Effect of ripeness on host-pathogen interactions of *Penicillium* spp. on stone fruits

by

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DECLARATION

I, Johannes Petrus Louw, declare that the thesis, which I hereby submit for the degree Doctor of Philosophy in 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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ABSTRACT

Stone fruits are susceptible to numerous postharvest pathogens. *Penicillium expansum* is a well-known concern to industry. Little information is available on other *Penicillium* spp. that cause decay of stone fruits. Pathogenicity of *P. digitatum* was recently described on pome fruits. Decay was associated with older fruit and specific cultivars. Preliminary trials revealed similarities on plum and nectarine. This led us to question the current understanding of pathogenicity and host specificity. Very little is known of the host-pathogen interactions of *Penicillium* spp. on stone fruits and less with regards to host ripeness. This study aimed to determine the pathogenicity of different *Penicillium* spp. on various nectarine and plum cultivars and further investigate the host-pathogen interactions of the most aggressive species on one plum and nectarine cultivar as model system. Different isolates, host ripeness levels, inoculum loads and storage conditions were considered. pH modulation and gene expression profiles of these *Penicillium* spp. were investigated. *Penicillium digitatum* was identified the most aggressive in terms of lesion diameter, followed by *P. expansum*, *P. crustosum* and *P. solitum*. Scanning electron microscopy provided additional information on different life stages of *P. digitatum*, *P. expansum* and *P. crustosum* on alternative hosts (nectarine, pear and lemon). Ripeness significantly increased lesion diameter and disease incidence of *P. digitatum* but not *P. expansum*. Irrespective of ripeness level, lesions caused by *P. expansum* were similar in size and disease incidence 100%. *Penicillium digitatum* was more sensitive to cold storage and greatly affected by inoculum load. Ripeness meaningfully affected pH modulation. Colonised tissue was acidic. A mechanism leading to alkalinisation of uncolonised tissue was discussed. This was specifically observed on plum. Higher pH at and

beyond lesion borders potentially facilitate invasion, maceration and colonisation (nutrient uptake and growth) by/during acidification. The pH of colonised tissue was similar to the initial pH of the host (prior to infection). Localised alkalinisation can be accomplished by ammonium/ammonia accumulation but areas further away from lesions can be due to host ripening directly (elicited) or indirectly (ethylene stress) caused by pathogen attack. Ripeness had a large effect on the gene expression (especially *PG*) of the *Penicillium* spp. on nectarine. *Penicillium digitatum* downregulated *ACCD* (only) whereas *P. expansum* upregulated *pacC* and *creA* on plum. With nectarine, *P. digitatum* upregulated *PG* and *creA* while *P. expansum* downregulated *PG* and upregulated *pacC*. Fresh plum (1d postharvest) was already a more suitable host based on lesion diameter, disease incidence and the nonsignificant increase in *PG* expression. Unlike what is known on citrus, *P. digitatum* showed an opportunistic lifestyle on the stone fruits depending on host and environmental conditions. Rapid decay caused by *P. digitatum* highlighted it as a concern to industry. Further research is needed to identify the trigger/s leading to the significant shift in lesion size and disease incidence of *P. digitatum* on riper fruit. Host resistance should be considered. More research is needed to investigate the dual mechanism of pH modulation and truly link *P. digitatum* with stone fruit losses in industry.

Chapter 1

General introduction

The major stone fruit crops originated from Asia and Europe. They are of nutritional and economic importance and favoured in numerous countries (Potter, 2013). Global plum (*Prunus* L. spp.), nectarine (*Prunus persica* (L.) Batsch var. *nucipersica* (Suckow) C. Schneider) and peach (*P. persica* (L.) Batsch var. *persica*) production volumes exceeded 37.06 million metric tons in 2016. Of this, over 2.91 million metric tons were exported (HORTGRO, 2017). These climacteric fruits are soft-fleshed, highly perishable and wound easily, thus characterising them with a limited market life and sensitive to several deterioration problems (Crisosto and Mitchell, 2011; Kader, 2011).

Numerous postharvest pathogens affect stone fruits and compromise the quality and safety of the produce. *Penicillium* Link includes some of the most important species in terms of losses and consumer safety (mycotoxins). *Penicillium expansum* Link is the most common species associated with losses and toxin production (i.e. patulin) (Ceponis *et al*., 1987; Pitt and Hocking, 2009; Snowdon, 2010; Wells *et al*., 1994). It has been reported to cause prune (*P. domestica* L.) losses of >50% if fruit is wounded (Ceponis and Friedman, 1957; Wells *et al*., 1994). Reports on other *Penicillium* spp. are few. Pathogenicity reports include *P. crustosum* Thom on peach (Restuccia *et al*., 2006), *P. chrysogenum* Thom on black plum (*Vitex doniana* Nielson) (Eseigbe and Bankole, 1996) and *P. digitatum* (Pers.) Sacc. on nectarine (Navarro *et al.*, 2011).

Peach and nectarine are physiologically and genetically very similar (Blake, 1932). Decay caused by *P. crustosum* can thus be expected on both fruit types. Background on decay and symptom development was unfortunately not reported. Restuccia *et al*. (2006) only provided disease incidence data (100% after 15d incubation). Lesions caused by *P. chrysogenum* on black plum (non-commercial fruit) were small (≥8mm after 8d incubation at 28±2ºC). Background on symptoms was again not reported (Eseigbe and Bankole, 1996). *Penicillium digitatum* was first isolated from commercial nectarine and plum in 1996 (Parlier, California, USA). It is unclear if isolates came from orchards or symptomatic fruit. No pathogenicity trials were conducted as this was not the study focus of Ma *et al*. (2003). It was Navarro *et al.* (2011) who first reported lesions caused by *P. digitatum* on nectarine (±1300mm^3 and ±1500mm^3 after 6d incubation at 25°C). Further symptom descriptions were not reported. Louw and Korsten (2014) recently identified *P. digitatum* pathogenic and highly aggressive on certain pome fruit cultivars.

The recent discoveries in new host-pathogen interactions prompted further investigation (Louw and Korsten, 2014; 2015; Vilanova *et al*., 2014). The fresh produce chain is an environment where the disease triangle is heavily influenced by man. Here, different fruit types from different countries can be handled and stored in the same facility (i.e. repack facility). Potential hosts on which pathogens will be able to survive, grow and reproduce will be available year round in the same environment (influence inoculum pressure). The genetic richness of any particular pathogen will be higher due to global trade. Hosts, especially climacteric fruits, will be physiologically older or riper (long distribution chains) and thus more susceptible (Louw and Korsten, 2014; 2015; Prusky *et al*., 2016; Vilanova *et al*., 2014). These and other factors build up to create a scenario where opportunistic pathogens can infect and colonise new hosts.

Penicillium digitatum was previously described as a pathogen distinctly associated with *Citrus* L. spp. (Pitt and Hocking, 2009). It is the most important postharvest pathogen of citrus fruits, able to cause rapid decay and greatly contribute to industry losses (Eckert and Eaks, 1989; Louw and Korsten, 2015; Marcet-Houben *et al*., 2012). It was only recently that *P. digitatum* was described highly aggressive (more than *P. expansum*) on certain apple, pear (Louw and Korsten, 2014), nectarine and plum (Louw and Korsten, 2016) cultivars. Whether it has always been a pathogen (undiscovered) or recently became one requires to be answered. *Penicillium digitatum* has been confirmed present in the environment of all the above-mentioned fruit types (Ma *et al*., 2003; Scholtz and Korsten, 2016) but no reports have linked it to stone or pome fruit losses. Decay can be associated with older or riper fruit (Louw and Korsten, 2014; Vilanova *et al*., 2014).

Fruit ripening results in the accumulation of sugar, increase in pH and decrease in antifungal compounds. In return, this affects host susceptibility and fungal pathogenicity (Prusky, 1996; Prusky *et al*., 2016). Many pathogens modulate the pH of hosts to regulate pathogenicity and virulence factors during infection and colonisation (Prusky *et al*., 2016). *Penicillium digitatum* and *P. expansum* acidify host tissue via the release of organic acids and/or uptake of ammonium/ammonia. Production and secretion of secondary metabolites (i.e. mycotoxins and pectolytic enzymes) will be upregulated and nutrients will be absorbed (Prusky *et al*., 2004; Sánchez-Torres and González-Candelas, 2003; Yao *et al*., 1996; Zhang *et al*., 2013). The pH, and carbon and nitrogen sources play an important role in the infection and colonisation process of *Penicillium* spp. (Prusky *et al*., 2016). An association between *P.*

digitatum and *P. expansum* colonisation and ethylene production was also made (Barad *et al.*, 2016; Chalutz and Lieberman, 1977; Jia *et al.*, 1999).

Many studies focus on the host-pathogen interaction of *P. digitatum* on citrus and *P. expansum* on apple (Barad *et al.*, 2016; López-Pérez *et al.*, 2015) but few have looked at *Penicillium* interactions on stone fruits. Since *P. digitatum* was recently confirmed pathogenic and highly aggressive on stone fruits (Chapter 3: Louw and Korsten, 2016), findings will be novel. This project was set forth to firstly determine the pathogenicity and aggressiveness of *Penicillium* spp. on nectarine and plum cultivars. Different isolates (pome and citrus supply chain environments), inoculum loads, host ripeness levels and storage conditions (ambient vs cold) will be assessed to better understand existing and new *Penicillium* disease interactions. Scanning electron microscopy will be used to examine and compare different disease stages of *Penicillium* spp. on known and new hosts. Lastly, the effect of plum and nectarine ripeness on the infection and colonisation of the most aggressive species (*P. digitatum* and *P. expansum*) will be determined at a physical (pH and firmness) and molecular (gene expression) level (Chapter 4 and 5).

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Chapter 2

Literature review: The concept and specificity of pathogenicity

ABSTRACT

The definition of pathogenicity has been challenged given new evidence regarding host specificity, host-pathogen interactions and next-generation technologies. Pathogenicity is applied across a myriad of disciplines with discrepancies and often incorrect interpretation and application. Several variations to the definition exist. This is largely due to a conceptual misunderstanding of the term since the interaction of the many factors involved in pathogenicity are complex and difficult to fully describe in a single definition. In this review, the way pathogenicity is and should be interpreted and applied within pathology was examined. The concept of pathogenicity has been and is largely still used with a pathogen centred view, even though the importance of a host-pathogen complex was described more than a century ago. The interaction of the host-pathogen-environment was later introduced to describe disease development (inadvertently the disease triangle). Time and the biotic environment also form part of this complex. For this review of pathogenicity, there was a need to revisit the definition and concept of pathogen and disease (disease pyramid). In 1929, Whetzel defined pathogenicity as "the ability of an organism (pathogen) to produce disease". Here, discrepancies exist at "ability", "organism", "produce" and "disease". Specificity of pathogenicity can be improved by using "infectious agent" to include biotic and abiotic agents with the ability to infect. A more general interpretation can be provided by using "cause" to include all disease interactions. Using "capacity" should be avoided since it is a quantitative term. Emphasis should be placed on the conceptual understanding that disease is a complex of which the pathogen forms part. The pathogen is vital for an infectious disease to occur although it can be affected by many other factors. A more specific and descriptive definition for pathogen and pathogenicity is proposed: pathogen - disease-causing infectious agent; pathogenicity - the capability of an infectious agent to solely or mutually cause disease.

