Penicillium* spp. infections on various commercial cultivars is not readily available for industry and fruit inspectors. Similar to green- and blue mould of citrus, there is a need to distinguish between decay caused by *P. digitatum* (distinct green mould) and the other blue mould causing *Penicillium* spp. as described in this study. This study further provides a more detailed description of blue mould symptoms over a range of apple and pear cultivars. *Penicillium expansum* and *P. crustosum* symptoms were frequently related to bull's-eye rot symptoms which are commonly associated with *Neofabraea* (Jacks.) spp. (Spotts *et al*., 1999; Gariepy *et al*., 2005). This may lead to the misidentification of the causal pathogen if no isolation and identification of the fungus is made. *Penicillium digitatum* symptom expression on pears and advanced symptom expression on apples were reported for the first time in this paper.

Pears were in general more susceptible and sensitive to *Penicillium* decay than apples. Pears are naturally more perishable (deteriorate faster) than apples (Kader, 2002), adding to susceptibility and sensitivity. *Penicillium expansum* and *P. crustosum* expressed pathogenicity over the cultivar ranges tested. *Penicillium expansum* was considered the most aggressive, except on 'Beurre Bosc', 'Beurre Hardy' and 'Rosemarie'. *Penicillium crustosum* closely mimicked the pathogenic and aggression profile of *P. expansum*, generally expressing the second highest aggressiveness on the majority of cultivars tested (excluding 'Beurre Bosc', 'Beurre Hardy' and 'Rosemarie'). *Penicillium solitum* covered a broader cultivar range, had a higher disease incidence and was more aggressive (larger lesions) on pear cultivars than on apple cultivars.

Penicillium spp. isolates from different environments (isolated from pear and citrus chains) did not produce significantly different lesion sizes on pears. This finding confirms that environmental isolates from different origins represent a source of natural inoculum (Spotts, 1986) for varying fruit types. The compilation of fruit from different origins, received in a central port or holding facility and sometimes even being re-packed prior to

further distribution in the same supply chain may increase the likelihood of cross-contamination. It also shows that *P. digitatum* and *P. expansum* originating from the citrus chain environment, produced similar lesion sizes and symptoms as the *P. digitatum* and *P. expansum* isolates from the pear environment.

Results from plug inoculated fruit were the most consistent and symptom expression, including sporulation, was rapid. It is however the inoculation method representing the most unlikely pathway of natural infections in orchards or within the handling and packing environments. The nesting effect is one of the few natural scenarios illustrating potential infection via this method (Kader, 2002). The method has been described in several papers (Spotts, 1986; Mirshekari *et al*., 2012). The rapid symptom expression with this method results from introducing a high inoculum dose of actively growing culture directly to wound sites.

Fruit inoculation via aerial dispersed conidia can be considered the most likely and natural rot for *Penicillium* to infect fruit in the supply chain, as *Penicillium* easily disseminates through air flow (Hart, 2006). Despite the advantages associated with the method, the success of inoculating fruit via aerial disseminated conidia in a chamber has rarely been reported (Amiri and Bompeix, 2005). Inoculation via air blown spores was experienced as the least preferred method due to inconvenience, being time consuming and sensitive to sample contamination (fruit more exposed pre-inoculation). These difficulties can be overcome by designing a chamber that is easy to sterilise and perform the inoculation in a biosafety cabinet to reduce outside contamination (Reponen *et al*., 1997; Lee *et al*., 2009).

Inoculating fruit with conidial suspensions was identified as the most effective inoculation method based on convenience and reproducibility over more than two decades (Kim *et al*., 1991; Peter *et al*., 2012). Fruit becoming infected via this inoculation pathway is likely in orchards (rain) and packinghouses (handling fruit in water) (Combrink *et al*., 1994; Sholberg and Haag, 1996). Vilanova *et al*. (2012) showed different conidial suspension concentrations of *P. expansum* (10^4 - 10^7 conidia/ml) had no significant effect on lesion growth rate on commercial mature or over-mature apples stored at 20°C and 85% RH. However, the higher concentrations produced symptoms earlier than the lower concentrations on immature and commercially mature pears. Associating the highest inoculum loads with plugs and lowest with inoculation via air, a similar effect (growth rate and visible symptom expression) was found as described by Vilanova *et al*. (2012). Although the inoculum concentrations did not

significantly impact on growth rate of mature and over-mature pears (Vilanova *et al*., 2012), it can be expected to influence disease incidence.

The cold storage test results showed that a change in environmental conditions can have a significant influence on pathogen aggressiveness and symptom expression. *Penicillium expansum* was the only species that produced large lesions under cold storage conditions that simulate commercial practices. The results correspond with findings from Vilanova *et al*. (2012), indicating that the pathogen can infect, invade and produce symptoms while in the cold chain. *Penicillium crustosum* was unable to produce large lesions while under cold storage conditions compared to the big lesions that developed under room storage conditions. *Penicillium* conidia are able to tolerate extremely low temperature conditions and germinate when conditions become favourable for growth (Sonjak *et al*., 2006; Smilanick and Mansour, 2007). This can occur when the cold chain is broken during the extended supply chain. *Penicillium crustosum* may thus be able to produce lesions on 'Golden Delicious' apples once the fruit exit cold storage. *Penicillium digitatum* is able to germinate at 4°C (Plaza *et al*., 2002) and *P. solitum* is able to produce symptoms at -1°C (Sanderson and Spotts, 1995), but the low sensitivity of 'Golden Delicious' towards the two *Penicillium* spp. made it difficult to evaluate temperature based disease sensitivity of the species on the apple cultivar. The growth patterns produced by *P. expansum* and *P. digitatum* in cold storage were similar to those described by Vilanova *et al*. (2012). The results correspond with Vilanova *et al*. (2012), although the higher temperature in this study (6.2±1.7°C vs. 0°C) resulted in a shorter lag phase and faster growth rate for *P. expansum*. Future research should include cold storage trials with a range of cultivars with varying susceptibility levels. This will allow the identification of temperature based disease sensitivity of specific *Penicillium* spp. depending on host susceptibility.

Fruit designated for export remains in the supply chain for an extended period of time and often is re-packed in facilities that do not necessarily comply with the same quality standards as is required in packinghouses at the beginning of the supply chain. It is therefore hypothesised that losses at the consumer-end of the chain can be partly due to improper handling, breaking of the cold chain and sometimes re-packing of fruit in facilities that do not have to comply with the same voluntary (but *de facto* compulsory) standards.

5. CONCLUSION

All tested species; *P. expansum, P. crustosum, P. digitatum, P. solitum* and *P. brevicompactum* were pathogenic on pears, but only the former four proved pathogenic on apples. *Penicillium digitatum*, a known citrus pathogen, caused lesions on several pear and apple cultivars tested. This is the first report of *P. digitatum* obtained from a citrus chain environment showing high aggressiveness (exceeding that of *P. expansum*) on pears ('Rosemarie', 'Beurre Bosc' and 'Beurre Hardy') and causing rot on apples ('Granny Smith' and 'Cripps Pink'). These pear cultivars have been reported by the fruit industry as the most prone to decay at the retail-end of the supply chain resulting in significant financial losses. Differences in aggressiveness and host specificity were found among the isolates. *Penicillium expansum* and *P. crustosum* were pathogenic on all cultivars tested whereas *P. digitatum* and *P. solitum* had narrower host cultivar ranges. *Penicillium expansum* and to a lesser extent *P. crustosum* are known as the more typical postharvest pathogens on pome fruit. *Penicillium digitatum* was identified as an opportunistic pathogen on pome fruit that can result in rapid pome fruit losses at the market-end of the supply chain depending on sanitation in the storage/shipping/marketing environments, temperature management during shipping and marketing, and host susceptibility as related to cultivar and fruit maturity.

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Chapter 4

Pathogenic *Penicillium* spp. on Citrus

ABSTRACT

Citrus fruit are exposed to numerous postharvest pathogens throughout the fresh produce supply chain. Well-known postharvest citrus fruit pathogens are *P. digitatum* and *P. italicum*. Lesser known pathogens include: *P. crustosum* and *P. expansum*. This study examined pathogenicity and aggressiveness of *Penicillium* spp. present in fresh fruit supply chains on various *Citrus* spp. and cultivars. The impact of different inoculation methods and storage conditions on decay were also assessed. *Penicillium digitatum* and *P. italicum* were the most aggressive *Penicillium* spp. on citrus, but aggressiveness varied significantly over the evaluated citrus range. Decay and tissue-response lesions caused by *P. crustosum* were observed on 'Nules Clementine', 'Nova', 'Owari Satsuma', 'Delta Valencia', 'Cambria Navel', 'Eureka' seeded and 'Star Ruby' for the first time. Likewise, these lesions caused by *P. expansum* were noted on 'Nules Clementine', 'Owari Satsuma', 'Delta Valencia', 'Midknight Valencia' and 'Eureka' seeded for the first time. Tissue-response lesions affect fruit quality and some *Penicillium* spp. sporulated from the lesions, causing the inoculated species to complete their life cycle. New citrus-*Penicillium* interactions were observed and the importance of monitoring inoculum loads of pathogens and non-host pathogens were highlighted.

1. INTRODUCTION

Citrus has an economic and nutritional importance world-wide (Liu *et al*., 2012). South Africa exported 64% of its citrus produced in 2011 as fresh fruit, earning a gross export total close to R6 billion (CGA, 2012). Decay caused by fungal pathogens contributes greatly to postharvest losses (Eckert and Eaks, 1989; Marcet-Houben *et al*., 2012). The most important postharvest pathogen of citrus, *P. digitatum* (Pers.:Fr.) Sacc., can account for up to 90% of total losses (Eckert and Eaks, 1989; Marcet-Houben *et al*., 2012). Additional *Penicillium* spp. of concern in the citrus industry are *P. italicum* Wehmer, Hedwigia and *P. ulaiense* Hsieh, Su & Tzean (Plaza *et al*., 2003; Pitt and Hocking, 2009; Barkai-Golan, 2001; Nunes *et al*., 2010; Marcet-Houben *et al*., 2012). Previous reports also indicate *P. crustosum* Thom (Garcha and Singh 1976; Arrebolla *et al*. 2010), *P. expansum* Link ex Gray (Vilanova *et al*., 2012b) and *P. fellutanum* Biourage (Sinha, 1946) to be citrus pathogens, however information associating these species with losses in citrus industries is lacking. Data on *P. fellutanum* have not been confirmed.

Little is known about the pathogenicity of *P. crustosum* on citrus. Garcha and Singh (1976) reported total decay of mandarins inoculated with *P. crustosum* after eight days. The cultivar, inoculum concentration and incubation temperature were not specified. The described symptoms included: watery spots which later extend deeper into the tissue, white mycelial tufts that turn bluish-green when sporulating and a fermented odor. Arrebolla *et al*. (2010) confirmed *P. crustosum* as pathogenic on citrus by reproducing lesions (17.25±5.2 mm diameter; incidence = 36%) on 'Valencia' oranges kept in modified atmosphere packaging at 25°C for 12 days. Unfortunately, the research focus did not further address the *P. crustosum*-citrus interaction and was mainly focused on control.

Macarisin *et al*. (2007) reported *P. expansum* infections on citrus (lemon, grapefruit and orange) and attributed the arrested infection to the host's production of reactive oxygen species. Vilanova *et al*. (2012b) were able to facilitate development of *P. expansum* decay on commercially mature and over-mature oranges ('Navelina' and 'Valencia') with high inoculum concentrations (10^6 and 10^7 conidia/ml). Lesion averages were 3 mm and 8 mm in diameter on 'Valencia' fruit inoculated with 10^6 and 10^7 conidia/ml, respectively. The 'Valencia' oranges were incubated for 17 days at 20°C and 85% RH. Lesions on 'Navelina' were larger, averaging from 10 to 35 mm after 11 days for the highest inoculum concentration.