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1. INTRODUCTION

The definition and interpretation of pathogenicity have been used inconsistently over time and across disciplines, frequently confused with closely associated terms such as virulence and aggressiveness. This resulted in arguments and extensive discussions within and between disciplines in which the terms are applied (Andrivon, 1993; Bos and Parlevliet, 1995; Casadevall and Pirofski, 1999; Shaner *et al*., 1992; Thomas and Elkinton, 2004; Watson and Brandly, 1949). An early application of pathogenicity in human and animal sciences was that of Young (1903); "the action of pathogenic bacteria in producing disease in man and other animals". In plant pathology, Whetzel (1929) defined pathogenicity as "the ability of an organism to produce disease". Shapiro-Ilan *et al*. (2005) preferred the definition of pathogenicity by Steinhaus and Martignoni (1970): "the quality or state of being pathogenic or the potential ability to produce disease." Fundamentally the definitions given by Whetzel (1929), and Steinhaus and Martignoni (1970) are still accepted today. However, pathogenicity still lacks a generic definition across disciplines and too many incorrect or incomplete interpretations skewed the principle concept. This can be rectified, at least to an extent, by combining the knowledge and experiences from the different disciplines in a way to benefit pathogenicity and pathology as a whole.

Casadevall and Pirofski (1999; 2000; 2001; 2007) expressed their concern with the pathogen centred view of pathogenicity and virulence. They noted that the current lexicon for microbial pathogenesis provides a definition intended to describe a pathogen based on causing disease or not. Pathogenicity is a complex phenomenon but the outcome of the host-microbe interaction is not incorporated in the definition. This must be understood to ensure the correct interpretation and application of the term. Duggar (1909) and Smith (1913) were among the first to note the role of the host in pathogenic interactions. Zinsser (1914) grouped microorganisms into three classes; pure saprophyte, pure parasite and half parasite. The later was specifically defined as a parasite with a low invasive power and infection only taking place depending on certain circumstances. This was indicative that infection of a host may be dependent on factors found outside the parasite. The importance of the environment in disease development started to become apparent in the mid-1930s (Stevens, 1960). Henle-Koch postulates (better known as Koch's postulates) were developed in 1890 and are still used today. It was one of the first assessments to link an infectious agent with a specific disease (Evans, 1976). Unfortunately, the postulates were pathogen centred and difficult to apply to nonculturable organisms or organism that caused disease only in some individuals,

hosts or circumstances (Isenberg, 1988). The pathogen centred view of pathogenicity and virulence was generally abandoned by the mid-20th century and later reviewed as an "expression or interaction of the host-parasite interaction" (Watson and Brandly, 1949).

Molecular Koch's Postulates were conceptualised to link bacterial pathogenesis with responsible disease-causing genes (Falkow, 1988). Unfortunately, the application of this expansion of the postulates largely remained pathogen centred (Gyles and Prescott, 2008). Another perspective included host factors by suggesting that an organism requires the ability to overcome the host defences to facilitate pathogenesis (Brubaker, 1985; Deitsch *et al.*, 1997). Host-pathogen interactions and the outcome of disease are dependent on the genetic makeup of each partaker however it is not completely sufficient to describe why and how disease develops in some cases. The presence of a pathogenic gene means very little if it is not expressed. For instance, what about quiescent pathogens, commensals or unknown pathobionts? Gene expression is also influenced by other pathogens, the host and environmental (biotic and abiotic) factors. Furthermore, all the genes involved in pathogenesis is not known, neither to what extent they function independently or synergistically. This review aims to revise how pathogenicity is understood and defined by revisiting and extrapolating the core principles applied in the definition. The focus was not to introduce a new definition. It was directed at expanding the interpretation of pathogenicity and encouraging the use of a more generic definition to improve accuracy and precision when applying the term.

2. IS THE DEFINITION OF PATHOGENICITY TOO BROAD?

Pathogenicity is defined and used by most researchers to include any organism able of causing disease. This broad definition would include an organism able to cause a disease to another organism that it is not necessarily associated with. Should the term be expounded to include any organism able of causing disease or limited to only organisms that interact and associate with a host for self-benefit (i.e. completion of their life cycle)? Hunt (1994) mentioned the need to consider toxin producing saprophytes capable of causing disease as pathogens. In the field of medical sciences, several authors highlighted the importance of commensal bacteria becoming pathogenic in immune impaired hosts (Brown *et al.*, 2012; Pallen and Wren, 2007; Pirofski and Casadevall, 2012). Some recently termed a symbiont (commensal or mutualist) with the potential of causing dysregulated inflammation resulting in disease under specific conditions a pathobiont. It was applied to the gut (Mazmanian *et*

al., 2008; Round and Mazmanian, 2009) and skin microbiota (Chen *et al*., 2018). Pathobiont seems restricted to inflammatory responses. If not, it is unclear if it will be applied in other disciplines as terms with similar meaning far outdates it.

In plant pathology, McNew (1960) mentioned that causal agents can range from facultative saprophytes where parasitism is incidental to facultative or obligate parasites with commensal tendencies. To add to the complexity, some diseases can be perceived as mutualistic or pathogenic depending on environmental conditions. For example, nodule-forming bacteria (*Rhizobium leguminosarum*) causing galls in legumes divert and live of host nutrients. In return, the bacteria provide nitrogen to the host via nitrogen fixation. This interaction can be regarded as mutualistic or commensal in a nitrogen deprived environment but pathogenic in a nitrogen-rich environment (McNew, 1960). Surely commensal or mutualistic symbionts that might have pathogenic abilities cannot be disregarded from the concept of pathogenicity since these organisms are directly associated with their host and receive mutual benefit from the interaction. Whether to include saprophytes might require some clarification.

Integrated lifestyles can include saprophytic stages, but the organisms are not limited by these (i.e. necrotrophs, commensals). Pure saprophytes, on the other hand, do not possess the ability to infect and the harm they might cause will be defensive (i.e. predators) or competitive (i.e. environmental benefits) in nature. Such interactions where there is a lack of interest (no benefit for either party or contact being preferably avoidable) and an absence of a host-pathogen interaction should not be considered in the definition of pathogenicity.

Duggar (1909) realised that many fungal diseases of plants are conditioned or directly affected by climatological factors. He mentioned that these factors can affect the host and parasite independently or influence the interrelations between them. McNew (1960) drew the first disease triangle, linking the pathogen, host and environment as factors for disease occurrence. In the context of plants, disease is the interaction between host-plant-metabolism, parasite or other symbiont physiology and the environment (McNew, 1960). Stevens (1960) placed emphasis on a host-pathogen-environment complex. Van der Plank's definition of aggressiveness [quantity of disease (Van der Plank, 1963; 1968)] depends primarily on the pathogen, but also the host and environment. Andrivon (1993) emphasised the need for a clear understanding of infectious disease (interaction between two organisms) for accurate definitions of pathogenicity-related terms such as virulence, avirulence,

aggressive and parasitic fitness. He defined infectious disease as a harmful alteration of a normal physiological state of a host due to it being challenged by a pathogen. Numerous scientists thereafter emphasised the importance of host-pathogen interactions (some including the environment) in pathogenicity and pathogenicity-related terms; i.e. Casadevall and Pirofski (1999; 2000; 2001), Thomas and Elkinton (2004) and Siegel (2012) to mention a few.

Will an organism interact with another without benefiting from it? Unintended exposure could coincidentally harm all parties. In host-pathogen interactions, the infectious organism or agent aims to benefit from the interaction. Commensals turning pathogenic are classic examples of this (Brown *et al.*, 2012; Pallen and Wren, 2007; Pirofski and Casadevall, 2012). Pathogenic organisms are usually parasitic but can be commensal; both lifestyles being beneficial for the pathogen. Can one, therefore, exclude an organism or agent based on a beneficial assumption? Do mutually disadvantageous host-pathogen interactions exist? For example, an organism that infects and causes disease of a host but dying in the process without benefiting (i.e. reproducing). Such interactions would not be continues, not as an infection and neither for the continuation and survival of the individual or its species. However, changing environments or circumstances might force such encounters. Many define disease with the need of continues irritation for it to result (Appendix A: Table 2.1). Thus, the dominating concept is that pathogenicity only includes infectious organisms or agents that would benefit from the interaction. Although this is pointed out in definitions of disease (term used in pathogenicity), it is not otherwise stated in definitions of pathogenicity (Appendix A: Table 2.1). Leaving pathogenicity open to include mutually disadvantageous host-pathogen interactions might not be problematic, but it is profoundly clear; an interaction is necessary and the role of the host in the life cycle of the pathogen has to be understood. Organisms fight for their own survival and will exploit or build relationships to ensure the continuation of their own species. If they themselves cannot continue, they will pay the ultimate price so that the next generation can.

3. WHAT IS A DISEASE?

It is difficult to specifically define disease (Godlee, 2011; Scully, 2004; Sharma, 2004). It is often confused with condition (symptom), injury or pathogen (cause) (Sharma, 2004). To be noted from the various definitions of disease (Appendix A: Table 2.1); it is a malfunctioning of host cells or tissue (abnormality) as a result of continuous irritation, is not

direct physical injury, express symptoms and can be caused by the environment, infectious agents, inherent defects (genetic) or a combination of these. Complexity is added as the lines between pathology, disability, ageing and psychiatric conditions are vague (Scully, 2004).

Disease and nutrition are synergetic. Undernutrition, stunting and the mere failure to achieve one's full genetic potential can lead to, or be as a result of, disease. Nutrition impacts on the health of the host thus affecting susceptibility. Nutrient uptake is influenced by many environmental factors whether physical, biological or social (Perkins *et al.*, 2016). With beings of advanced intelligence and conscience (i.e. humans), the state of healthy or diseased can even be influenced by one's psyche.