Penicillium spp. are ubiquitous organisms, commonly found in air, water, soil, indoor and in numerous fresh and processed food products (Frisvad and Samson, 2004; Pitt and Hocking, 2009). These characteristics and intricate fruit trade networks contribute to the dissemination of the fungus, often resulting in high inoculum build-up in these diverse environments of the fruit handling and marketing chain. Fruit trade networks can result in various fruit types, originating from different countries, being handled, transported and stored together (Vermeulen *et al*., 2006). These fruit can harbor different pathogens which can lead to cross-contamination when handled together. This exposes citrus fruit to typical postharvest pome fruit pathogens (i.e. *P. crustosum* and *P. expansum*) and pome fruit to the typical postharvest citrus pathogens (i.e. *P. digitatum* and *P. italicum*). Recently, Louw and Korsten (2014) reported *P. digitatum* pathogenic on pome fruits and Vilanova *et al*. (2012b) reported decay of oranges caused by *P. expansum*.

The aim of this study was to provide better understanding of the infective potential of *Penicillium* spp. in the citrus supply chain and confirm the pathogenicity and aggressiveness of different *Penicillium* spp. on various *Citrus* spp. and cultivars. The significance of different inoculation methods and storage conditions on decay development was also assessed.

2. MATERIALS AND METHODS

Fugal cultures. The isolates of *P. digitatum*, *P. italicum*, *P. crustosum* and *P. expansum* used in this study are listed in Table 4.1. With the exception of *P. solitum* that was replaced by *P. italicum*, they are the same isolates used in Chapter 3 and described by Louw and Korsten (2014). Criteria for selecting the isolates and species were based on their presence in export chains (pome and citrus) and pathogenic potential on citrus. Cultures were single spore isolated, grown on malt extract agar (MEA) (Merck, Biolab Diagnostics (Pty) Ltd, Johannesburg, South Africa) and incubated in darkness at 25°C for three weeks prior to fruit inoculation studies. The species were consistently used throughout the study.

Fruit origin and handling. Commercially harvested and graded citrus fruit obtained for the trials originated from commercial export farms in the Eastern Cape Province. Mandarin cultivars were ‘Nules Clementine’ (*Citrus clementina* hort. ex Tanaka), ‘Nova’ (hybrid: *C. clementina* x ‘Orlando’ tangelo (*C. paradisi* Macf. x *C. tangerina* hort. ex Tanaka)) and ‘Owari Satsuma’ (*C. unshiu* Marcow.). Sweet orange (*C. sinensis* L. Osbeck) cultivars

included 'Navel' ('New Hall', 'Palmer' and 'Cambria') and 'Valencia' ('Midknight' and 'Delta'). The lemon and grapefruit cultivars were 'Eureka' seeded (*C. limon* (L.) Burm. f.) and 'Star Ruby' (*C. paradisi* Macf.) respectively.

Table 4.1. *Penicillium* isolates used in the citrus trials

Isolate code	*Penicillium* spp.	Fruit chain	Date	Country of origin	Source (location of isolation)
PdC	*P. digitatum*	Citrus	2009/ 2010	Netherlands	Floor of distributer/repack facility. Re-introduced into plums and isolated from lesions (2011).
PdP	*P. digitatum*	Pear	2011	United Kingdom	Small waste bin of repack facility.
PiC	*P. italicum*	Citrus	2009/ 2010	Germany	Air of distributer/repack facility/cold room
PiP	*P. italicum*	Pear	2011	United Kingdom	Air of repack facility receive area.
PcC	*P. crustosum*	Citrus	2009/ 2010	Germany	Air, walls or floors of packhouse.
PcP	*P. crustosum*	Pear	2011	South Africa	Wall of packhouse holding area.
PeC	*P. expansum*	Citrus	2009/ 2010	Germany	Wall of distributer/repack facility. Re-introduced into apples and isolated from lesions (2011).
PeP	*P. expansum*	Pear	2011	United Kingdom	Air of cold storage facility.

Fruit were inoculated at two different laboratories depending on the seasonal availability of fruit. The first inoculations took place in a laboratory at the citrus packinghouse in the Eastern Cape and the second was done at the University of Pretoria (UP) facilities. Fruit inoculated at the packinghouse were 'Nules Clementine', 'Nova', 'Owari Satsuma', 'New Hall Navel', 'Palmer Navel' and 'Eureka' seeded. They were delivered directly after commercial handpicking by pickers. Fruit were inoculated one, two or three days after delivery, depending on availability and seasonality. 'Owari Satsuma' was the only cultivar that was stored (nine days at ±4°C) prior to inoculation. Fruit inoculated at UP were 'Cambria Navel', 'Midknight Valencia', 'Delta Valencia', 'Eureka' seeded and 'Star Ruby'. They were cargo shipped from Port Elizabeth to Johannesburg (transported under cargo holding conditions and cold stored on arrival), collected within 24hr and transported from Johannesburg to Pretoria where trials started a day thereafter. Fruit used in trials were physiologically mature according to the natural quality standards for export (South African Department of Agriculture, Forestry and Fishery, 2011) without any postharvest treatment.

Confirming *Penicillium* spp. pathogenicity on citrus. An initial pathogenicity trial was conducted to determine the pathogenicity of major postharvest *Penicillium* spp. pathogens encountered in citrus fruit storage and handling chains. Each isolate from the citrus chain (Table 1) was inoculated into mandarin ('Nules Clementine'), sweet orange ('New Hall Navel'), lemon ('Eureka' seeded) and grapefruit ('Star Ruby') fruit. Fruit were surface-sterilised prior to inoculation by dipping in 0.002% sodium hypochlorite solution for more than five min and allowed to air dry on a surface-sterilised table overlaid with paper towels. A set of 10 fruit were wounded (1.5 mm x 1.5 mm x 2 mm) on opposite sides by piercing into the pericarp prior to inoculation. Each inoculated fruit represented a replicate. A metal wire protruding from a cork was used for wounding to ensure wounds were uniform. Wounds were inoculated with conidial suspensions at 6.3 x 10^4 conidia/ml. Un-inoculated fruit were included as control. Conidial suspensions were prepared in sterile Ringers solutions (physiological saline solution, Merck) and 0.05% Tween 80 (Associated Chemical Enterprises, Johannesburg). Concentrations were determined using a haemocytometer. Ten microliter conidial suspension was deposited with a micropipette into each wound. A strip of Paraflim was taped around the fruit, covering the wounds to avert cross-contamination. Fruit were arranged in a completely randomised design (CRD) on a disinfected table and incubated for seven days under ambient conditions (20-22°C). Lesions were measured on the third, fifth and seventh day post-inoculation. Horizontal and vertical (calyx axis vertical) measurements were taken. The mean of control wounds was subtracted from the diameter of decay and tissue-response lesions. Tissue-response lesions were lesions caused by hypersensitive response (HR) reactions (infection was arrested after reaching a certain size). The experiment was repeated.

A comparative pathogenicity trial was conducted using *Penicillium* spp. environmental isolates from both fruit supply chains (Table 4.1). The aim of the trial was to determine if a citrus environment harbours similar isolates with respect to pathogenicity and aggressiveness as those from a pear supply chain environment. The methodology used was similar to the initial *Penicillium* spp. trial. Five mandarin fruit ('Nules Clemetine') were inoculated with each *Penicillium* spp. isolate. Five wounded, but un-inoculated fruit served as controls. Fruit were arranged in a CRD and the experiment was repeated. Incubation and data recording was as previously described.

Comparison of inoculation methods. The three methods used to inoculate 'Golden Delicious' apples in Chapter 3 were also evaluated on 'Eureka' seeded lemons. This allowed

the selection of the most suitable method for citrus fruit inoculation trials. The methods include inoculation via conidial suspensions, plugs (MEA) or aerial dispersed conidia. Methodology for inoculating fruit was as described in Chapter 3 and Louw and Korsten (2014), except for inoculation via air. Inoculation via air, unlike in Chapter 3, was completed with the inoculation chamber assembled inside a biosafety cabinet (Lee *et al*., 2009; Reponen *et al*., 1997) and an open sterile MEA plate (65 mm) positioned among the fruit in the chamber to observe conidial dissemination after setup run. Similar to Chapter 3, only citrus environment isolates were used in this trial and in succeeding trials, based on the absence of isolate significant differences. Ten 'Eureka' seeded lemons were wounded and inoculated with each *Penicillium* spp. for each method. Control fruit were also included for each method. Incubation, data recording, randomisation (CRBD), trial repeat and subtraction of wounds were as described in Chapter 3.

***Penicillium* decay of lemons under cold storage conditions.** The effect of cold storage conditions on decay development caused by *Penicillium* spp. was evaluated on 'Eureka' seeded lemons. Two sets of 10 surface-sterilised fruit were inoculated with each *Penicillium* spp. via conidial suspensions. Each inoculated fruit counted as a replicate. Wounds were covered with Parafilm as described earlier. One set of fruit was incubated under ambient conditions for seven days (21.9±0.4°C; 43.6±4.6% RH) and another under refrigerated conditions for 26 days (5.0±0.6°C; 86.4±4.4% RH). Lesion diameters for ambient incubated fruit were measured as described earlier. Measurements for fruit stored in the cold room started on the first day of lesion development (observation) and continued every second day thereafter up to the 26th day. Means of control wounds were also calculated to subtract from means of measured lesions. The experiment was repeated and arranged in a CRBD. Wounded, but un-inoculated fruit served as controls.

***Penicillium* spp. aggressiveness on citrus cultivars.** The aggressiveness of each *Penicillium* spp. was assessed on mandarin ('Nules Clementine', 'Nova' and 'Owari Satsuma'), sweet orange ('New Hall Navel', 'Palmer Navel', 'Cambria Navel', 'Midknight Valencia' and 'Delta Valencia'), lemon ('Eureka' seeded) and grapefruit ('Star Ruby') fruit. Ten surface-sterilised fruit from each cultivar was inoculated with each *Penicillium* spp. via conidial suspensions. Each inoculated fruit represented a replicate. Fruit were randomised (factorial arrangement on a CRD) and the experiment was repeated. The incubation of fruit and data collection was as described in the initial trial.

Re-isolation from fruit, identification and preservation. Isolates were made from two fruits from each *Penicillium*-cultivar interaction from each experiment in every trial. Variables were also involved in the case of the isolate comparison, inoculation method and cold room trials: different isolates, inoculation methods or incubation condition. In these cases, two isolates were also made for each *Penicillium*-cultivar interaction for each variable from both experiments in a trial. Isolates (MEA) were incubated as previously described. Sufficient growth from pure cultures revealed visual similarities among cultured isolates. Regardless, one culture from each cultivar-*Penicillium* interaction and variable was similarly identified (PCR-RFLP and sequencing) and preserved (two water- and two cryo-preservations per culture) as described in Chapter 3. The only difference from Chapter 3 was the use of a different thermocycler for PCRs and PCR yield was not verified on an agarose gel with a 1-kb ladder/marker. The *Penicillium* beta-tubulin (β-tubulin) gene was amplified in a CFX Connect™ Real-Time PCR Detection System (Bio-Rad, Singapore) using the Bt2a and Bt2b primers (Glass and Donaldson, 1995) and EvaGreen® dye (Biotium Inc., Hayward, USA). PCR yields are verified as PCR cycles progress. The PCR cycles were similarly; 95ºC for three min, 35 cycles of 94ºC for 30 sec followed by 57ºC for 45 sec and 72ºC for two min, and a final elongation step of 72ºC for seven min. The thermocycler (Eppendorf® Masterycler® Pro S, Hamburg, Germany) used for sequencing reactions was as described in Chapter 3.

Statistical analysis. SAS software (version 9.2; SAS Institute Inc., Carry, NC, USA) was used for statistical analysis of data. Each inoculated fruit accounted for a replicate. The two wounds on each fruit provided four sub-samples (two wound; each with a horizontal and vertical diameter). Respective means of control wounds were subtracted from lesions prior to statistical analysis. When the least-square mean t-test analysis revealed similarity between experiments in trials, experiments were pooled. Means were separated using Fisher's Least Significant Difference (LSD). When means follow with $\pm x$; x is standard deviation. Bars on graphs illustrate standard deviation.

Disease incidence (%) and lesion diameters (mm) from the pathogenicity trial were recorded to calculate disease intensity. Disease intensity combines disease incidence with disease severity to express disease concern linked to each *Penicillium* spp. on a specific crop. Tissue-response lesions, although they are not typical fruit decay lesions and infection is arrested, were considered because they have an important impact on fruit quality and in some cases became significantly large. Only lesions significantly larger than the mean of the

control wound diameters were considered for calculating disease intensity. Disease intensity was previously described and used by Van Eeden and Korsten (2013) and Louw and Korsten (2014). Disease intensity = [(d x F)/(D x T_n)] x 100; d - degree of disease severity assessed (mean lesion diameter); F – frequency (number of lesions); D - maximum lesion diameter measurable; T_n - total number of fruit, in our case lesions, examined.

3. RESULTS

Confirming *Penicillium* spp. pathogenicity on citrus. No significant difference was observed between the independent pathogenicity experiments (P = 0.7). Only *P. digitatum* and *P. italicum* caused large lesions on mandarin ('Nules Clementine'), sweet orange ('New Hall Navel'), lemon ('Eureka' seeded) and grapefruit ('Star Ruby'). Tissue-response

Table 4.2. Pathogenicity of *Penicillium* spp. on citrus

Cultivar	*Penicillium* spp.	Means (mm) of lesions[a]	Incidence (%) (significant lesions)[b]	Mean (mm) of only significant lesions[a,b]	Disease intensity (%)[a,b]
Nules Clementine	*P. digitatum*	84.2 ± 21.8 a	100.0	84.2 ± 21.8	91.3
	P. italicum	34.3 ± 16.4 b	80.0	35.9 ± 15.5	31.1
	P. crustosum	2.6 ± 2.8 c	5.6	9.1	0.6
	P. expansum	1.0 ± 2.0 c	5.0	10.7	0.6
	Control	0.1 ± 0.2 c	-	-	-
New Hall Navel	*P. digitatum*	81.0 ± 26.8 a	95.0	84.9 ± 21.2	78.2
	P. italicum	33.1 ± 7.8 b	79.0	33.1 ± 7.8	25.4
	P. crustosum	1.1 ± 2.6 c	0	-	-
	P. expansum	0. 9 ± 1.8 c	0	-	-
	Control	0.1 ± 0.2 c	-	-	-
Star Ruby	*P. digitatum*	119.8 ± 20.7 a	90.0	119.8 ± 20.7	81.3
	P. italicum	32.1 ± 9.6 b	85.0	32.1 ± 9.6	20.6
	P. crustosum	2.4 ± 2.5 c	16.7	7.9 ± 0.5	1.0
	P. expansum	1.4 ± 1.5 c	5.0	9.3	0.4
	Control	0.1 ± 0.2 c	-	-	-
Eureka seeded	*P. digitatum*	84.4 ± 10.9 a	90.0	84.4 ± 10.9	84.9
	P. italicum	46.4 ± 6.9 b	85.0	46.4 ± 6.9	44.0
	P. crustosum	0.4 ± 1.1 c	0	-	-
	P. expansum	0.1 ± 0.1 c	0	-	-
	Control	0.1 ± 0.1 c	-	-	-

Disease intensity = [(d x F)/T_n x D] x 100; D values: 'Nules Clementine' = 92.25 mm, 'New Hall Navel' = 103.13 mm, 'Star Ruby' = 132.51 mm, 'Eureka' seeded = 89.55 mm.
a, Mean of the control diameters were subtracted from the mean of the measured diameters;
b, Only measurements significantly larger than the mean of the control diameter were included in calculating these figures.
Means followed by ±*x*; *x* refers to standard deviation.

lesions included in disease intensity were caused by *P. crustosum* and *P. expansum* on 'Nules Clementine' and 'Star Ruby' (Table 4.2). Additional tissue-response lesions caused by *P. expansum* and *P. crustosum* considered worth mentioning were observed on 'Nules Clementine'; *P. crustosum* = 7.2±2.3 mm lesion diameter (*ld*) (22.2%), 'New Hall Navel'; *P. crustosum* = 8.1 mm *ld* (5.9%) and *P. expansum* = 4.9±1.0 mm *ld* (11.1%), 'Star Ruby'; *P. crustosum* = 6.7±2.3 mm *ld* (27.8%) and *P. expansum* = 5.0±3.8 mm *ld* (15.0%) and 'Eureka' seeded; *P. crustosum* = 4.3±2.9 mm *ld* (10.5%).

Independent experiments comparing citrus- and pear chain environment isolates on mandarins, were non-significant different ($P = 0.8$). Importantly, significant differences were not found when *Penicillium* spp. lesion sizes of pear and citrus isolates were compared (Fig. 4.1). Three t-groupings formed; *P. digitatum* isolates, *P. italicum* isolates and the remainder of the *Penicillium* spp. isolates grouped with the control. It was again noted that *P. crustosum* and *P. expansum* were able to produce tissue-response lesions on 'Nules Clementine'. Lesions caused by pear isolates were 2.1±0.7 mm (10.5%) and 2.4±1.1 mm (10.5%) respectively. Lesions caused by citrus isolates were 4.6±1.7 mm (41.2%) and 2.1±0.7 mm (11.1%) respectively. In these cases mean of control wounds were already subtracted from the lesion diameters.

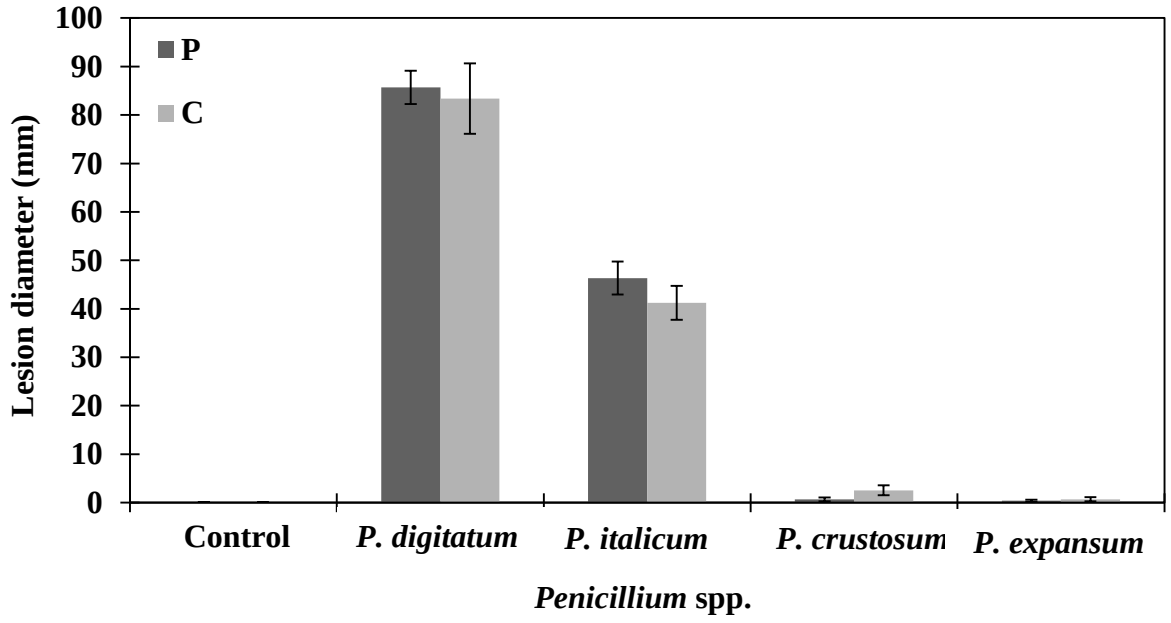

Fig. 4.1. Lesion diameters caused by different *Penicillium* spp. isolates on 'Nules Clementines' (seven days incubation at ambient conditions); **P,** Pear supply chain isolate; **C,** Citrus supply chain isolate. Bars illustrate standard deviation.

Comparison of inoculation methods. Results from the independent experiments were pooled (P = 0.6). The mean lesion sizes produced by the inoculation methods differed significantly (P < 0.0001). The plug method received a separate t-grouping from the conidial suspension and air inoculation methods. However, *P. digitatum* inoculated via the conidial suspension was significantly different compared to inoculations via air, but not plugs. Decay lesions of *P. expansum* and *P. crustosum* were produced on 'Eureka' seeded when inoculation took place via the plug method (Fig. 4.2 and Fig. 4.3). The *P. expansum* and *P. crustosum* lesions displayed a rapid initial lesion growth rate, but rates decreased as decay progressed. The other methods were only able to facilitate tissue-response lesions from *P. expansum* and *P. crustosum* inoculations (Fig. 4.3). Inoculation via air (cultured plate as source of inoculum) delivered one *P. crustosum* (1.1 mm) and six *P. expansum* (3.9±5.4 mm) lesions and inoculated via conidial suspensions delivered one *P. expansum* (2.4 mm) lesion (mean of control wound already subtracted). Some lesions were small, but the inoculated species were able to sporulate (Fig. 4.3).

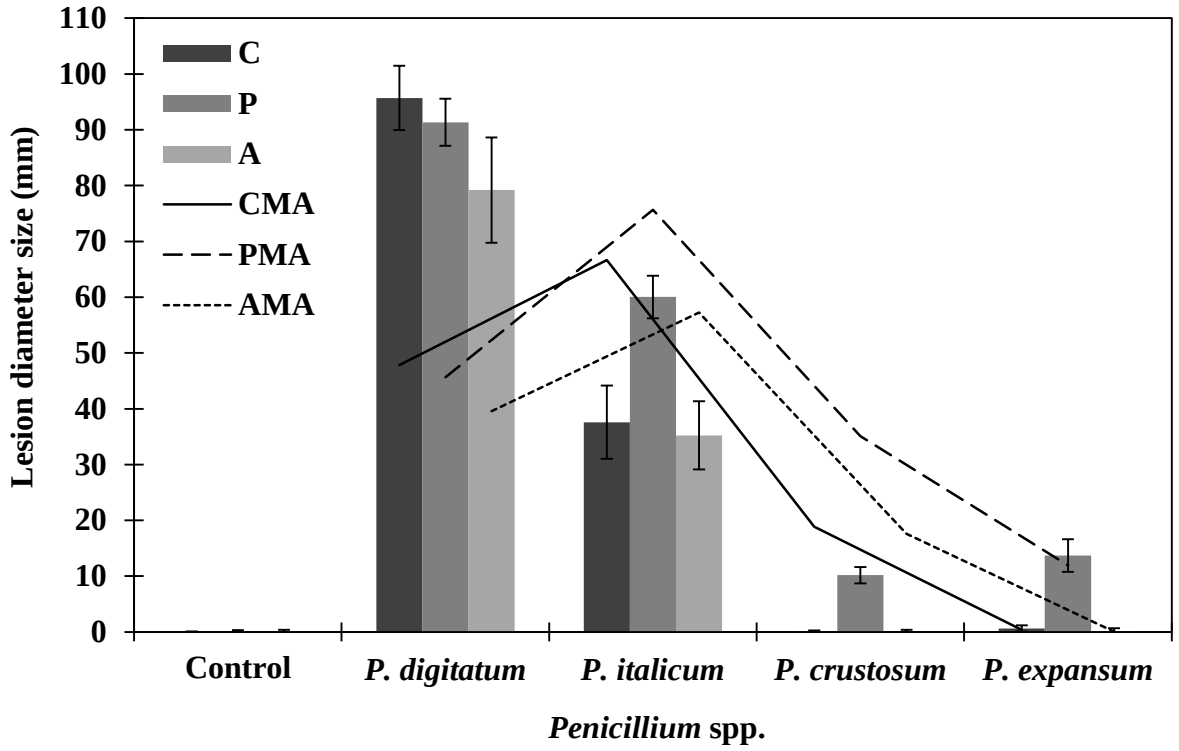

Fig. 4.2. Seven day lesion diameters of *Penicillium* spp. inoculated into 'Eureka' seeded using three different inoculation methods; **C,** Inoculation via conidial suspensions (**CMA,** Conidial suspension inoculation moving average); **P,** Inoculation via plugs (**PMA,** Plug inoculation moving average); **A,** Inoculation via air (**AMA,** Air inoculation moving average). Bars illustrate standard deviation.