Disease can be caused by abiotic and/or biotic factors (Sharma, 2004). Basically, only three factors are required for a disease to occur; organism, environment and time. This, of course, would exclude all infectious diseases, but disease none the less. Environment (space) and time are the only abiotic factors and can never be removed from a scenario. Physically, the host, pathogen and other biotics can be removed from an environment, but never time and space. These latter two factors can only change, they cannot be avoided. They should form the basis when starting to consider disease occurrence and development. The host forms the response organism. When considering infectious diseases, the pathogen becomes the causal agent and other biotics influential agent/s. Surely all five factors (two abiotic and three biotic) are needed to consider all diseases (Sharma, 2004) and the measure by which they interact change the disease effect (Casadevall *et al.*, 2011).

To draw an illustration as representative for all diseases, one must consider all five factors of disease. In plant pathology, McNew (1960) conceptualised the first disease triangle using six factors; seasonal development and inherent susceptibility of the host, prevalence and virulence of the pathogen, duration of the infection period and severity of environmental factors. The extent of disease was represented by the height of the peak of the triangle. Today the disease triangle is depicted in a much simpler form (Fig. 2.1A). All three factors are connected; each serving as a qualitative requirement for triangle formation (disease occurrence) and the surface area quantitatively representing the amount of disease. McNew already described time in the disease triangle but Stevens (1960) explicitly mentioned it as the fourth dimension required for plant disease to occur and develop. Zadoks (1972) and Van der Plank (1975) further enforced its importance. With time (i.e. season, age, life cycles, vector presence, incubation period) added, the disease pyramid was formed. Later the role of

humans (i.e. cultural practices, disease control, environmental impact) as a factor was considered and sometimes used instead of time (Francl, 2007; Sharma, 2004). The disease pyramid, including all five factors (Fig. 2.1C), has the disease triangle as foundation, time as a perpendicular line arising from the centre and humans as the peak. Here, the amount of disease is represented by volume. As the peak, the human factor will be connected with all of the other factors (Sharma, 2004). Disease will thus only occur in a susceptible host and under favourable environmental conditions, depending on human influence and time limitations.

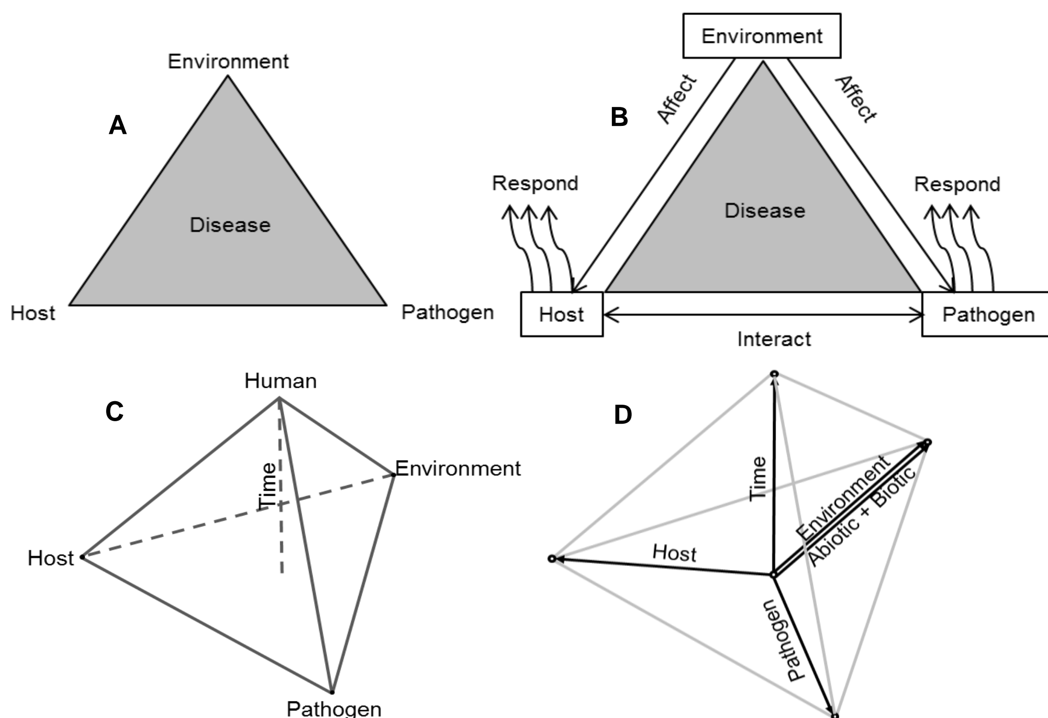

Fig. 2.1 Disease triangle (**A, B**) and disease pyramid (**C**, **D**).

The concept behind the disease triangle and pyramid is commonly understood in plant pathology (Francl, 2007; Scholthof, 2007). With this said, many plant pathologists do not include the human factor in the disease pyramid. They argue that domesticated plants and many crops already have their identity intimately interwoven with humans (husbandry). The human factor is thus already indirectly represented in the pyramid and arguably forms part of the pathosystem environment. Other biotics such as animals and vectors are not included in the pyramid since they are only essential for some diseases to occur. This is especially the case where vectors are used by the pathogen for transmission and multiplication. In such cases, it can be helpful to include the vector on the side between the host and pathogen vertices (Francl, 2007).

A comprehensive picture of the disease pyramid that stretches across all related disciplines and includes all diseases is needed. Not only humans, other animals and vectors, but all other disease affecting biotics must be included. It is hard to find an organism not affected by another (McNew, 1960). Some diseases depend on biological synergism (Watson and Brandly, 1949). The current disease pyramid with all five factors is somewhat confusing. It is unclear how the factors qualitatively form the pyramid as the removal of human or time will still allow the formation of the pyramid (disease occurrence) and yield volume (amount of disease)? Human and time are thus interchangeable? Or based on the understanding that time cannot be removed but human can, makes human a non-essential factor for disease occurrence (which makes sense)? If human is the peak on top of time, an odd concept to begin with, how does it quantitatively contribute to volume? From the diagram, how are all of the factors connected with one another? Based on this and the consideration that humans are part of the pathosystem environment (Francl, 2007); a slight alteration to the disease pyramid is proposed (Fig. 2.1D). Humans are included with all other biotics, one of the interwoven factors of the environment. Environment is logically split into abiotic and biotic. The abiotic environment, similar to time, cannot be removed but only change. The biotic environment can include organisms that positively (i.e. vector, disease complex, secondary infection, immune suppresser) or negatively (i.e. health or growth promoter of the host, antagonist or competitor of the pathogen) affect disease occurrence and severity. In addition, in this pyramid, all factors are connected at the centre and move outward to increase volume.

The way the environment exercises force on the host is vastly different from that of the pathogen (Fig. 2.1B). How do the five factors of the disease pyramid interact or affect each other and what is the intrinsic aim of each towards disease development or advancement? This will give guidance in understanding disease and identifying the cause of disease. The abiotic factors (environment and time) are without aim (neutral), thus they negatively or positively affect disease without intention. Neither of them can be removed, only change. In many cases the one/s affected (host or pathogen) cannot change the abiotic factors itself, they can only alter their own state or the immediate environment to counter the abiotic effects (escape or adapt). The environment is the most powerful controlling factor in pathogenic diseases (Stevens, 1960). The biotic environment is usually aimed at self-benefit, whether the biotics contribute or counter disease depends on how they will be affected by it. In some cases, the biotic (i.e. vector) can be used by the pathogen without it benefitting from the interaction (commensal). In competition, some biotics can defend the host without intention

by purely competing with the pathogen for nutrients and space in or around the host. Point being, biotics can intentionally or unintentionally affect disease in a positive or negative way. The host will always aim to protect itself, trying to overcome or prevent disease. The pathogen will always aim to benefit from the interaction, thus directly orientated to cause and to advance disease.

4. WHAT IS A PATHOGEN?

The use of the term pathogen was introduced late in the 1880s, meaning a microbe that can cause disease (Casadevall and Pirofski, 2014). Most authors/researchers restrict the use of "pathogen" to only living causes of disease, some including viruses under living organisms, others not (Cowling and Horsfall, 1979), and some include abiotic factors as well (Bateman, 1978; Cowling and Horsfall, 1979; Dunster and Dunster, 1996). According to Cowling and Horsfall (1979), abiotic pathogens would include pollutants, nutrient deficiencies and imbalances, and water and temperature extremities. Additionally, they included insects and parasitic higher plants as biotic pathogens. It is hard to agree with some of these propositions set forth, especially with the broad view of abiotic factors called pathogens. The fact that something can cause disease does not imply that it must be called a pathogen. Also, regarding such abiotic factors as pathogens would place confusion on the disease triangle or pyramid as some environmental factors can then also be pathogen factors. Pathogens may be biotic or abiotic (Bateman, 1978). This statement is not totally untrue. Whether Bateman (1978), Arneson (unknown date) and Dunster and Dunster (1996) included biotic or abiotic into the definition of pathogen to accommodate agents such as viruses or all abiotic agents in the term is not totally clear. One thing is certain, nonliving infectious agents such as viruses and viroids, generally and obviously regarded as pathogens, must be included as pathogens and be considered in the definition of pathogenicity. Some definitions fail to include or correctly include these agents in the definition of a pathogen (Appendix A: Table 2.1). There is a need to reconsider the use of terms such as "any agent" and "organism" in the use of pathogen since it will impact on the interpretation of the definition of pathogenicity. Based on current understandings (i.e. need for infection, need for host-pathogen interaction, commensals turning pathogenic) and the dominating concept that pathogens are primarily living entities, but viruses and viroids need to be included, the use of "infectious agents" instead of "any agent", "agent", "an entity", "microorganism" or "organism" is promoted. "Infectious agent" would include disease-causing groups of microbes (fungi, bacteria and viruses) and parasites [helminths (worms) and protozoa] (Murphy and Weaver, 2017), but

need not be limited to these. “Agent” is very general term and can be used to express an idea of any context where a specific term is not required (Merriam-Webster Inc., 1984). Parasitic higher plants, oomycetes, mollicutes and viroids are all groups that meet the requirements of “infectious agent”. This insertion will also exclude all biotics unable to infect and other abiotic agents (environmental) from the definition of pathogen. From literature, the dictionary definition of Merriam-Webster (2015) comes the closest in meeting the requirements and Schmidt-Posthaus and Wahli (2015) already applied “infectious agent” to pathogenicity (Appendix A: Table 2.1).

5. THE CONSTRUCT OF PATHOGENICITY

Whetzel’s proposed definition of pathogenicity from 1929 is simple, applicable and valued across disciplines (Andrivon, 1995; Hunt, 1994). Numerous variants were introduced but their adoption in literature has been sporadic (Appendix A: Table 2.1). During the review of definitions, it remains rudimental to preserve simplicity while increasing precision. The definition originally proposed by Whetzel (1929) will be used as reference to assess the construct of pathogenicity. It provides a generic basis for adoption in all disciplines.