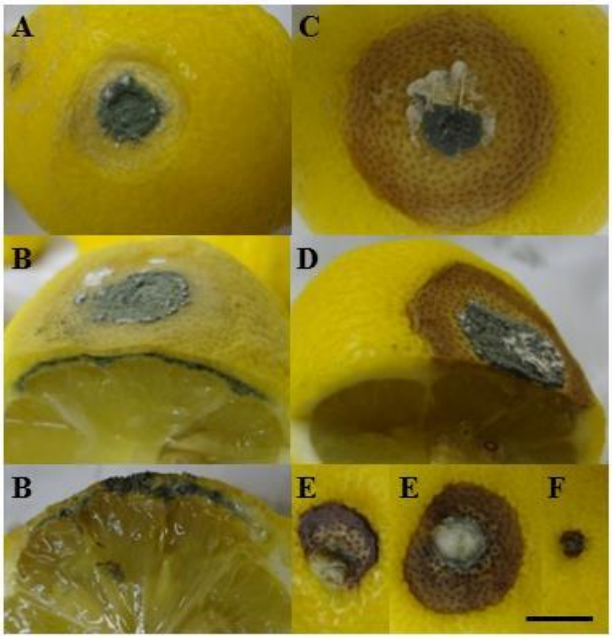

Fig. 4.3. Decay and tissue response lesions caused by *Penicillium crustosum* and *Penicillium expansum* on 'Eureka' seeded inoculated via three different methods; **A-B,** seventh day *P. crustosum* lesions via plug inoculation; **C-D,** seventh day *P. expansum* lesions via plug inoculation; **E,** 14th day *P. expansum* lesions via air inoculation; **F,** 14th day *P. expansum* lesions via suspension inoculation. Bar = 10 mm.

Inoculation via conidial suspensions were found to be convenient and less time consuming, but results produced varied more in comparison to plug inoculation results. Inoculation via plugs delivered the highest incidence (*P. digitatum* and *P. italicum* = 100.0%, *P. crustosum* = 90.0%; *P. expansum* = 94.9%), measurements from repeats deviated the least, symptom expression was rapid and well-defined, *P. italicum* lesions were significantly larger and it was the only method yielding well-defined *P. crustosum* and *P. expansum* decay lesions on citrus (Fig. 4.2 and Fig. 4.3). Inoculation via air was the most sensitive to contamination and least convenient compared to the other methods. In addition, inoculation via air also revealed biased results when directly using cultures as source of inoculum (can also be expected form inoculation via plugs). Disease incidence of *P. digitatum* was low (20.0%) when inoculated via air and using plates as source of inoculum. Upon completing air inoculations using an infected lemon covered with conidia (seven days ambient incubation) as source of inoculum, a *P. digitatum* disease incidence of 82.5% was achieved. This

incidence was comparable to that achieved with inoculations via conidial suspension (84.2%) and the mean of the lesion size was still similar to that obtained from inoculation via air using a *P. digitatum* culture plate as source of inoculum (76.4±19.9 mm vs. 79.2±18.9 mm).

***Penicillium* decay of lemons under cold storage conditions.** Lesion development was significantly influenced by temperature. The individual experiments for the cold storage trial did not differ significantly on the seven day incubation period (P = 0.5). However, contamination emerged within the third week of the first experiment (predominantly only fruit stored at cold conditions). Only results from the second experiment will be discussed. *Penicillium digitatum* and *P. italicum* were able to cause lesions under cold storage conditions (5.0±0.7°C; 86.4±4.5% RH). The lesions were 43.8±5.6 mm and 19.9±6.9 mm respectively after 26 days cold storage and 96.2±16.3 mm and 33.9±11.0 mm respectively after seven days ambient storage (mean of control wounds already subtracted). No lesions were observed from *P. crustosum* inoculated lemons stored at any condition. A single tissue-response lesion caused by *P. expansum* developed under ambient conditions after seven days incubation (ld = 6.9 mm).

Lesion growth rates were calculated from the first day of lesion development to the last day of measurement. Growth rates at ambient and cold conditions for *P. digitatum* were 13.8 mm/day and 3.4 mm/day respectively and for *P. italicum* were 4.8 mm/day and 1.4 mm/day respectively. The growth rate of lesions caused by *P. digitatum* and *P. italicum* was correspondingly reduced by 75.5% and 70.7% due to the cold conditions. The largest lesions at cold storage (43.8±15.6 mm and 19.9±6.9 mm respectively) were respectively delayed by 21 and 20 days. The first signs of lesion development under cold conditions from *P. digitatum* inoculated fruit was observed on day 13/14, whereas *P. italicum* started to cause lesions a day earlier. Alternatively, *P. digitatum* was the first to cause lesions under ambient conditions (day 1/2) and *P. italicum* only a day thereafter. The earliest mycelia and conidia were observed on day 21/22 under cold storage conditions vs. day four/five under ambient conditions for both *P. digitatum* and *P. italicum*.

***Penicillium* spp. aggressiveness on citrus cultivars.** Results from independent experiments were not significantly different and thus pooled (P = 0.7). The interaction effect of the *Penicillium* spp. on the different cultivars showed significant difference (P < 0.0001). Large lesions were caused by *P. digitatum* and *P. italicum* over the whole citrus range evaluated (Fig. 4.4). Lesion sizes produced did however vary significantly. In general the

lesions caused by *P. digitatum* decreased in size: lemon > mandarin > sweet orange > grapefruit. Aggressiveness of *P. digitatum* varied more over mandarin cultivars (each cultivar grouped in separate t-groupings) and less over sweet orange cultivars (some 'Navel' and 'Valencia' did not differ significantly). The aggressiveness of *P. italicum* was more consistent over the citrus range than that of *P. digitatum*. The means of almost all lesions caused by *P. italicum* were grouped together or in related t-grouping, except for those on 'Owari Satsuma' and 'Cambria Navel'. The largest mean of lesion diameters caused by *P. digitatum* and *P. italicum* was respectively produced on 'Eureka' seeded and 'Owari Satsuma'.

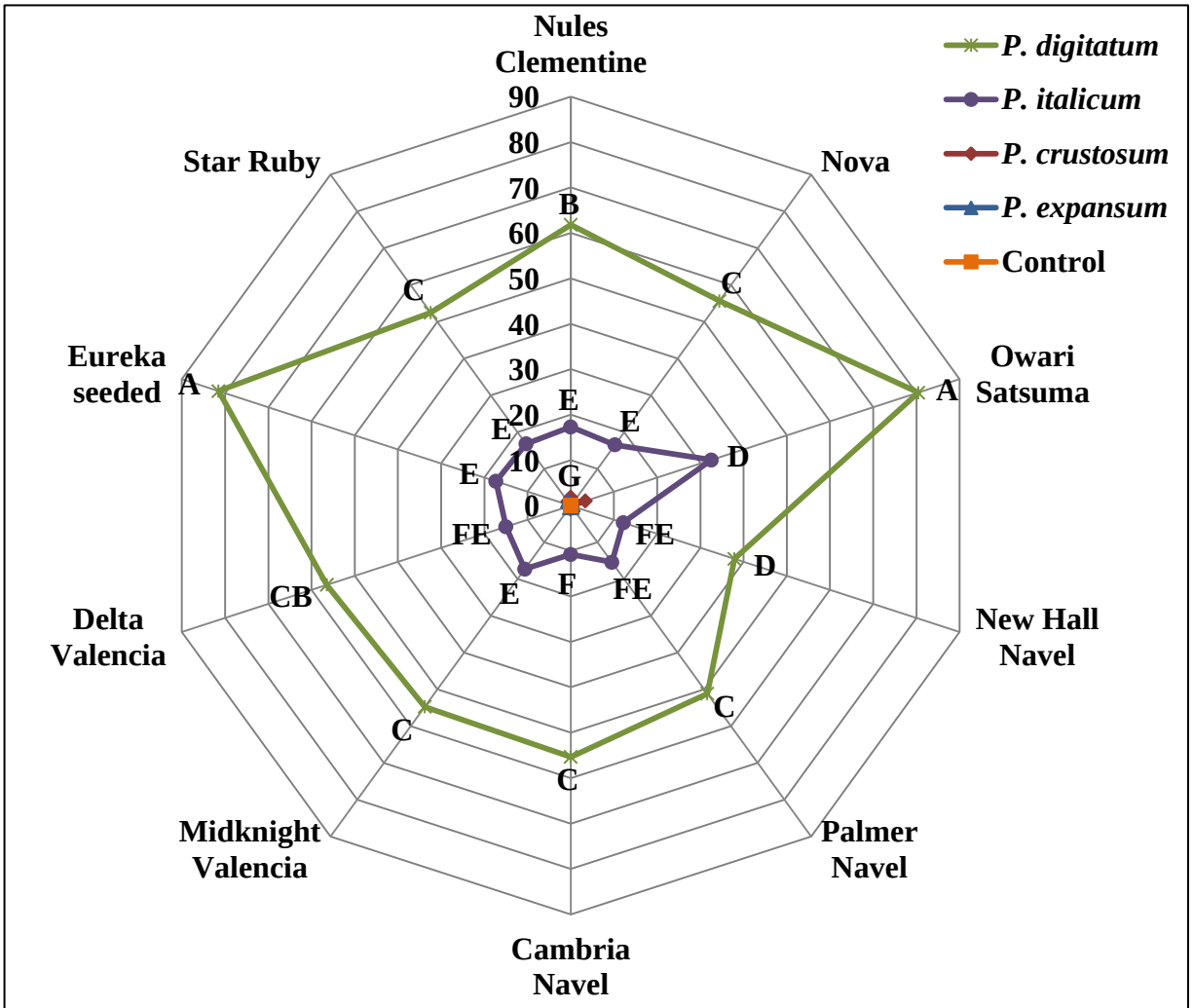

Fig. 4.4. Decay and tissue-response lesion diameters (mm) caused by *Penicillium* spp. on various citrus cultivars (incubated under ambient condition for five days). Letters that are not the same are significantly different.

The means of lesions caused by *P. crustosum* and *P. expansum* were not significantly different compared to the control on the fifth day of incubation (Fig. 4.4). However, some lesions caused by *P. crustosum* and *P. expansum* (decay and tissue-response lesions) were significantly different on the seventh day of incubation. Most lesions were small and developed at low incidences Table 4.3.

Table 4.3. Citrus-*Penicillium* disease interactions with incomplete incidence (<100%) after seven days incubation

***Penicillium* spp.**	**Cultivar**	**Mean of significant lesions[a,b]**	**Incidence (%)[b]**
P. crustosum	Nules Clementine	9.1 ± 2.9	29.7
	Nova	11.9 ± 1.7	10.8
	Owari Satsuma	11.0 ± 2.9	48.3
	Delta Valencia	6.0 ± 0.7	8.8
	Cambria Navel	7.1	2.6
	Star Ruby	7.9 ± 0.5	8.1
P. expansum	Nules Clementine	13.3 ± 1.1	6.1
	Owari Satsuma	9.0 ± 1.7	19.4
	Midknight Valencia	7.7 ± 0.4	7.1
	Delta Valencia	14.8	2.6

a, Mean of the control diameters were subtracted from the mean of the measured diameters;
b, Only measurements significantly larger than the mean of the control diameter was included in calculating these figures.
Means followed by ±*x*; *x* refers to standard deviation.

***Penicillium* symptom expression on citrus cultivars.** Additional symptom characteristics caused by the *Penicillium* spp. were distinguished on the citrus range evaluated. Lesions caused by *P. digitatum* and *P. italicum* radiated with a watery soaked appearance as infection progressed. Infected rind (pericarp) tissue lost its smoothness and became more susceptible to mechanical damage. *Penicillium italicum* exhibited darker infected tissue on mandarin cultivars and 'Eureka' seeded than on sweet orange cultivars and 'Star Ruby' (darkening more localised around the inoculation sites) (Fig. 4.5). White mycelail growth later started to radiate from the infected tissue, followed by sporulation (dark olive green conidia for *P. digitatum* and blue conidia for *P. italicum*).

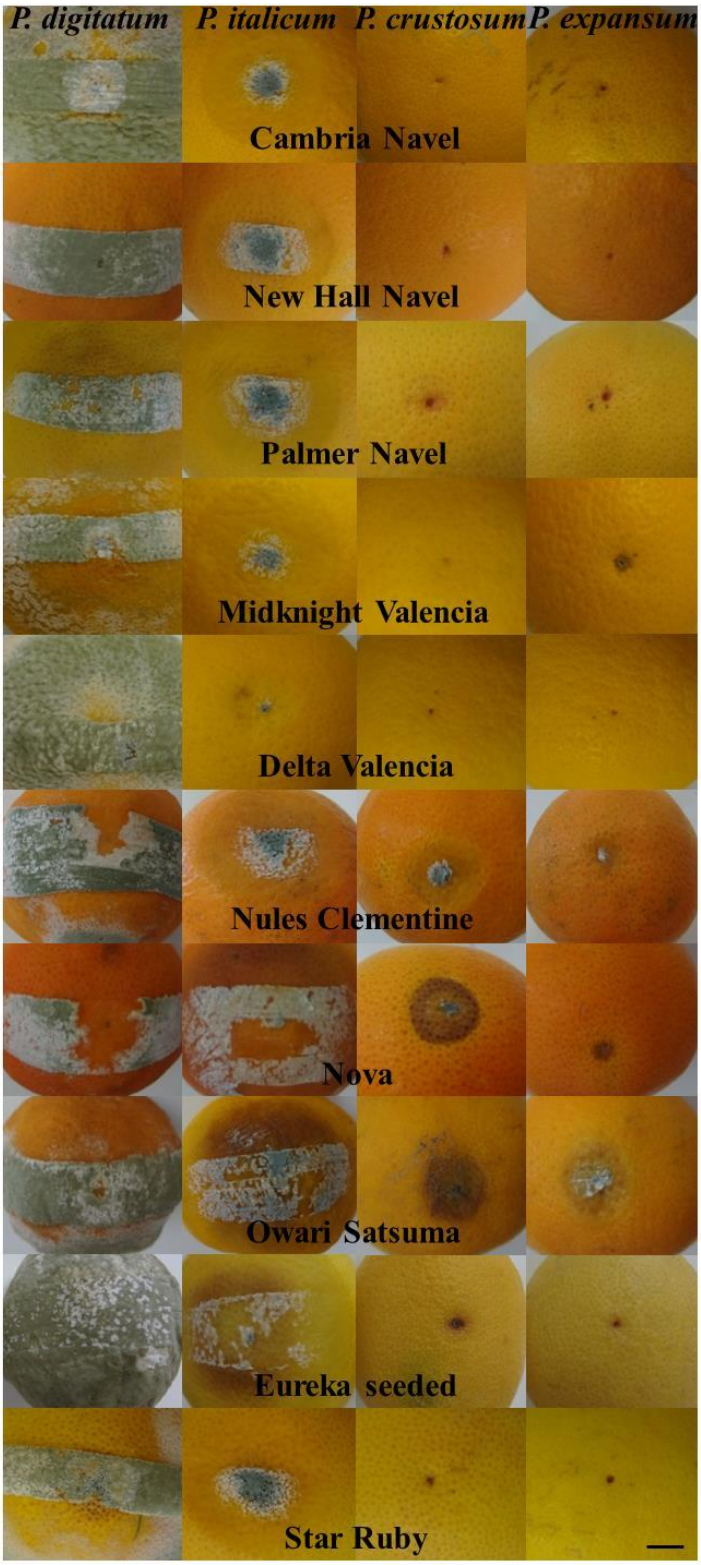

Fig. 4.5. Symptom expression of *Penicillium* spp. (columns from left to right: *P. digitatum*, *P. italicum*, *P. crustosum*, *P. expansum*) on citrus after seven days incubation at ambient temperatures. Bar = 10 mm.

Decay lesions caused by *P. crustosum* and *P. expansum* on 'Eureka' seeded (plug inoculated) and some mandarin cultivars (conidial suspension inoculated) had a sunken-in appearance and infected/affected rind tissue was harder, drier and browner than healthy tissue. This only diverged with 'Nules Clementine' and 'Owari Satsuma' in which cases the lesions were softer than before mentioned. Lesions of *P. crustosum* were in general lighter coloured (Fig. 4.3), but not consistently so over the tested citrus range (Fig. 4.5). The browning of lesions varied, depending on numerous factors (*Penicillium* spp. used, cultivar host and presumably environmental factors). Tissue-response lesions (typical HR reactions) were characterised as small, hardened, dark and sunken symptoms. Some HR reactions became relatively large compared to typical symptom characteristics expected from such reactions (Table 4.3 and Fig. 4.3).

Mycelial growth and sporulation of *P. crustosum* and *P. expansum* from decay lesions were occasionally restricted to close proximity of the inoculated sites. Both species were also able to sporulate at some tissue-response lesions, despite the small size of the lesions (Fig. 4.3 and Fig. 4.5). This caused difficulties when trying to characterize such lesions, especially when they were also significantly larger than controls. In the case of inoculation via plugs, sporulation of *P. crustosum* from decay lesions was also limited to close proximity of the inoculated site when observing the lesions from the fruit surface (on the exocarp). However, underneath the exocarp, sporulation took place within the spongy mesocarp and endocarp of infected pericarp tissue. Even fruit pulp/juice vesicles were affected (browning). Sporulation underneath the exocarp of infected *P. expansum* lemons was not observed, although the infected rind tissue was drier and harder than *P. crustosum* infected tissue (Fig. 4.3). *Penicillium crustosum* and *P. expansum* produced mycelia and sporulated from some lesions on 'Nules Clementine' (*P. crustosum* mycelial growth on day three and sporulation on day five; *P. expansum* mycelial growth on day five and sporulation day seven) and 'Owari Satsuma' (*P. crustosum* mycelial growth and sporulation on day four/five; *P. expansum* mycelial growth and sporulation on day six/seven). Only *P. crustosum* produced mycelia and conidia on 'Nova' (day two/three) and 'Delta Valencia' (day six/seven). Conidia produced by *P. crustosum* were pale turquoise no. 4 and conidia from *P. expansum* were light blue no. 4 with a tint grayer in colour (Fig. 4.3 and Fig. 4.5).

The first signs of *P. digitatum* and *P. italicum* mycelial growth and sporulation were detected on day four/five on nearly all cultivars inoculated. It was only detected earlier on 'Nules Clementine' (day three). The colour or shade of conidia produced from the lesions was not continuously uniform over the citrus range. The shade of green of the *P. digitatum* conidia varied. 'Cambria Navel', 'Midknight Valencia', 'Delta Valencia' and 'Star Ruby' displayed conidia of dark olive-green no. 4. 'New Hall Navel', 'Palmer Navel' and 'Nova' resulted in the production of dark olive-green conidia. Conidia produced by *P. digitatum* from lesions on 'Owari Satsuma' and 'Eureka' seeded were dark olive-green with a tint of more gray and lastly conidia from lesions on 'Nules Clementine' were dark sea-green no. 4 coloured. *Penicillium italicum* conidial colour was more consistent ranging from light blue no. 3 to light blue no. 4, depending on the amount of conidia grouped (Fig. 4.3 and Fig. 4.5).

Isolate identity confirmation. Re-isolated *Penicillium* spp. from the infected sites was confirmed as the previously inoculated species by PCR-RFLP and sequencing. The β-tubulin gene sequences were submitted in GenBank (Table 4.4).

Table 4.4. Accession numbers as allocated by GenBank for the β-tubulin gene sequences of *Penicillium* isolated from citrus

Isolate no.	Species identity	Accession no.
C57	*P. digitatum*	KF952540
C55	*P. digitatum*	KF952539
C51	*P. digitatum*	KF952538
C45	*P. italicum*	KF952537
C41	*P. italicum*	KF952536
C37	*P. italicum*	KF952535
C29	*P. crustosum*	KF952534
C27	*P. crustosum*	KF952533
C23	*P. crustosum*	KF952532
C15	*P. expansum*	KF952531
C11	*P. expansum*	KF952530
C6	*P. expansum*	KF952529

4. DISCUSSION

Penicillium crustosum and *P. expansum* were pathogenic on some cultivars with varying incidences and aggressiveness. Both species caused decay lesions on lemons and mandarins, but only tissue-response lesions on most of the remaining cultivars evaluated. Conidia were produced on decay and some of the tissue-response lesions (Fig. 4.3 and Fig. 4.5), allowing *P. crustosum* and *P. expansum* to complete their life cycles in these cases. Additionally, the small lesions will affect marketability of fruit. These species are well-known deciduous fruit pathogens (Louw and Korsten, 2014), but very few reports have described them as citrus pathogens and none has described the species as pathogens on a broad citrus host range or provided complete symptom descriptions (Garcha and Singh, 1976; Arrebolla *et al*., 2010; Macarisin *et al*., 2007; Vilanova *et al*., 2012b). This also links citrus with the potentially harmful toxins associated with the species; citrinin (nephrotoxin), communesin B (cytotoxic), patulin (multiple range), penitrem A (neurotoxin), roquefortine C (neurotoxin), terrestric acid (cardiotoxin) and others (Frisvad and Samson, 2004; Frisvad *et al*., 2004; Pitt and Hocking, 2009).

Garcha and Singh (1976) conducted cross inoculation studies, revealing complete *P. crustosum* decay of mandarins within eight days. They were the first to report *P. crustosum* pathogenic on citrus. They confirmed Koch's postulates and identified *P. crustosum* via morphologically techniques used at that time. Symptoms described were similar to those reported in this study (shallow/sunken lesions, mycelial growth as clumps and the bluish-green colour of conidia). The fermented odour noted by Garcha and Singh (1976) was not observed in this study and the symptom illustrations could not be compared due to the vagueness of their images. A more recent study concerning *P. crustosum* on citrus reported a mean lesion diameter of 17.3±5.2 mm at 36.0% incidence (Arrebolla *et al*., 2010). Their study focused mainly on biocontrol screening and therefore did not elaborate on the lesions produced by *P. crustosum*.

Our findings confirmed *P. crustosum* pathogenic on mandarins (Garcha and Singh, 1976), but only tissue-response lesions were noted on 'Valencia' oranges (Arrebolla *et al*., 2010). Lesions were also smaller and disease incidence lower. Differences with Garcha and Singh (1976) on mandarins can be ascribed to: different incubation conditions (higher relative humidity (RH) of 90%, temperature unmentioned), inoculum concentration (not specified), inoculation method (cotton pad wetted with conidial suspension and placed in a pinpricked

fruit for 48h), fruit age (market fruit) and cultivar. Differences noted by Arrebolla *et al*. (2010) on 'Valencia' are likewise credited to: different incubation conditions (higher temperature of 25°C, humidity unspecified), inoculum concentration (higher inoculum concentration of 3 x 10^6 conidia/ml), inoculation method (dipping of wounded fruit in conidial suspension for three minutes) and incubation period (12 days). Vilanova *et al*. (2012b) have shown that conidial concentrations can impact lesion growth rate, initial lesion development and disease incidence. Disease incidence can be influenced by inoculum concentration to such an extent that certain pathogens can be overlooked if inoculum concentrations are too low. Our study is the first to report *P. crustosum* pathogenic on 'Nules Clementine', 'Nova', 'Owari Satsuma' and 'Eureka' seeded. It is also the first tissue-response lesions caused by *P. crustosum* reported on 'Delta Valencia', 'Cambria Navel' and 'Star Ruby'. Symptoms on the mandarin cultivars and 'Eureka' seeded were well-documented.

Macarisin *et al*. (2007) inoculated lemons, grapefruit and oranges (cultivars not mentioned) with 20 µl of *P. digitatum* and *P. expansum* conidial suspensions (10^5 conidia/ml). Fruit were incubated at 20°C in darkness in covered plastic trays with moistened filter paper. They found that *P. expansum* was able to germinate and temporarily grow in the peel wounds of the three inoculated citrus group. Growth progressed until the plant defence-related oxidative burst was triggered leading to HR, averting infection or invasion (Macarisin *et al*., 2007). They additionally reported that *P. digitatum* is able to infect citrus because the species is able to suppress the host's defence-related oxidative burst. Based on this, Macarisin *et al*. (2007) treated fruit with citric-, ascorbic- and oxalic acids and enzyme catalase (suggestive H_2O_2 production suppressors) before inoculation with *P. expansum*. This resulted in lesions caused by *P. expansum* on all three citrus groups. They were the first to report on the pathogenic potential of *P. expansum* on citrus, although lesions produced by the non-host pathogen were artificially stimulated. No symptom descriptions were provided for the lesions.