“The ability of an organism (pathogen) to produce disease”

Based on this and considering the other definitions of pathogenicity (Appendix A: Table 2.1), discrepancies were detected at “ability”, “organism”, “produce” and “disease”.

5.1 ABILITY VS CAPABILITY VS CAPACITY

“Ability”, “capability” and “capacity” are terms with subtle differences and frequently used interchangeably resulting in confusion (Bernstein, 1998; Merriam-Webster Inc., 1984). We can observe this in our comparative analysis of pathogenicity (Appendix A: Table 2.1). The confusion is emphasised by the inability of some dictionaries to properly distinguish the definitions (Appendix B: Table 2.2). First off, “ability” and “capability” are considered qualitative terms whereas “capacity” is traditionally used quantitatively (Merriam-Webster Inc., 1984). “Ability” is traditionally used in reference to a person (animated) while “capacity” and to a lesser extent “capability” is used in general for animate and inanimate objects (Garner, 2009; Merriam-Webster Inc., 1984). Additionally, “ability” and “capability” are understood as innate and acquired characteristics where “capacity” is only innate (Bernstein, 1998; Merriam-Webster Inc., 1984). Although pathogenicity is understood as innate and used in reference to animate entities, it isn’t enough to limit the use of “ability”,

"capability" or "capacity" in its definition. The fact that pathogenicity is largely accepted as a qualitative term (Shapiro-Ilan *et al*., 2005; Thomas and Elkinton, 2004), does restrict the use of capacity.

"Ability" and "capacity" are very closely considered. "Capability" is used in reference to possessing qualities or qualifications needed for a work or achievement of a given end (Merriam-Webster Inc., 1984). Capacity thus denotes development whereas ability generally denotes actual power. The fact that an entity possesses the needed qualities or qualifications (are able in it), suggest that the entity can complete the required work or achievement. This also makes capacity the preferred choice for something that requires a large degree of precision (AMA Style Insider, 2005; Garner, 2009; Merriam-Webster Inc., 1984). For instance, you are able to write but are you capable of writing a thesis? Pathogen and pathogenicity are terms that require precision thus making "capability" the preferred choice in their definitions. However, the science community more frequently makes use of "ability" instead of "capability" (Fig. 2.2). Based on this and the similarity between them indicate that although "capability" is preferred, "ability" cannot be disregard from the definitions.

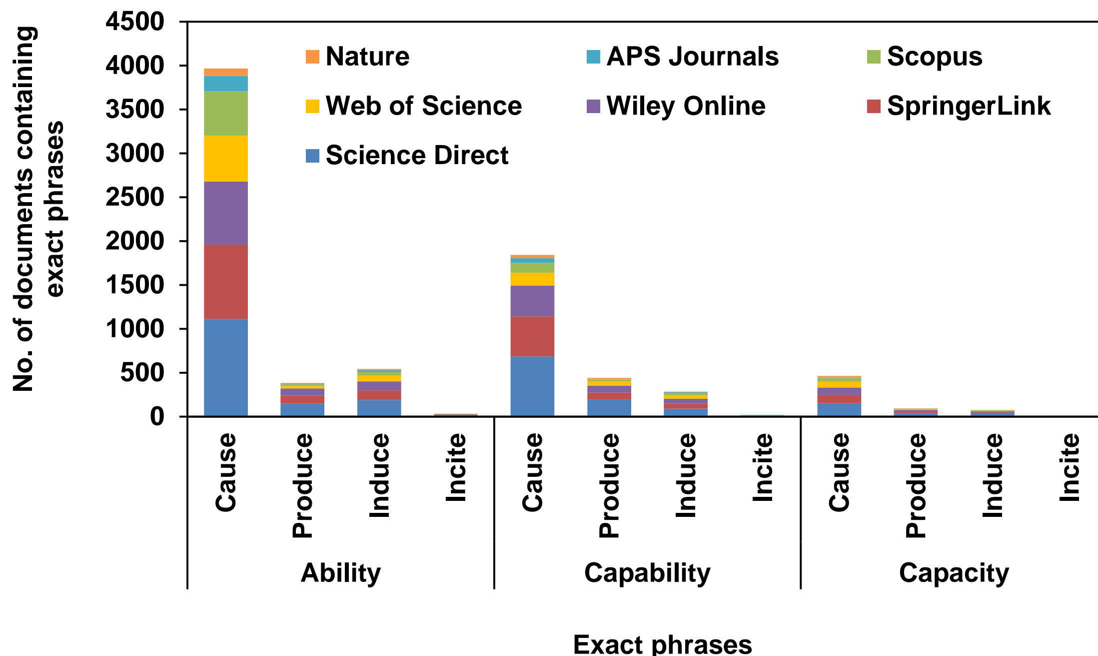

Fig. 2.2 Search results of exact phrases for pathogenicity from scientific databases. [1]Exact phrases: "ability/capability/capacity to cause/produce/induce/incite disease" or "able/capable to cause/produce/induce/incite disease" or "able/capable/capacity of causing/producing/ inducing/inciting disease". [2]NCBI (i.e. PubMed) databases were not included; unable to perform adjacent searching.

5.2 ORGANISM VS MICROORGANISM VS PARASITE VS PATHOGEN VS INFECTIOUS AGENT

Numerous studies use the term "organism" when defining pathogenicity, however, some authors refer to "microorganism", "parasite", "pathogen" and more recently "infectious agent" (Appendix A: Table 2.1). Although "organism" is non-restrictive and excludes non-living agents (i.e. viruses and viroids, although debatable, are generally still regarded as non-living), insertion of "microorganism" and "parasite" might limit the use of pathogenicity too much, whereas the insertion of "pathogen" seems unhelpful. One can just as easily use "pathogenic" in the definition of pathogen as "pathogen" in the definition of pathogenicity. Sequentially an organism or agent can only be called a pathogen if it is pathogenic (capable of causing disease).

The use of "microorganism" can be ruled out based on pathogenesis associated with helminths; parasitic worms capable of causing serious disease (Wakelin, 1996). Bateman (1978) and Agrios (2005) clearly distinguished between parasitism and pathogenesis. A parasite is commonly defined as an organism that lives in (endoparasitic) or on (ectoparasitic) another living organism called a host from which it obtains its nourishment (Appendix A: Table 2.1). The uptake of nutrients by the parasite from the host (parasitism) usually results in reduced growth (Agrios, 2005; Mendgen and Hahn, 2002; Northrop-Clewes and Shaw, 2000). In numerous cases some parasites cause harm to their hosts by not only obtaining nutrients from them, but also by the secretion of harmful metabolites (i.e. degrading enzymes, toxins) from the parasite itself (Mendgen and Hahn, 2002) or from the host plant due to response to parasitic stimuli (Overmyer *et al*., 2000; Ryals *et al*., 1996). The damage caused by these parasites are not necessarily proportional to the nutrients removed by them (damage ≠ nutrient removal) (Agrios, 2005; Mendgen and Hahn, 2002), numerous symptoms occur (disease) and thus they are referred to as pathogens. Agrios (2005) placed emphasis on host-pathogen interactions within the definition of pathogenicity by substituting "organism" with "parasite". Keep in mind, all parasites are not pathogens and all pathogens are not parasites (Bateman, 1978; McNew, 1960). "Parasite" exclude non-living agents such as viruses and lifestyles that can potentially turn pathogenic such as commensals (Brown *et al*., 2012; Pallen and Wren, 2007; Pirofski and Casadevall, 2012). While categorising pathogenicity to include only certain organisms will prove useful, the wrong insertion may restrict its use too much and further cause confusion between disciplines.

Hunt (1994) referred to research by Leukel (1948) and Steinberg (1950) so that the definition of pathogenicity should not be limited to the extent that toxin-producing saprophytes are excluded. Pathogenicity should not be limited to only parasites, but the remark to include saprophytes causing disease via toxins is too vague. These articles referred to by the author describe organisms far more complex than saprophytes. An excerpt from the summery of Leukel (1948): “It is possible that *P. circinata* is a saprophyte like most other species of the genus and that it grows on cells killed by the toxin”. This suggests that a *Periconia circinata* (L. Mangin) Sacc. is a necrotroph of sorghum as clearly pointed out by Laluk and Mengiste (2010) from research conducted by Nagy *et al*. (2007) and Stergiopoulos *et al*. (2013) from research by Dunkle and Macko (1995). Steinberg (1950) showed that *Bacillus cereus* can cause frenching symptoms of tobacco by the release of diffusates. Today *B. cereus* refers to a grouping of closely related species with a complex and ambiguous taxonomy. Numerous strains fall within these groupings and are known to produce a vast variety of harmful or beneficial metabolites depending on the relationship. *Bacillus cereus* is thus not limited to a saprophytic lifecycle, but permits commensal, mutualistic or pathogenic interactions, depending on the strain, host and environment (Ceuppens *et al*., 2013). Although it was not the principle of the debate between Andrivon (1993; 1995) and Hunt (1994), they drew attention to a common concern in the application of pathogenicity; the definition of pathogenicity lacks “pathogen” restriction.

There is thus a need to restrict organisms and agents that share their life cycle with the host and draw benefit from the interaction. This should be considered in the definition of pathogenicity. Symbiont might be a potential substitute but unfortunately lacks a standard definition (Appendix A: Table 2.1), is used indiscriminately with mutualism and is inapplicable in some biological systems (Bronstein, 2015; Douglas, 2010). Attention is again drawn to the term promoted earlier; “infectious agent”. This term is relatively new and not commonly defined as a phrase (Appendix A: Table 2.1). When broken down, the medical definition of “agent” is something that produces or is capable of producing an effect and can be a chemically, physically, or biologically active principle, and “infectious” is the capability of causing or transmitting an infection (Appendix B: Table 2.2). An infectious agent would thus include any agent capable of infecting and causing or producing disease (Appendix A: Table 2.1). Many scientists already use “agent” in the definition of pathogen and some make reference to infection in the definition of pathogenicity (Appendix A: Table 2.1). Using

"infectious agent" would accurately limit pathogen and pathogenicity to only the agents understood as causing disease and being pathogenic.