Vilanova *et al*. (2012b) were able to observe well-defined lesions caused by *P. expansum* on citrus ('Valencia' and 'Navelina' oranges) without employing chemical pre-treatments such as those used by Macarisin *et al*. (2007). Rot caused by *P. expansum* was only observed after inoculating fruit with high conidial concentrations (no lesions at 10^4 or 10^5 conidia/ml). Lesion diameters were 3 mm (10^6 conidia/ml) and 8 mm (10^7 conidia/ml) after 17 days incubation (20°C; 85% RH). Much larger lesions were recorded on 'Navelina'; up to 35mm in diameter within 11 days. The more suitable RH for decay could have contributed to

advanced lesion sizes (Amiri and Bompeix, 2005; Sugar, 2009) and symptom development (mycelial growth and sporulation). Vilanova *et al*. (2012b) additionally reported orange-red coloured reactions around inoculation sites on the flavedo when *P. expansum* was unable to infect. The albedo tissue underneath the inoculated sites was dead. The reactions were concentration-dependent and prominent when inoculating fruit with high inoculum concentrations (10^7 conidia/ml). No reactions were observed from fruit inoculated with 10^4 conidia/ml. This was the first report where *P. expansum* caused decay of oranges under specific conditions.

We observed lesions on 'Valencia' oranges, but not on 'Navel' oranges, after inoculation with *P. expansum* (Vilanova *et al*., 2012b). The lower inoculum concentrations used in our study may have been too low to facilitate infections. Our study is the first to report *P. expansum* pathogenic on lemons ('Eureka' seeded) when inoculated via plugs (high concentration) and mandarins ('Nules Clementine' and 'Owari Satsuma') when inoculated via conidial suspensions (low concentration). It is also the first tissue-response lesions caused by *P. expansum* recorded on 'Valencia Midknight' and 'Valencia Delta' after direct inoculation with a low inoculum concentration (6.3×10^4 conidia/ml). Symptoms on 'Eureka' seeded and mandarins in our study presented similarity to symptoms illustrated by Vilanova *et al*. (2012b) on 'Valencia' and 'Navelina' oranges.

The well-known postharvest citrus pathogens, *P. digitatum* and *P. italicum* (Holmes and Eckert, 1999), were confirmed as being the most aggressive *Penicillium* spp. on all the citrus cultivars in terms of. decay and incidence. Lesions caused by *P. digitatum* decreased in size from mandarins and 'Eureka' seeded to sweet oranges and 'Star Ruby', confirming citrus susceptibility increasing from grapefruit, oranges, lemons to mandarins for most diseases (Reuther *et al*., 1989). This was not observed with *P. italicum* which expressed itself as a general pathogen over the entire citrus range evaluated in this study.

Aggressiveness of *P. digitatum* varied more over mandarin than over sweet orange cultivars. This could be due to a larger variance found among the mandarin cultivars tested. The mandarin cultivars differed on species level (according to Tanaka classification (Tanaka, 1969 and 1977)), whereas sweet oranges cultivars all belonged to the same *Citrus* sp. 'Owari Satsuma' was expected to be the most susceptible mandarin cultivar (industry observation), but in our study the fruit were stored for a prolonged period, which could have contributed to the larger lesions observed. Larger lesions caused by *P. digitatum* and *P. italicum* were

expected on 'Navel' oranges than on 'Valencia' oranges (industry observation), but this was not observed in this study. The smallest lesions were observed on 'New Hall Navel' and the largest on 'Delta Valencia' in this study. This can be based on differing incubation environments, but susceptibility alone depends on multiple factors. Susceptibility varies among cultivars and can be influenced by scion wood or rootstocks, cultural practices, harvest season, water and nutrient status of tree, fruit maturity and the postharvest environment (Eckert and Eaks 1989).

Symptoms recorded in this study added descriptions to rather un-described *P. crustosum* and *P. expansum* symptoms (Garcha and Singh, 1976; Vilanova *et al*., 2012b). Symptoms of *P. digitatum* and *P. italicum* are well-known (Fawcett and Klotz, 1948; Snowdon, 1990), but not necessarily over a range of citrus cultivars. Little *P. italicum* symptom variance was observed over the citrus ranges: conidial colour was relatively constant, but lesion darkness and time required for initial mycelial growth and sporulation differed to some level. Regarding *P. digitatum*, lesion darkness was more constant over the citrus range, but this was not the case with conidial colour.

Different isolates (citrus- and pear chain isolates) were not significantly different when lesion sizes were compared, indicating that disease severity was not significantly affected by the type of isolate. However, decay problems should be connected to the most likely origin of the inoculum sources (i.e. confinements with high inoculum loads) so that problem areas can be highlighted in the supply chain. Introduction of *P. crustosum* and *P. expansum* conidia from pome fruit into an environment where citrus fruit are handled or re-packed can result in cross-contamination and cross-infection of citrus fruit, especially when inoculum levels are high (Vilanova *et al*., 2012b). The probability of cross-infection of *P. digitatum* from citrus to pome fruit has also been projected by Louw and Korsten (2014). This aspect is important as different fruit types are often handled in the same environment during periods of overlapping seasons and towards the end of the chain (i.e. citrus and pome fruit).

Results from inoculation via plugs were the most consistent and symptoms were well-defined. The unique results produced with the plug method can be attributed to the direct inoculation of a concentrated inoculum dose in an open wound (Vilanova *et al*., 2012b). The role inoculum load plays in incompatible host-pathogen interactions (non-host pathogens) has been highlighted on citrus (Chapter 4; Vilanova *et al*., 2012b) and apples (Chapter 3; Vilanova *et al*., 2012a; Vilanova *et al*., 2014).

The inoculation of fruit via aerially dispersed conidia is the most likely pathway for natural *Penicillium* infections (see Chapter 3). This method still requires further modifications before it can be regarded as a suitable postharvest inoculation method for inoculating fruit/vegetables with pathogens capable of air dissemination. The method was found to be the least convenient and most sensitive to (cross-contamination and the inoculum source) in this study. The culturing of *Penicillium* spp. on artificial media can result in advanced growth and/or sporulation for certain species (i.e. *P. expansum* and *P. crustosum* grew faster and sporulated more abundantly than *P. digitatum* on MEA within the same incubation period and conditions). Sensitivity of the method to contamination was overcome by inoculating fruit in the inoculation chamber set up inside a biosafety cabinet (Lee *et al*., 2009; Reponen *et al*., 1997). An easier to sterilise inoculation chamber and making use of infected fruit as source of inoculum are proposed improvements for the method. Fruit as source of inoculum serve as a more natural infection source and pathway, and can eliminate biased interaction found with culturing. An alternative source of inoculum can be dry conidial masses (conidia/grams), added to the chambers to standardise inoculum loads disseminated in chambers. Specifying atmospheric inoculum loads (conidia/cm^3) required for each species that can cause decay will prove beneficial for industry to manage facility hygiene programmes.

Despite the advantages associated with inoculation via plugs and air, inoculation via conidial suspensions was still currently regarded as the most suitable inoculation method for inoculating citrus. Improvement for inoculation via air may lead to the method being preferred for *Penicillium* inoculation studies.

Environmental conditions impacted *P. digitatum* and *P. italicum* aggressiveness and symptom expression. *Penicillium digitatum* caused the largest lesions, and *P. expansum* and *P. crustosum* caused no lesions under the cold conditions. Vilanova *et al*. (2012b) reported large lesions caused by *P. expansum* on oranges (‘Valencia’ *ld* = ±45 mm; ‘Navelina’ *ld* = 70-100 mm) after 75 days incubation under cold storage conditions (4°C). This was only achieved when fruit were inoculated with concentrations of 10^6 and 10^7 conidia/ml and not 10^4 and 10^5 conidia/ml. Our results support the finding that low inoculum concentrations were unable to cause lesions under cold storage on citrus (Vilanova *et al*., 2012b). The lower concentrations (6.3 x 10^4 conidia/ml) were however able to cause lesions on apples under cold storage (Louw and Korsten, 2014), demonstrating that *P. expansum* and *P. crustosum* are opportunistic pathogens of citrus.

This study has highlighted the importance of controlling inoculum levels within the fruit chain. Understandably, control practices have been established to control or attempt to control a broad postharvest *Penicillium* pathogen range. However, industry has not seriously considered the former non-host pathogens, *P. crustosum* and *P. expansum*, a concern on citrus. This can be a new emerging field due to the more extensive distribution systems. The lack of attention to prevent or lower the increase of inoculum levels in geneal and also of non-host pathogens specifically may contribute to market-end losses. The handling and storage of different fruit species within the same environment (Vermeulen *et al*., 2006) may thus lead to increased inoculum levels of these pathogens, thus increasing inoculum pressure and contributing to decay (Vilanova *et al*., 2012b). Future studies should investigate the link among market-end losses, the causal agents involved and inoculum levels and sources.

5. CONCLUSION

All tested species (*P. digitatum, P. italicum, P. crustosum* and *P. expansum*) were pathogenic on citrus, although pathogenicity and aggressiveness varied over the citrus range. *Penicillium digitatum* and *P. italicum* were pathogenic over the entire citrus range, exhibiting high levels of aggression. Specific conditions and cultivars were required for *P. crustosum* and *P. expansum* to express decay, high aggression and proper symptom development. The species were regarded as opportunistic pathogens on citrus, dependent on the inoculum levels, inoculation pathway, host susceptibility and environmental conditions (cold chain management). This is the first report demonstrating decay lesions caused by *P. crustosum* on 'Nules Clementine', 'Nova', 'Owari Satsuma' and 'Eureka' seeded, and tissue-response lesions on 'Delta Valencia', 'Cambria Navel' and 'Star Ruby'. It is also the first reported decay lesions caused by *P. expansum* on 'Nules Clementine', 'Owari Satsuma' and 'Eureka' seeded, and tissue-response lesions on 'Midknight Valencia' and 'Delta Valencia'. Additionally, varying aggression of *P. digitatum* and *P. italicum* over a broad citrus host range and further symptom descriptions on citrus-*Penicillium* infections have been reported in this study. Varying aggression over the citrus range displayed *Citrus* spp. and cultivar related *Penicillium* decay concerns in the fruit industry, thus highlighting areas requiring additional attention. Future studies should focus on market-end fruit susceptibility and inoculum loads further down the fruit chain.

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Chapter 5

General Discussion

The genus *Penicillium* includes numerous species contributing to postharvest losses of fresh produce (Pitt and Hocking, 2009). Of these species, *P. expanusm*, *P. crustosum*, *P. solitum*, *P. digitatum* and *P. italicum* are of significant importance when dealing with postharvest losses of pome and citrus fruit (Eckert and Eaks, 1989; Sanderson and Spotts, 1995; Spotts *et al*., 1998; Kim *et al*., 2005; Barkai-Golan, 2001; Nunes *et al*., 2010; Marcet-Houben *et al*., 2012). A few shortcomings regarding the pathogenicity and aggressiveness of these species were identified in this study. The potential of cross-infection taking place between pathogens of pome and citrus fruit were evaluated, since the pathogenic species mentioned have been isolated from both citrus and pome fruit environments.

Penicillium digitatum is strongly associated with citrus, causing a destructive postharvest rot on the fruit (green mould). The citrus pathogen has never before been reported to cause decay on pome fruit, although reports have indicated the pathogenic potential of the species on the fruit (Sanderson and Spotts, 1995; Vilanova *et al*., 2012a). This study was the first to demonstrate *P. digitatum* as a destructive postharvest pathogen of pome fruit (Louw and Korsten, 2014). Decay on some pear cultivars ('Beurre Bosc', 'Beurre Hardy' and 'Sempre Rosemarie') was more aggressive than observed from the dominant postharvest *Penicillium* pathogen of pome fruit (*P. expansum*). *Penicillium digitatum* required specific conditions to cause decay. A connection between the maturity of fruit and the susceptibility of specific cultivars was observed. Although fruit physiology was not tested, older fruit were much more susceptible to *P. digitatum*. The correlation between older pome fruit and *P. expansum* was not as strongly observed as with *P. digitatum*. A recent paper (Vilanova *et al*., 2014) has confirmed results reported in this study. High inoculum levels and over-mature fruit are associated with *P. digitatum* decay of apples ('Golden Smoothee').