5.3 CAUSE VS PRODUCE VS INCITE VS INDUCE

These terms were seriously considered and debated within the plant pathology community. "To produce disease" and "to induce disease" were the earliest references to what we understand as pathogenicity (Fig. 2.3). Concern linked with "produce" is that it strongly adds to the pathogen centred view of pathogenicity. Disease is not produced by the pathogen; it is an interaction and an outcome. Most researchers regard the pathogen as able to cause disease (Appendix A: Table 2.1; Fig. 2.3), but some (i.e. Walker) argued that "cause" imply the pathogen as the sole cause of disease and the role of the environment is disregarded. Based on this the terms "incite", "incitant" or "causal organism" were preferred over "cause" because it refers to a causal complex in which the pathogen forms part (Sharma, 2004). Many (i.e. Agrios, Horsfall and Dimond, Luttrell) again rejected the thought and emphasised that the pathogen is the immediate cause of a specific disease but disease expression is determined in part by the host, environment and pathogen factors, thus leaving "incite"

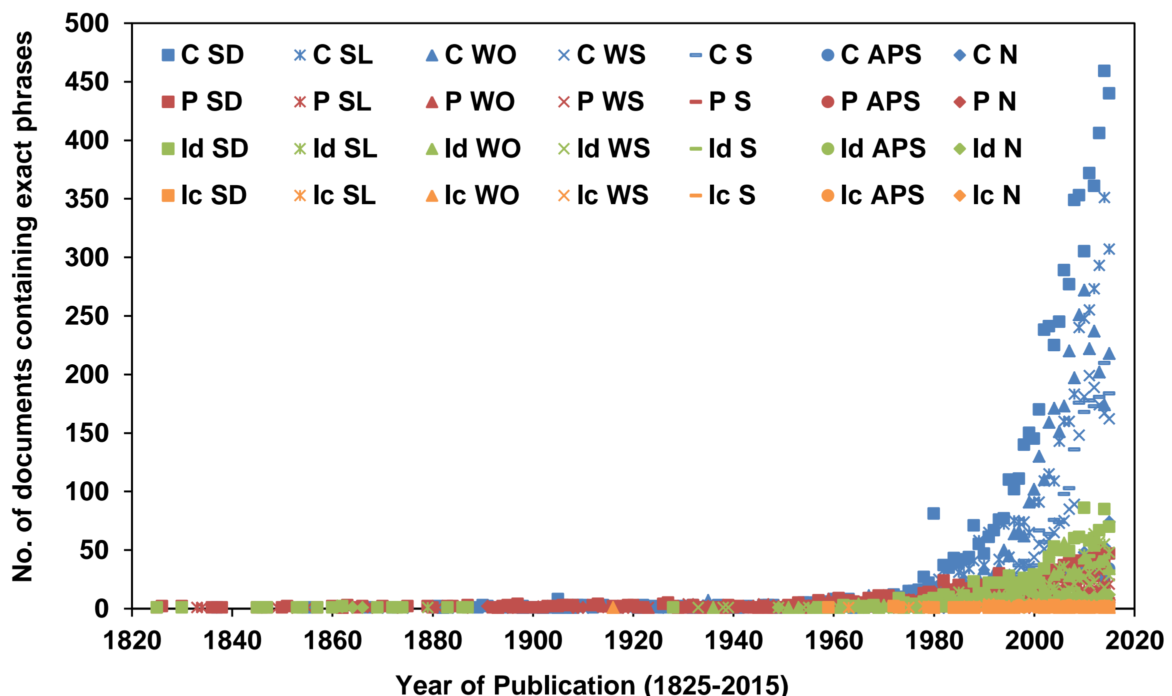

Fig. 2.3 Search results of exact phrases used in pathogenicity over nearly two centuries. [1]Exact phrases: **C,** "to cause disease"; **P,** "to produce disease"; **Id,** "to induce disease"; **Ic,** "to incite disease". [2]Databases: **SD,** Science Direct; **SL,** SpringerLink; **WO,** Wiley Online; **WS,** Web of Science; **S,** Scopus; **APS,** American Society of Phytopathology Journals; **N,** Nature. NCBI (i.e. PubMed) databases were not included; unable to perform adjacent searching.

unnecessary and "cause" sufficient. The pathogen is the basic cause and characterises the disease whereas the environment is not (Luttrell, 1954; Sharma, 2004). Also, "to incite disease" has never truly been adopted, and "to produce disease" and "to induce disease" fall behind "to cause disease" in modern-day literature (Fig. 2.3).

"Induce" was introduced into medicine and physics long before 1954 with an extension of denotation similar to "incite" in plant pathology (Luttrell, 1954; Fig. 2.3). Luttrell (1954) mentioned the use of "cause", "incite", "induce" and also "precipitate" would depend on certain situations and disease interactions. Cowling and Horsfall (1979) later went on to reject "cause" and "incite" and replaced it with "induce" in plant pathology. They rejected "cause" based on the understanding that it is rare to find a pathogen with a capacity to affect its host, without being affected by the environment. Rejecting "cause" based on this is flawed since pathogenicity is qualitative (Shapiro-Ilan *et al.*, 2005; Thomas and Elkinton, 2004). It is unavoidable that environmental factors affect disease, whether the effect of a specific environment increases, decreases or nullifies disease means nothing if the disease can occur in another environment. The fact that disease can occur (qualitative) is the crux of the matter, not the fact that it can be affected (quantitative). "Incite" was regarded as misleading and adversative to the concept of the definition of disease and pathogen. Pathological diseases will not continue without the pathogen. This is of course with exception to irreversible and/or fatal damage caused by the pathogen. According to Cowling and Horsfall (1979) "induce" recognises the importance of both the environment and pathogen as major factors of disease. Considering, "induce" would more strongly refer to a complex but it is hard to see how it raises the importance of the environment in the matter. "Induce" is a more specific term (Luttrell, 1954), if used it pertains to the pathogen as an influencer/stimulator of the host to react and cause symptoms. For example, it is accurately applied to hypersensitive response (HR); the pathogen induces HR of a host (Agrios, 2005; Overmyer *et al.*, 2000; Ryals *et al.*, 1996; Singh *et al.*, 1995).

In some textbooks confusion is created by giving two different definitions for pathogenicity in different chapters, using "induce disease or hypersensitive response" in one (Singh *et al.*, 1995) and "ability to cause disease" in another (Rudolph, 1995). In their defence, throughout the textbook (Pathogenesis and Host Specificity in Plant Disease) "induce" or "elicit" was strongly linked to HR and "cause" to disease. Other authors use the terms interchangeably depending on the definition of pathogenicity or pathogen. Agrios (2005) used "pathogen to cause disease" for pathogenicity and "entity that can incite disease"

for pathogen. The dictionary by Dunster and Dunster (1996) used "capability to cause disease" for pathogenicity but "disease-inducing organism or abiotic agent" for pathogen. Cowling and Horsfall (1979), who stressed the use of "induced" for the definition of pathogen, still used "cause disease" when referring to viruses, viroids, and plasmids in their textbook: "…viruses, viroids, and plasmids cause disease by…" and "They all reproduce and cause disease by…"

6. WHAT IS THE CAUSE?

The reason for discrepancies among the use of "cause", "incite" and "induce" is based on difficulties to identify the reason (causer) of disease. In the broad spectrum of disease, all factors (disease pyramid) can be involved, but all need not be present depending on the type of disease. The mode and degree to which each contributes to disease can vary. This would then place more emphasis on the major contributing factor/s of a specific disease. As pointed out by Luttrell (1954), this will heavily influence the use of the terms "cause", "incite", "induce" and "precipitate" with regards to the effect the pathogen will have in a specific disease. In some cases, the role of the pathogen might even seem insignificant with respect to symptom expression but play a detrimental role in the initialisation or stimulation thereof. The use of "cause" might appear too broad for some diseases and "induce" too specific for others. "Cause" has a more general meaning (all diseases considered) and used more frequently than the other terms (Fig. 2.3). This makes "cause" more suitable for use in pathogen and pathogenicity. Equally important, the use of "disease" in the definitions should be enough proof to the reader that many factors and interactions are involved (complex). It is not possible to elaborate on all the aspects included in a single definition. When approaching disease, one must always consider the five factors affecting it.

The presence of the pathogen in the host does not mean that the host is diseased, even if it is susceptible (i.e. quiescent pathogen or pathobiont). It does, however, imply that the host is at risk and depending on its resistance and other factors disease can result. Pathogenicity is only considered when disease develops. Symptoms are conditions of disease. They are very important to perceive and evaluate disease (Appendix A: Table 2.1). Symptom: "subjective evidence of disease or physical disturbance; something that indicates the presence of bodily disorder" (Merriam-Webster, 2015). If disease is identified and defined by signs and/or symptoms, and signs and symptoms can be influenced by other disease-causing factors

(disease pyramid), the same pathogen can cause multiple diseases. Is a pathogen linked to a disease or a disease to a pathogen?

An infectious disease includes the involvement of a pathogen (Agrios, 2005; Andrivon, 1993) and pathogenicity is a qualitative term (Shapiro-Ilan *et al*., 2005; Thomas and Elkinton, 2004). However, if a specific disease can still occur in the absence of the pathogen, it is not only an infectious disease. How should this be considered in pathogenicity? The amount of disease (quantity) is not regarded and the "pathogen" will not be needed for the disease occurrence or will not play a role in defining the disease. If the disease changes (signs and/or symptoms) due to the absence of the pathogen, the pathogen will be seen as the cause of the original disease, but not the second. Similar, if the disease changes due to the presence of the pathogen, the pathogen will be seen as the cause of the second disease, but not the original. The removal of the cause will result in the removal of the defined disease?

Addressing pathogenicity with only Koch's postulates in mind could prove unsuccessful when approaching some disease interactions (i.e. opportunists) (Shapiro-Ilan *et al*., 2005). Identifying the cause of a disease in the lab is not as simple as just removing the cause (Table 2.3). In cases where the environment and pathogen can cause the same disease and severity only increases when both are involved, both cause the disease (independently). If the pathogen is sensitive to the environment, the correct environment is fundamental for the pathogen to infect and cause disease. In cases where both the pathogen and environment effects influence the host to such an extent that disease will never occur if both aren't involved (not dealing with pathogens that are sensitive to the environment), it becomes difficult to discern. Would the disease persist if the diseased host is removed from that environment? If so, the pathogen is the cause. If not, the pathogen is an opportunist that requires an immune impaired host to cause disease. In other words, pathogenicity is regulated by host health (resistance)? It could even be a commensal or mutualistic organism turning pathogenic when the host is under stress (environmental, other biotics or age). An environment that suppresses (affect) the immunity of the host does not make it the cause of the infectious disease, but it does affect how it is perceived. The fact that low nutrients, cold weather or pollutants impair host health only makes it more susceptible; the pathogen is the direct cause of the disease. Opportunists specifically require other factors to cause host stress (a hurdle effect) so that they can cause disease (Shapiro-Ilan *et al*., 2005). Regardless, singling the pathogen out as the sole cause of diseases still seems unlikely.