Penicillium expansum, *P. crustosum*, *P. solitum* and *P. brevicompatum* pathogenicity were also evaluated on pome fruit. *Penicillium expansum* and *P. crustosum* were the most aggressive on the majority of apple cultivars assessed ('Royal Gala', 'Granny Smith', 'Golden Delicious', 'Topred' and 'Cripps Pink'), but not all pear cultivars (only 'Packham's Triumph' and 'Forelle'). *Penicillium digitatum* was the most aggressive on three pear cultivars tested ('Beurre Bosc', 'Beurre Hardy' and 'Sempre Rosemarie') and caused decay on some apple cultivars ('Granny Smith' and 'Cripps Pink'). *Penicillium solitum* was the least aggressive species over the evaluated cultivar range, unable to cause decay on most apple cultivars (possibly only 'Granny Smith' and 'Cripps Pink'). The pathogenicity of *P.*

brevicompatum was confirmed on pears, but not on apples. *Penicillium brevicompatum* was not included in further studies.

The potential of *P. crustosum* and *P. expansum* to cause decay lesions and tissue-response lesions was demonstrated over a broad *Citrus* spp. and cultivar range. Previous papers have reported *P. crustosum* decay on mandarin (Garcha and Singh, 1976) and 'Valencia' oranges (Arrebolla *et al*., 2010). *Penicillium expansum* can infect lemons, grapefruit and oranges when host defences are compromised by ascorbic, citric and oxalic acids (Macarisin *et al*., 2007), and cause decay of oranges ('Navelina' and 'Valencia') (Vilanova *et al*., 2012b). The report of Garcha and Singh (1976) is old and the research focus of Arrebolla *et al*. (2010) did not elaborate on the citrus-*P. crustosum* interaction. Infections demonstrated by Macarisin *et al*. (2007) did not include all commercial *Citrus* spp. and the occurrence of natural decay was not reported. Vilanova *et al*. (2012b) only reported on *P. expansum* rot of oranges. High inoculum concentrations were required to cause decay. All this highlighted the need for further research on the pathogenicity of *P. crustosum* and *P. expansum* on citrus.

This study was the first to demonstrate *P. crustosum* and *P. expansum* decay on the mandarin cultivars; 'Nules Clementine', 'Nova' and 'Owari Satsuma', and 'Eureka' seeded lemons. Garcha and Singh (1976) did not mention the mandarin cultivar on which they reported *P. crustosum* decay. This is important, since mandarins compose of numerous *Citrus* spp. (according to Tanaka classification (Tanaka, 1969 and 1977)) and hybrids, which may differ in susceptibility. Additional tissue-response lesions reported in this study was observed from *P. crustosum* on 'Delta Valencia', 'Cambria Navel' and 'Star Ruby', and from *P. expansum* on 'Delta Valencia' and 'Midknight Valencia'. Reports on the tissue-response lesions were novel in terms of cultivar level. Arrebolla *et al*. (2010) and Vilanova *et al*. (2012b) did not specifically state the Valencia cultivar, Macarisin *et al*. (2007) did not provide cultivar names and Vilanova *et al*. (2012b) reported decay on a different Navel cultivar. The tissue-response lesions reported in this study was also via direct inoculation with low inoculum concentrations (6.3×10^4 conidia/ml). Host susceptibility, fruit maturity and inoculum load have been connected with *P. crustosum* and *P. expansum* decay of citrus (Vilanova *et al*., 2012b). *Penicillium crustosum* and *P. expansum* decay of citrus links the crop with the potential harmful mycotoxins associated with the species.

Penicillium digitatum and *P. italicum* were reported as the most aggressive *Penicillium* pathogens on all evaluated *Citrus* spp. and cultivars. Mandarin cultivars were observed as the

most susceptible, followed by lemons, oranges and grapefruit. Aggressiveness of *P. digitatum* varied more over mandarin than over sweet orange cultivars. This may be due to the large *Citrus* spp. variance observed within the mandarin group. Unlike *P. digitatum*, *P. italicum* expressed general aggression over the full citrus host range.

This study added symptom descriptions to the newly described host-pathogen combinations. Symptom descriptions of *P. digitatum* on apples and pears were first reported from research in this study (Louw and Korsten, 2014). However, infection reactions have previously been reported by Vilanova *et al*. (2012a) on apples. They recently gave symptom descriptions for *P. digitatum* rot on 'Golden Smoothee' (Vilanova *et al*., 2014). *Penicillium digitatum* typically produced lime green conidia (green mould) from white mycelia on infected and rotten tissue (brown). Sporulation on cultivars varied, thus displaying symptom variance from cultivar to cultivar and from apples to pears. Symptom descriptions of *P. crustosum* and *P. expansum* on citrus are rather un-described or new (Garcha and Singh, 1976; Vilanova *et al*., 2012b). In the case of sporulating lesions, blue conidia (blue mould) were produced on white mycelia.

Penicillium isolates from different environments (citrus- and pear chains) showed no significant difference in terms of lesion sizes on pome ('Beurre Hardy') and citrus ('Eureka' seeded) fruit. The origin of isolates thus had no significant impact on the severity of decay. Introduction of *P. digitatum* conidia from infected citrus into a pome fruit environment and *P. crustosum* and *P. expansum* conidia from infected pome fruit into a citrus environment can contribute to fruit decay. This becomes considerable if fruit are handled or re-packed together, especially near or at the end of the supply chain. Higher losses can result as inoculum levels increase, cross-infections occurs and fruit become more susceptible (riper after transportation) (Janisiewicz *et al*., 2008; Kader, 2011; Vilanova *et al*., 2012a, 2012b, 2014).

Different inoculation methods were evaluated in the study: inoculation via plugs, air and conidial suspensions. Inoculation via plugs was the most effective in terms of yielding the highest incidence, lowest variance, well-definable symptoms and in the case of citrus, prominent *P. crustosum* and *P. expansum* lesions on 'Eureka' seeded lemons. It is although the least probable pathway for natural infections to occur. The unique results produced with the method can be attributed to the direct inoculation of a concentrated inoculum dose in an open wound (Vilanova *et al*., 2012b). Although inoculation via aerial dispersed conidia is the

most likely pathway for natural *Penicillium* infections to occur, the method still require further modifications. The method was experienced as inconvenient, time consuming, sensitive to contamination (after wounding, but prior to inoculation) and biased when using cultured plates as source of inoculum. Improvements proposed were to set up the inoculation chamber inside a biosafety cabinet for fruit inoculations (Lee *et al*., 2009; Reponen *et al*., 1997), use a more robust and easier to sterilise inoculation chamber, and make use of infected fruit as source of inoculum. Fruit as source of inoculum would simulate natural infections and remove biased interactions found with culturing. Despite the advantages associated with inoculation via plugs and air, inoculation via conidial suspensions remained the most suitable for inoculating fruit at the time.

Environmental conditions impacted significantly on *Penicillium* decay. *Penicillium expansum* caused rot of apples ('Golden Delicious') and *P. digitatum* and *P. italicum* caused rot of citrus ('Eureka' seeded) under cold storage conditions. No infections were observed from the remaining *Penicillium* spp. evaluated (*P. crustosum*, *P. solitum* and *P. digitatum* on apples, and *P. expansum* and *P. crustosum* on citrus). This may indicate that the previously thought non-host pathogens of the crops are opportunistic and require specific conditions to cause decay. However, the fruit type and/or cultivars selected to observe condition based disease sensitivity in this study was less susceptible than observed with other cultivars. Further evaluation on more susceptible cultivars may be needed.

This study has demonstrated the pathogenic ability of previously thought incompatible pathogens on non-hosts. The importance of inoculum levels, environmental conditions and host susceptibility (cultivar and maturity) in these interactions has been highlighted (Vilanova *et al*., 2012a, 2012b; Louw and Korsten, 2014; Vilanova *et al*., 2014). This may lead us to theorise that all non-host pathogens causing hypersensitive responses can potentially become pathogenic on non-hosts once conditions are favourable (host susceptibility and inoculum levels are high). The disregard or lack of attention to prevent or lower the increase of inoculum levels of even non-host pathogens in the fruit chain may pose a spoilage risk to industries. Future studies should examine the link between host susceptibility as influenced by maturity and the pathogenic potential of non-host pathogens. Investigating market-end losses, the causal agents involved, and inoculum levels and sources will also provide answers to the industry to prevent decay at the retail-end.

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WEBSITES USED:

http://dx.doi.org

Appendices

APPENDIX A

Table 6.1. Harvest periods of apple cultivars in South Africa

Cultivars	Dec	Jan	Feb	Mar	Apr	May	Jun
Anna	Mid						
Akane		Mid-Late					
Braeburn				Mid/Late			
Canvada	Mid-Late						
Cox's Orange Pippin			Mid				
Cripps' Pink (Pink Lady R)					Mid	Early	
Cripps' Red (Sundowner R)						Early/Mid	
Drakenstein		Mid					
Delkistar			Mid-Late				
Early Red One			Late	Early			
Elegant					Early-Mid/Late		
Empire			Late	Early			
Fiesta			Early-Mid				
Flavorglo			Mid-Late				
Fuji Akifu				Mid	Mid/Late		
Fuzi Irradiated				Mid/Late			
Gala			Mid				
Golden Delicious			Mid	Mid			
Golden Del Emla			Late	Early			
Golden Delicious 1729			Late	Early			
Golden Gift			Early-Mid				
Granny Smith				Mid	Mid		
Jonagold			Mid				
Jonathan			Early-Mid				
Lady Williams							Mid
Oregon Spur			Mid-Late				
Panorama Golden			Early-Mid				
Paula Red		Mid-Late					
Red Chief			Late	Early			
Royal Beaut		Late		Mid			
Royal Gala		Late		Early			
Smoothee			Late	Early			
Starking			Mid	Mid/Late			
Starkrimson				Mid			
Summerred		Mid-Late					
Superchief			Mid-Late				
Sweet Comelly		Mid-Late					
Topred			Late	Early			

FPEF, 2010a; Fruits.co.za., 2011a; SAFE, 2011a

Seasons are dependent on annual climate and region, and may thus vary. Colour of blocks characterise skin colour of fruit.

Table 6.2. Harvest periods of pear cultivars in South Africa

Cultivars	Dec	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug
Buerre Bosc		Late		Mid-Late					
Beurre Hardy		Late	Mid						
Comice			Mid	Early-Mid					
Flamingo		Late	Mid						
Forelle			Early	Late					
Packham's Triumph		Early		Early					
Red D'Anjou		Late	Mid						
Rosemarie		Early	Mid						
Sempre		Early	Mid						
Starkrimson		Early-Late							
Williams Bon Chretien	Mid		Mid						
Abate Fetel			Early-Late						
Conference			Early-Late						
Concorde			Early-Late						
Golden Russet Bosc		Late	Mid						
Doyenne du Comice		Late	Mid						
Red Williams Bon Chretien		Late	Mid						
Bon Rouge			Mid						

FPEF, 2010g; SAFE, 2011g; Dole SA, 2011

Seasons are dependent on annual climate and region, and may thus vary. Colour of blocks characterise skin or rind colour of fruit.