Table 2.3 Trial simulation to determine the possible cause/s of disease

All present				No P				Different E				No P and different E				Results and Discussion
H	**E**	**P**	**D**	**H**	**E**	**P**	**D**	**H**	**E**	**P**	**D**	**H**	**E**	**P**	**D**	
1	1	1	1	1	1	0	0	1	2	1	0	1	2	0	0	P=D1; P sensitive[#] to E2
1	1	1	1	1	1	0	0	1	2	1	0	1	2	0	1	P=D1 and E2=D1 but P+E2≠D1; P and E2 neutralise each other's disease causing effects (possibly P sensitive[#] to E but promotes H resistance to E2)
1	1	1	1	1	1	0	0	1	2	1	0	1	2	0	2	P=D1 and E2=D2 but P+E2≠D1/D2; P and E2 neutralise each other's disease causing effects (if P beneficial[$] to H, although P sensitive[#] to E2 it still promotes H resistance to E2)
1	1	1	1	1	1	0	0	1	2	1	1	1	2	0	0	P=D1; P not sensitive to E (1 or 2)
1	1	1	1	1	1	0	0	1	2	1	1	1	2	0	1	P=D1 and E2=D1; only P or E2 needed for D1; P+E2 might increase D1 severity
1	1	1	1	1	1	0	0	1	2	1	1	1	2	0	2	P=D1 and E2=D2 but P+E2=D1; P not sensitive to E (1 or 2) and D1 overshadows* D2 (if P beneficial[$] to H, P possibly improves H resistance against E2 effects)
1	1	1	1	1	1	0	0	1	2	1	2	1	2	0	0	P=D1 and D2 but E determines which; E affect P and/or H, altering symptom expression (symptom expression sensitive to E)
1	1	1	1	1	1	0	0	1	2	1	2	1	2	0	1	P=D1 and E2=D1 but P+E2=D2; E2 affects P and/or H, altering symptom expression (D2 overshadows* and is possibly more severe than D1)
1	1	1	1	1	1	0	0	1	2	1	2	1	2	0	2	P=D1 and E2=D2 but D2 overrules D1; P sensitive[#] to E2 or D2 overshadows* D1
1	1	1	1	1	1	0	1	1	2	1	0	1	2	0	0	E1=D1 irrespective of P; P not pathogenic
1	1	1	1	1	1	0	1	1	2	1	0	1	2	0	1	E1=D1 and E2=D1 but P+E2≠D1; P sensitive[#] to E1 but neutralises effects of E2 (possibly beneficial[$] to H, improving resistance against E2 effects)
1	1	1	1	1	1	0	1	1	2	1	0	1	2	0	2	E1=D1 and E2=D2 but P+E2≠D2; P sensitive[#] to E1 but neutralises effects of E2 (possibly beneficial[$] to H, improving resistance against E2 effects)
1	1	1	1	1	1	0	1	1	2	1	1	1	2	0	0	E1=D1 and P=D1; only P or E1 needed for D1 to occur but P+E1 probably increase D1 severity
1	1	1	1	1	1	0	1	1	2	1	1	1	2	0	1	E1=D1 and E2=D1 irrespective of P; P not pathogenic (H possibly sensitive to E)
1	1	1	1	1	1	0	1	1	2	1	1	1	2	0	2	E1=D1, P=D1 and E2=D2 but P+E2=D1; P not sensitive to E (1 or 2), E1+P might increase D1 severity and D1 overshadows* D2 (H possibly sensitive to E)
1	1	1	1	1	1	0	1	1	2	1	2	1	2	0	0	E1=D1 and P=D2 but D1 overrules D2; P sensitive[#] to E1 or D1 overshadows* D2
1	1	1	1	1	1	0	1	1	2	1	2	1	2	0	1	E1=D1 and E2=D1 and P=D2; P sensitive[#] to E1 (if P beneficial[$] to H, P possibly improves H resistance against E2 effects)
1	1	1	1	1	1	0	1	1	2	1	2	1	2	0	2	E1=D1 and E2=D2 irrespective of P; P not pathogenic
1	1	1	1	1	1	0	2	1	2	1	0	1	2	0	0	P=D1 and E1=D2 but D1 overrules D2; P sensitive[#] to E2 and symptoms of D1 overshadows* D2 (if P beneficial[$] to H, P possibly improves H resistance against E1 effects)
1	1	1	1	1	1	0	2	1	2	1	0	1	2	0	1	P=D1 and E2=D1 but P+E2≠D1, and E1=D2 (D1 overrules D2); P and E2 neutralise each other's disease causing effects (possibly P sensitive[#] to E2 but promotes H resistance to E2 effects) and D1 overshadows* D2
1	1	1	1	1	1	0	2	1	2	1	0	1	2	0	2	P=D1 and E1=D2 and E2=D2 but P+E2≠D1/D2 (D1 overrules D2); P and E2 neutralise each other's disease causing effects (P sensitive[#] to E2 but promotes H resistance to E2 effects) and D1 overshadows* D2 (if P beneficial[$] to H, P possibly improves H resistance against E1 effects)
1	1	1	1	1	1	0	2	1	2	1	1	1	2	0	0	P=D1 and E1=D2 but D1 overrules D2; P not sensitive to E (1 or 2) and D1 overshadows* D2 (if P beneficial[$] to H, P possibly improves H resistance against E1 effects)
1	1	1	1	1	1	0	2	1	2	1	1	1	2	0	1	P=D1 and E2=D1 but E1=D2 (D1 overrules D2); only P or E2 needed for D1 to occur but P+E2 will probably increase D1 severity and D1 overshadows* D2
1	1	1	1	1	1	0	2	1	2	1	1	1	2	0	2	P=D1 but E1=D2 and E2=D2 (D1 overrules D2); P not sensitive to E (1 or 2) and D1 overshadows* D2 (if P beneficial[$] to H, P possibly improves H resistance against E effects)
1	1	1	1	1	1	0	2	1	2	1	2	1	2	0	0	P=D2 and E1=D2 but P+E1=D1; E1 affects P and/or H resulting, altering symptom expression (D1 overshadows* and is possibly more severe than D2)
1	1	1	1	1	1	0	2	1	2	1	2	1	2	0	1	E1=D2 but P+E1=D1 and E2=D1 but P+E2=D2; P affects H, altering symptoms caused by E (possibly dealing with a sensitive H)
1	1	1	1	1	1	0	2	1	2	1	2	1	2	0	2	P=D1 but E1=D2 and E2=D2 (D1 overrules D2); P sensitive[#] to E2 and D1 overshadows* D2 (if P beneficial[$] to H, P possibly improves H resistance against E1 effects)

H, Host; **E,** Environment; **P,** Pathogen; **D,** Disease; **=,** cause; **0,** not present; **1-2,** Environment, pathogen and disease one or two.
*Overshadows, P+E results in combining, replacing or changing of symptoms that appears as another disease; [#]P sensitive, Certain E conditions prevents P from causing disease; [$]P beneficial, P can cause symptoms (appear like D) but beneficial to the H.

Disease is always a complex but "cause" is not. In terms of infectious diseases, one or many factors can be involved in the cause of disease but the pathogen is essential. Removing the pathogen will always result in the removal of the infectious disease. Some ways to control disease are by removing (i.e. quarantine or destruction) or disrupting the life cycle (i.e. environmental or biotics such as vectors) of the pathogen or increasing host resistance (i.e. environmental, biotics such as probiotics and plant growth promoting rhizobacteria, or genetics). However, the pathogen is not the sole cause of all diseases. The definition requires that the pathogen (an infectious agent) has the ability to cause (verb) disease. It doesn't require the pathogen to be the sole or exclusive cause (noun) of a disease. Nor does it require that the pathogen will cause disease in every case, simply because disease depends on other factors. This is unfortunately easily misunderstood, giving reason for many to include "potential ability" in the definition of pathogenicity (Appendix A: Table 2.1). The fact that the environment can cause a similar disease, contribute to one or fundamentally participate in causing one removes nothing from the role of the pathogen. The pathogen still has to be able to cause disease, even if the cause is mutual. The pathogen is uniquely and exclusively the infectious agent in a disease complex (pyramid) of which it is the sole or mutual cause. No other factor fulfils this role. In case of a mutual cause, the other factor/s must participate with the pathogen in causing the disease (affect the host) (Sharma, 2004) and participation must be qualitative (disease won't occur without).

7. CONCLUSION

Nearly a century later pathogen and pathogenicity still lack specificity and retain a generalised pathogen centred context. Pathogen and pathogenicity are qualitative terms specifically pertaining to the involvement of the infectious agent in a disease complex. The pathogen is vital for disease to occur although it can be affected by many other factors. The fact that these factors can increase, decrease or prevent disease doesn't remove "the capability to cause disease" from the pathogen if disease can result in another situation. However, some cases where factors uniquely and mutually cause disease with the pathogen cannot be avoided. The use of terms already applied in previous definitions was encouraged. Specificity can be increased by using "infectious agent" to include all biotic and abiotic agents with the ability to infect. "Infectious agent" can also satisfy the need to refer to a complex as "infectious" implies an interaction and "agent" is frequently used to imply an action of go-between. Generality can be retained by using "cause" to include all disease interactions. Much care must be taken not to use capacity (quantitative) as an alternative in

the definition of pathogenicity; it will fundamentally change its use and understanding. Emphasis is placed on the conceptual understanding that disease is a complex of which the pathogen forms part. It is not necessary to add much to the definitions as disease is enough proof that a complex is being dealt with. An infectious disease complex normally, if not always, has five factors involved (disease pyramid). Some of these factors are essential for disease occurrence or development, some unavoidable and others influential. Generic definitions for pathogen and pathogenicity are proposed. Hopefully these definitions will stretch across disciplines and considered with the concept of a disease complex (five factors involved), reduce confusion when applying the terms. Pathogen: disease-causing infectious agent. Pathogenicity: the capability of an infectious agent to solely or mutually cause disease.

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Chapter 3

Postharvest decay of nectarine and plum caused by *Penicillium* spp.

ABSTRACT

Stone fruits are highly perishable and susceptible to numerous postharvest pathogens. *Penicillium expansum* is a well-known pathogen of stone fruits but little is known about other *Penicillium* spp. that could potentially cause decay. This chapter aims to determine pathogenicity profiles of *P. expansum*, *P. crustosum*, *P. solitum* and *P. digitatum* on selected nectarine and plum cultivars, and in part examine the disease cycle within new fruit-*Penicillium* interactions to observe the potential of the pathogens to cross-infect. Lesions caused by *Penicillium* spp. isolated from the pear and citrus handling environments did not differ on nectarine. *Penicillium digitatum* was the most aggressive species on most nectarine and plum cultivars evaluated. Decay was associated with older fruit (longer stored). The highest aggression was observed on 'Nectargold', 'May Glo' and 'African Rose'. *Penicillium expansum* and *P. crustosum* had the highest disease incidences and were the second and third most aggressive species respectively. *Penicillium solitum* caused small lesions. Its role as a postharvest pathogen in the supply chain is thus negligible. Scanning electron microscopy confirmed infection and provided new information on the growth and reproduction of *P. expansum, P. crustosum* and *P. digitatum* on infected nectarine, pear and lemon. Infected pear and lemon fruits can serve as cross-infection sources for stone fruits in the fresh produce chain. To our knowledge, this is the most complete description of disease caused by *P. digitatum*, *P. crustosum* and *P. solitum* on nectarine and plum. Rapid decay caused by *P. digitatum* highlighted the potential of the species to contribute to losses in the stone fruit industry. Future research should investigate the presence and impact of *P. digitatum* in the stone fruit supply chain. The role of fruit ripeness on fruit-*Penicillium* interactions also requires further investigation.

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1. INTRODUCTION

Nectarine and plum are climacteric fruits that are highly perishable and easily wounded, leading to a short shelf-life and representing high-risk crops in terms of postharvest decay (Crisosto and Mitchell, 2011; Kader, 2011). South Africa (SA) is a small, but important stone fruit producer that traditionally exports a significant volume of its crop. Fresh nectarine (*Prunus persica* (L.) Batsch var. *nucipersica* (Suckow) C. Schneider), peach (*P. persica* (L.) Batsch var. *persica*) and plum (*Prunus* L. spp.) exports reached 73 721 metric ton (~26% of total production) during the 2015/16 season. Plum occupied the largest export volume (~73%). Export of these fruit types contributed over R1.49 billion in net export realisation for SA over the 2015/16 season (HORTGRO, 2016).

Numerous postharvest pathogens can cause decay of stone fruits. *Monilinia*, *Rhizopus*, *Penicillium*, *Alternaria*, *Botrytis*, *Cladosporium*, *Colletotrichum* and *Stigmina* include some of the most common fungal disease causing genera (Snowdon, 2010). According to Wells *et al*. (1994) and Snowdon (2010), brown rot is the most important disease of stone fruits, although *Penicillium expansum* Link can cause significant losses when fruit is wounded (i.e. >50% prune losses) (Ceponis and Friedman, 1957; Wells *et al*., 1994). *Penicillium expansum* is the only *Penicillium* spp. associated with nectarine (*P. persica* var. *nucipersica*) and plum (*Prunus* spp.) losses (Pitt and Hocking, 2009). Other reported *Penicillium* pathogens of stone fruits include *P. crustosum* Thom on peach (*P. persica* var. *persica*) (Restuccia *et al*., 2006), *P. chrysogenum* Thom on black plum (plum-like fruit of *Vitex doniana* Nielson) (Eseigbe and Bankole, 1996) and *P. digitatum* (Pers.) Sacc. on nectarine (Navarro *et al*., 2011). Lesions caused by *P. chrysogenum* on black plum were small (≥8mm) after 8d incubation (28±2ºC) and the fungus was infrequently isolated (19%) from rotten fruit. No further disease symptoms were provided (Eseigbe and Bankole, 1996).

Penicillium digitatum has been isolated from commercial nectarine and plum (Parlier, California, USA, 1996). The isolates were stored in a culture collection but very little information was provided and no further research was conducted to conclude the species pathogenic. Ma *et al*. (2003) only made use of the isolates to develop nested PCR assays. Navarro *et al*. (2011) were the first to report on lesions caused by *P. digitatum* on nectarine. Artificial inoculation of ‘Flavela’ and ‘Flanoba’ resulted in infection volumes of roughly 1300mm^3 and 1500mm^3 respectively after 6d incubation at 25ºC. No information was provided on the symptoms.

Different fruit types often have overlapping export seasons and are usually handled and stored together from the point of distribution up to the point of sale (PPECB, 2013; Vermeulen *et al*., 2006). Complex fresh produce chains are also characterised by products from different countries. These factors create an environment with a higher level of risk to inoculum build-up, cross-contamination, cross-infection and ultimately fruit losses. Risk can be even higher at the end of a season (higher inoculum loads) and end of the fresh produce chain (increased susceptibility of older/riper fruit), causing non-host pathogens to be a threat (Louw and Korsten, 2014; 2015; Vilanova *et al*., 2012a; 2012b; 2014). For instance, when infected citrus increase inoculum levels of *P. digitatum* (high decay prevalence) in a facility where pears are handled, *P. digitatum* can reach the pears via aerial dispersal or other means of cross-contamination (i.e. handling), infect, cause decay and contribute to losses (Louw and Korsten, 2014; 2015). This chapter investigates the pathogenicity and aggressiveness of *Penicillium* spp. isolated from citrus and pome fruit supply chain environments on nectarine and plum cultivars, and partly examine the disease cycles (infection to reproduction) of *Penicillium* spp. on nectarine and alternative hosts (lemon and pear) via scanning electron microscopy (SEM). Observing this segment of the disease cycle on different hosts might prove helpful to illustrate the potential of inoculum build-up, cross-contamination and cross-infection.

2. MATERIALS AND METHODS

Fungal cultures. *Penicillium* spp. isolates used in this chapter were selected from pear (2010/2011) and citrus (2009/2010) export chain studies (South Africa to European Union). The isolates were the same as used by Louw and Korsten (2014). The identities of the isolates were confirmed via sequencing (*β-tubulin*). Isolates of *P. expansum* (P.eC and P.eP), *P. crustosum* (P.cC and P.cP), *Penicillium solitum* Westling (P.sC and P.sP) and *P. digitatum* (P.dC and P.dP) were selected from each chain (the last letter of the isolate code denotes the chain: C, citrus; P, pear). Cultures were prepared by single-spore isolation, plated on malt extract agar (MEA) (Merck, Biolab Diagnostics (Pty) Ltd, Johannesburg, SA) and incubated in darkness at 25°C for two to three weeks.

Fruit origin and handling. Postharvest practices for nectarine (*P. persica* var. *nucipersica*) and Japanese plum (*P. salicina* Lindl.) cultivars collected for trials differed (Table 3.1). The fruits were harvested at a mature stage according to industry guidelines (DAFF, 2013a; 2013b). Cultivars collected in 2011/12 were from farms and packhouses in

the Waterberg region of Limpopo Province (one of the major production regions in SA). Cultivars collected in 2012/13 were from the local market (Tshwane Fresh Produce Market) and originated from the Western Cape Province (the major production region in SA). Nectarine and plum are not commercially treated with any postharvest fungicides. Immediately after collection and transport, fruits were placed into cold storage (4–7°C; ±60% RH) at the University of Pretoria (UP) plant pathology laboratories.

Table 3.1 Postharvest handling and storage practices of stone fruit cultivars

Fruit	Season	Region[1]	Cultivar	Postharvest practices	Lag period[2]
Nectarine	2011/12	WL	NE 3-48-49	Packhouse: single layer packed (D76N) (Class 1). Fruit harvested and cold stored 6 days before collection.	½
		WL	ARC NE-5 (Nectargold)	Packhouse: single layer packed (D76N) (Class 1). Fruit harvested and cold stored 4 days before collection.	1
		WL	Sunburst	Orchard: handpicked (Class 1) and directly placed into cooler boxes on day of collection.	½
		WL	Sunlite	Packhouse: jumble packed. Fruit harvested same day as collection.	½
		WL	NE 6-4-31	Packhouse: single layer packed (D76N) (Class 1). Fruit harvested and cold stored 4 days before collection.	½
	2012/13	PWC	Bright Pearl	Tshwane Fresh Produce Market (TFPM): single layer packed (D82N) (Class 1).	1/5
		TWC	May Glo	TFPM: single layer packed.	½
		PWC	Flavortop	TFPM: single layer packed (D82N) (Class 1).	1/5
		WWC	Alpine	TFPM: single layer packed (Class 1).	½
Plum	2011/12	WL	Honey Star	Packhouse: closed-top traypack (D05I) (Class 1). Fruit harvested and cold stored 2 days before collection.	½
		WL	ARC PR-4 (African Rose)	Packhouse: single-layer open-top prepack (6kg) (Class 2). Fruit harvested and cold stored 7 days before collection.	½
	2012/13	PWC	ARC PR-4 (African Rose)	TFPM: open-top traypack (Class 1).	½
		WWC	Pioneer	TFPM: open-top traypack (Class 1).	½
		TWC	Fortune	TFPM: open-top traypack.	1/5
		RWC	Sun Kiss (African Pride)	TFPM: open-top traypack (M05D) (Class 1).	1/5

[1]Region (origin): **WL,** Waterberg, Limpopo Province; **PWC,** Prins Alfred Hamlet, Western Cape Province (WCP); **TWC,** Tulbagh, WCP; **WWC,** Wellington, WCP; **RWC,** Robertson, WCP. [2]Number of days from fruit collection to inoculation.

Confirming pathogenicity and comparing citrus isolates to pear isolates. Fruit handling and inoculation were similar as described by Louw and Korsten (2014; 2015). Pathogenicity was determined by inoculating (6 x 10^4 conidia/ml) five Sunburst nectarines and five 'ARC PR-4' ('African Rose') (2011/12) plums with each *Penicillium* sp. Citrus chain isolates of *P. expansum*, *P. crustosum*, *P. solitum*, and *P. digitatum* was used for inoculation. Conidial suspensions were prepared in sterilised Ringers solution (Merck) containing 0.05% Tween 80 (Associated Chemical Enterprises, Johannesburg). Fruits were surface sterilised by dipping into 0.0018% sodium hypochlorite solution for up to ten min and allowed to air dry. Wounding (1.5 x 3mm) was done on opposite sides (two wounds; each on a side) by aseptically piercing the fruit surface with a sterile micropipette tip (20–200µl). Inoculation was via pipetting 20µl of conidial suspension into each wound site. Controls were included (only wounded). Inoculated/wound sites were taped with Parafilm to prevent cross-contamination at the early stage of the trial and during measurements. Randomisation was done on a disinfected table and incubation at room temperature conditions (23.70±0.23°C; 59.73±4.57% RH) for up to 7d. The horizontal and vertical (calyx axis vertical) diameter of lesions were recorded three, five and 7d post-inoculation.