Table 6.3. Harvest periods of nectarine cultivars in South Africa

Cultivars	Oct	Nov	Dec	Jan	Feb
African Glo		Early-Mid			
Alpine	Late		Early/Mid		
Arctic Star			Early-Mid		
August Glo				Mid	Mid
August Red					Mid-Late
Bella Rosa				Mid	
Crimson Blaze		Late	Mid		
Crimson Giant			Early-Mid		
Crimson Glo		Mid-Late			
Donnarine			Early	Early/Mid	
Fantasia				Early-Late	
Fiesta Red	Late		Early		
Flamekist					Mid-Late
Flavorine		Late	Early/Mid		
Flavortop				Early-Mid	
July Red			Late	Mid	
Margaret's Pride			Early	Early	
Mayglo	Mid/Late	Early/Mid			
Olympia		Late	Early/Mid		
Red Jewel			Late	Mid	
Royal Gem	Late	Late			
Royal Glow	Late	Late			
Ruby Sweet			Mid/Late	Early	
SGNE3027 (Diva)		Early-Mid			
Silver Fire		Late	Early		
Sky	Late				
Stark Sunglo				Early/Mid	
Sunglo				Early-Mid	
Sungrand			Late	Mid	
Sunlite		Late	Mid/Late		
Zaigina					Early-Late
Zeeglo			Mid	Mid	

FPEF, 2010e; African Trade Mark, 2010a; Fruits.co.za., 2011d
Seasons are dependent on annual climate and region, and may thus vary. Colour of blocks characterise skin colour of fruit.

Table 6.4. Harvest periods of plum cultivars in South Africa

Cultivars	Nov	Dec	Jan	Feb	Mar	Apr	May
African Rose	Late	Early					
Angeleno				Mid		Early	
Amber Jewel (Teak Gold)			Early-Mid/Late				
Castalina		Late	Mid				
Casselman				Mid			
Flavour Fall						Mid	Early/Mid
Flavour King			Late	Early/Late			
Fortune		Late	Mid-Late				
Gaviota		Late	Late				
Golden Kiss (African Pride)			Mid				
Harry Pickstone			Early-Mid/Late				
Hiroma Red		Mid	Early				
Honey Star		Mid					
Sapphire	Late		Mid				
Sunkiss		Mid	Mid				
Souvenir		Late	Late				
Ruby Red			Early/Mid-Late				
Laetitia			Early/Mid		Early		
Lady Red			Early/Mid-Late				
Lady West			Early-Late				
Laroda			Early-Mid				
Larry Ann				Early-Late			
Pioneer	Mid	Mid					
Purple Majesty (Red)		Mid	Early				
Purple Majesty (Black)		Early-Mid					
Ruby Red		Late	Late				
Santa Rosa		Late	Mid				
Satsuma		Mid	Late				
Songold			Mid		Early		
Southern Belle					Early	Early	
Sunbreeze (Songold)			Mid-Late				
Sundew (African Pride)		Mid	Late				
Sunplum II (Black Diamond)		Late	Mid				
Sun Supreme			Mid				
Simka			Late	Early			

FPEF, 2010h; Dole SA, 2011; Fruits.co.za, 2011e; SAFE, 2011h

Seasons are dependent on annual climate and region, and may thus vary. Colour of blocks characterise skin colour of fruit.

Table 6.5. Harvest and availability periods of grape cultivars in South Africa

Cultivars	Nov	Dec	Jan	Feb	Mar	Apr	May
Prime Seedless	Early		Mid				
Sugra Twelve (Coachella)	Late		Late				
Sugra Thirteen (Midnight Beuty)	Late		Late				
Flame Seedless	Mid		Late				
Sugraone	Late			Mid			
Victoria	Mid			Early/Mid			
Regal Seedless	Late			Early/Mid			
Autumn Royal				Mid/Early	Mid/Early		
Thompson Seedless		Early			Mid		
Sunred Seedless			Mid			Early	
Red Globe		Early				Mid	
Alphonse Lavallee		Late			Mid		
La Rochelle		Late			Late		
Crimson Seedless			Early				Mid
Bonheur			Mid		Late		
Dauphine				Late			Early
Barlinka				Mid			Late
Dan-ben-Hannah	Late			Mid			
Black Gem	Late		Early				
Ralli Seedless	Mid			Mid			
Waltham Cross			Mid		Late		
Majestic			Late	Late			
Muscat Supreme			Mid	Mid			
Ebony Star				Mid	Early		
Sundance			Early	Mid			
Bien Donne			Early/Mid	Mid			
Superior Seedless	Late		Late				

FPEF, 2010b; Fruit.co.za., 2011c; SAFE, 2011c; SATI, 2011; Rekopane-estates, 2011
Seasons are dependent on annual climate and region, and may thus vary. Colour of blocks characterise skin colour of fruit.

Table 6.6. Harvest and availability periods of citrus cultivars in South Africa

Cultivars	Mar	Apr	May	June	July	Aug	Sep	Oct
Oranges								
Autumn Gold					Early	Late		
Cara Cara			Late			Mid/Late		
Delta Seedless				Mid				Early
Lane Late					Early		Mid	Late
Midknight				Late				Early
Navel		Late					Mid	
Navelina				Early	Mid			
Newhall				Early	Mid			
Palmer			Early	Late				
Robyn					Early		Mid	
Tomango				Early-Mid	Mid			
Valencia				Mid			Mid	
Washington			Ealy/Mid			Mid		
Grapefruit								
Marsh		Late				Mid		
Ruby Red (Rosé)		Late				Early		
Star Ruby		Mid					Late	
Lemons								
Eureka	Early/Mid							Early
Mandarins								
Clemengold					Mid	Mid		
Clemenpons		Early-Late						
Clementine			Mid	Early				
Clemlate					Mid	Early		
Fairchild				Late	Mid			
Mandarins			Early				Late	
Marisol		Mid	Mid					
Minneola				Mid		Mid		
Nova		Late			Early/Mid			
Nules		Late			Mid			
Satsuma		Early	Late					

Karsten, 2009; African Trade Market, 2010b; FPEF, 2010c, 2010d, 2010f, 2010i; Dole SA, 2011; Fruit.co.za., 2011b; SAFE, 2011b, 2011d, 2011e, 2011f; Summer citrus, 2011
Seasons are dependent on annual climate and region, and may thus vary. Colour of blocks characterise rind colour of fruit.

APPENDIX B

Research *e*-Xtra*

Pathogenic *Penicillium* spp. on Apple and Pear

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Abstract

Louw, J. P., and Korsten, L. 2014. Pathogenic *Penicillium* spp. on apple and pear. Plant Dis. 98:590-598.

Numerous *Penicillium* spp. have been associated with postharvest fruit spoilage. This study investigates pathogenicity and aggressiveness of selected *Penicillium* spp. previously isolated from South African and European Union fruit export chains. *Penicillium expansum* was the most aggressive and *P. crustosum* the second most aggressive on all apple cultivars ('Royal Gala', 'Granny Smith', 'Golden Delicious', 'Topred', and 'Cripps Pink') and two pear cultivars ('Packham's Triumph' and 'Forelle') tested. *P. digitatum* was the most aggressive on 'Beurre Bosc', 'Beurre Hardy', and 'Sempre' ('Rosemarie') pear cultivars and the third most aggressive on Granny Smith and Cripps Pink apple cultivars. To our knowledge, this is the first report where *P. digitatum* has been described as aggressive on certain pome fruit cultivars. These pear cultivars are also the most commonly associated with decay on the export markets, resulting in considerable end-market losses. *P. brevicompactum* was detected as pathogenic on pear but was not further evaluated in the study. *P. solitum* covered a broader cultivar range, expressed higher disease incidence, and was more aggressive (larger lesions) on pear cultivars than on apple cultivars. This study provides new information on host specificity and the importance of pathogenic *Penicillium* spp. isolated from various environments in the shipping and marketing channels.

The South African pome fruit industry is economically important in terms of global trade. The industry produces over 1.1 million tons of fruit, of which 46.6% are exported as fresh produce, earning close to $431.6 million in net export realization in 2012 (9). Postharvest losses have been reported as high as 50% in developing countries (5,21,29). Significant losses have been attributed to decay caused by *Penicillium* spp. (12,14,16,19,22,32).

A report by Sanderson and Spotts (22) indicates that numerous *Penicillium* spp. naturally inhabit pome fruit environments (packinghouses and storage facilities) and are prominent on fruit bins. Of these species, *Penicillium expansum* Link, *P. crustosum* Thom, and *P. solitum* Westling have been described as the most important on apple (*Malus domestica* L. Borkh.) and pear (*Pyrus communis* L.) in causing decay (14,22,32). Other pathogenic species reported include *Penicillium aurantiogriseum* Dierckx, *P. brevicompactum* Dierckx, *P. commune* Thom, *P. griseofulvum* Dierckx, *P. verrucosum*, Dierckx, and *P. carneum* (Frisvad) Frisvad (2,12,16,18,23). However, these species have less often been associated with decay of pome fruit.

Several other *Penicillium* spp. isolated from pome fruit environments (2,22) are best known as pathogens on other fruit crops. These include *P. digitatum* (Pers.) Sacc. on citrus (8) and *P. glabrum* (Wehmer) Westling on pomegranate (3). Complex fruit trade networks result in a large variety of fruit from different countries being retained together in storage or holding facilities (34). In addition, citrus and pome fruit are sometimes repacked in the same facility to remove decaying fruit. This practice can potentially introduce a wider range of pathogens to the hosts that would not otherwise encounter these pathogens. Recently, Vilanova et al. (35) reported *P. digitatum* as able to infect 'Golden Smoothee' apple but with lesions not developing beyond the initial infection site. Infectivity was described as limited to specific fruit maturity conditions (commercial and over-mature) and inoculum load, resulting in peel infection reactions of up to 6 mm in diameter (including the wound site).

The lack of transparency, more specifically toward the end of the supply chain, makes it difficult to assess the impact of pathogen presence and the inoculum load. Costs associated with market-end losses are often passed back to the farmer without verification of the causal agent or possible point of contamination. End-of-the-season fruit, long-term storage, and extended transit periods can result in physiologically older end-market produce that may be more susceptible toward decay (4,10,15,36). In this context, opportunistic pathogens encountered further down the supply chain may infect fruit previously considered nonhosts for those pathogens.

The aim of this research project was to evaluate representative dominant *Penicillium* spp. isolated from various environments in the citrus and pome fruit supply chains and to determine pathogenicity, aggressiveness, and host specificity of these isolates. This knowledge will provide a better understanding of the causal agents, inoculum buildup, and fruit decay at the market end of the supply chain.

Materials and Methods

Fungal cultures. The *Penicillium* spp. isolates were obtained from the fungal culture collection at the University of Pretoria. Isolates came from pear (2010–11) and citrus (2009–10) export chain studies (*unpublished data*) (Table 1). The criterion for initial selection of species from the culture collection for the present study was based on the identified species being well-known pathogens or the species representing dominance in the supply chain environment. Cultures of the isolates for experimental trials were prepared by single-spore isolation. Cultures were grown for up to 3

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APPENDIX C

Plant Disease

plant disease

Pathogenicity and host specificity of *Penicillium* spp. on citrus

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Pathogenicity and host specificity of *Penicillium* spp. on citrus

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Citrus fruit are exposed to numerous postharvest pathogens throughout the fresh produce supply chain. Well known postharvest citrus fruit pathogens are *P. digitatum* and *P. italicum*. Lesser known pathogens include: *P. crustosum* and *P. expansum*. This study examined pathogenicity and aggressiveness of *Penicillium* spp. present in fresh fruit supply chains on various citrus species and cultivars. The impact of different inoculation methods and storage conditions on decay were also assessed. The most aggressive species were *P. digitatum* and *P. italicum* on all citrus cultivars, but aggressiveness varied significantly over citrus species and cultivars. Decay and tissue response lesions caused by *P. crustosum* were observed on 'Nules Clementine', 'Nova', 'Owari Satsuma', 'Delta Valencia', 'Cambria Navel', 'Eureka' seeded and 'Star Ruby' for the first time. Likewise, *P. expansum* decay and tissue response lesions were noted on 'Nules Clementine', 'Owari Satsuma', 'Delta Valencia', 'Midknight Valencia' and 'Eureka' seeded for the first time. Tissue response lesions affect fruit quality and some sporulated in the wounds, resulting in completion of their life cycle. New citrus-*Penicillium* interactions were observed and the impotence of monitoring inoculum loads of pathogens and non-host pathogens were highlighted.

Additional keywords: Green mold, blue mold, virulence, disease intensity, PCR-RFLP.