Penicillium spp. isolates from two different environments (citrus and pear export chains) were compared on nectarine to evaluate similarity in pathogenicity and aggressiveness of the isolates from the different backgrounds. Five surface sterilised Bright Pearl nectarines were inoculated with conidial suspensions of each isolate of *P. expansum*, *P. crustosum*, *P. solitum*, and *P. digitatum*. Preparation of conidial suspensions, sterilisation, inoculation, incubation, randomisation and recording of data here and for the following trial were as described earlier.

Aggressiveness of *Penicillium* spp. on nectarine and plum cultivars. Nectarine and Japanese plum cultivars (Table 3.1) were inoculated with conidial suspensions of *P. expansum*, *P. crustosum*, *P. solitum* and *P. digitatum*. Isolates from the citrus export chain environment were used. Ten surface sterilised fruit of each cultivar were inoculated with each *Penicillium* spp. (10 fruit for every unique cultivar-*Penicillium* combination). Infected wounds (%), lesion diameter (*ld*) and symptom expression were recorded after 3d, 5d and 7d incubation. Nectarine and plum cultivars from season 2012/13 (Table 3.1) were evaluated for first signs of mycelial growth and sporulation. Forty lesions were evaluated per cultivar-*Penicillium* interaction.

Scanning Electron Microscopy. Colonisation and sporulation differences between the most aggressive of the three pathogens assessed in this chapter (*P. expansum*, *P. crustosum* and *P digitatum*) were evaluated using SEM on nectarine, lemon and pear. Lemon and pear fruits were added to compare infection of nectarine with that of alternative hosts. Some of these host-pathogen associations were recently reported (Louw and Korsten, 2014; 2015) and potentially serve as cross-contamination sources for stone fruits in the fresh produce chain. Fruits available during the same period [‘Crimson Glo’ nectarines (retail bought), ‘Forelle’ pears (retail bought) and ‘Eureka seeded’ lemons (end-market and non-treated)] were wound-inoculated on the same day at two-to-three sites with 20µl conidial suspension of *P. expansum*, *P. crustosum* and *P. digitatum*. Two sets of three fruit per fruit type were inoculated with each *Penicillium* sp. Control fruits were included (only wounded). One set was incubated for 24h and the other for 48h. Each set of fruit was randomised. Inoculated sites were cut out (5mm x 5mm) after incubation, placed into fixing solution [2.5% Glutaraldehyde ($OCHCH_2CH_2CH_2CHO$) in 0.075M phosphate buffer, pH 7.4] and held overnight in a refrigerator. The next day, samples were rinsed (x3) with 0.075M phosphate buffer for 10min, treated with 0.5% aqueous osmium tetroxide (OsO_4) (SPI Supplies Division Structure Probe, Inc., West Chester, USA) for 2h in a fume hood and rinsed another three times with phosphate buffer (10min each). Samples were dehydrated by submergence in an increasing range of ethanol concentrations [30%, 50%, 70%, 90%, 100% (x3)]. Samples were submerged for 10min in each concentration, except for the final 100% step in which case samples were held prior to critical drying in a Bio-Rad E3000 critical point dryer (Bio-Rad, Watford, UK). Samples were mounted on an aluminium stub, coated with carbon in an EMITECH K950X carbon coater (EM Technologies Ltd, Ashford, UK) with a BOC Edwards EXT 70H 24V pump (BOC Ltd, Crawley, UK) and viewed using a Zeiss Ultra Plus SEM (Ultra High Resolution FEG SEM) equipped with a Gemini column (Carl Zeiss NTS GmbH, Oberkochen, GER).

Scan sites of $0.04mm^2$ (size of a large block of a haemocytometer) were evaluated under SEM and scored using an index to provide quantitative information on mycelial, conidiophore and spore development for each interaction. This allowed for comparisons between the life stages of different *Penicillium* spp. on different hosts. Index: **1** – mycelia cover ≤5% of scan area, conidiophores ≤2, single conidia on a phialide; **2** – mycelia cover >5% and ≤25% of scan area, >2 and ≤5 conidiophores, chains of two conidia; **3** – mycelia cover >25% and ≤50% of scan area, >5 and ≤10 conidiophores, chains of three conidia; **4** –

mycelia cover >50% and ≤75% of scan area, >10 and ≤20 conidiophores, chains of four conidia; **5** – mycelia cover >75% of scan area, >20 conidiophores, chains of five or more conidia.

Reisolation from fruits, preservation and identification. Two-to-three fruit per cultivar-*Penicillium* spp. interaction were used for reisolating fungi from all experiments, excluding the SEM samples. Isolations were made on MEA plates and incubated as previously described. Cultures were purified and observed for phenotypic similarities. A single culture from any of the two to three fruit was preserved (water -and cryo-preservation) and identified by PCR-Restriction Fragment Length Polymorphism (PCR-RFLP) assay and sequencing, as described by Louw and Korsten (2014; 2015). PCR-RFLP allowed for molecular grouping of the *Penicillium* spp. Three representatives for each unique PCR-RFLP pattern were submitted for sequencing at the DNA Sequencing Facility of the Faculty of Natural and Agricultural Sciences at UP to confirm species identity.

Statistical design and data analysis. All trials, excluding SEM, were repeated using a complete randomised design (CRD). Trials comparing different *Penicillium* spp. isolates and fruit cultivars had factorial arrangements. Four measurements (two inoculation sites, each with horizontal and vertical diameter measurements) were taken from each fruit (including wounds on control fruit). The four measurements were averaged to account as one rep. Wound diameters (means) were subtracted from lesion diameters, allowing lesions to be expressed without the wounding effect. Data (lesion diameters with wounds subtracted) were subjected to analysis of variance (ANOVA) in Statistical Analysis System (SAS) (version 9.2; SAS Institute Inc., Carry, NC, USA). Bartlett's test for homogeneity was used to reveal similarity among trial repeats (independent experiments). Nonsignificant differences resulted in trial repeats being pooled. Means were separated using Fisher protected Least Significant Difference.

3. RESULTS

Confirming pathogenicity and comparing citrus isolates to pear isolates. The diameter of lesions from independent pathogenicity experiments were not significantly different (P = 0.91). All *Penicillium* spp. were observed pathogenic on both nectarine and plum cultivars (Table 3.2). Based on lesion sizes, the interactions between fruit type and *Penicillium* spp. were significantly different (P < 0.0001). *Penicillium expansum* caused the largest lesions on Sunburst, *P. digitatum* the second largest and *P. crustosum* and *P. solitum*

thereafter. Disease incidence was low for *P. digitatum* and *P. solitum* on Sunburst. Only 25% of lesions caused by *P. digitatum* on nectarine were significantly larger than control fruit. The largest lesion was 83.90±8.49mm in diameter (diameter of wound subtracted) after 7d incubation. *Penicillium digitatum* caused the largest lesions on African Rose, thereafter *P. expansum*, *P. crustosum* and *P. solitum*. Disease incidence was 100% for all *Penicillium* spp. on the plum cultivar.

Table 3.2 Lesions caused by *Penicillium* spp. on nectarine and plum after 7d incubation

Cultivar	*Penicillium* spp.	Lesion diameter (mm)*	Incidence (% lesions)
Sunburst nectarine	*P. expansum*	44.95±8.25b	100
	P. crustosum	30.10±5.17d	100
	P. solitum	5.43±2.00fe	50
	P. digitatum	36.36±22.75c	26.32
	Control	0±0.26f	
African Rose plum	*P. expansum*	47.16±2.69b	100
	P. crustosum	32.98±4.22dc	100
	P. solitum	7.34±2.53e	100
	P. digitatum	58.43±2.72a	100
	Control	0±0.08f	

*Mean lesion diameter ± standard deviation of 10 fruit. Means of wounds from control fruits were subtracted from lesion diameters. Letters that are not the same are significantly different ($P < 0.05$) according to Fisher protected Least Significant Difference.

Independent experiments of the isolate comparison trial were not significantly different ($P = 0.72$). However, a distinct difference was noted for *P. digitatum* in the independent experiments (Fig. 3.1). Both isolates of *P. digitatum* failed to cause lesions on Bright Pearl in the initial experiment, yet large lesions were produced in the second experiment [citrus isolate *ld* = 24.28±2.65mm (20%); pear isolate *ld* = 22.95±3.10mm (60%)]. Because only mean disease severity data are reported in Figure 3.1, results from *P. digitatum* in the first experiment (no lesions) are not observable. Disease incidence for *P. expansum* and *P. crustosum* were 100% for both isolates, whereas that for *P. solitum* was 65% (citrus isolate) and 70% (pear isolate). Control fruit yielded no lesions. Citrus and pear chain isolates were not significantly different based on *ld* ($P = 0.60$).

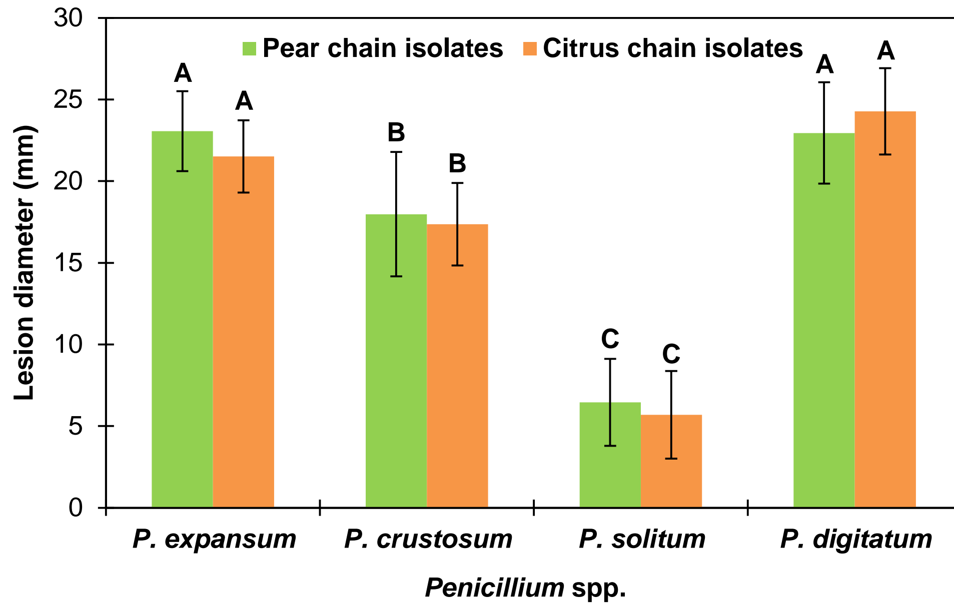
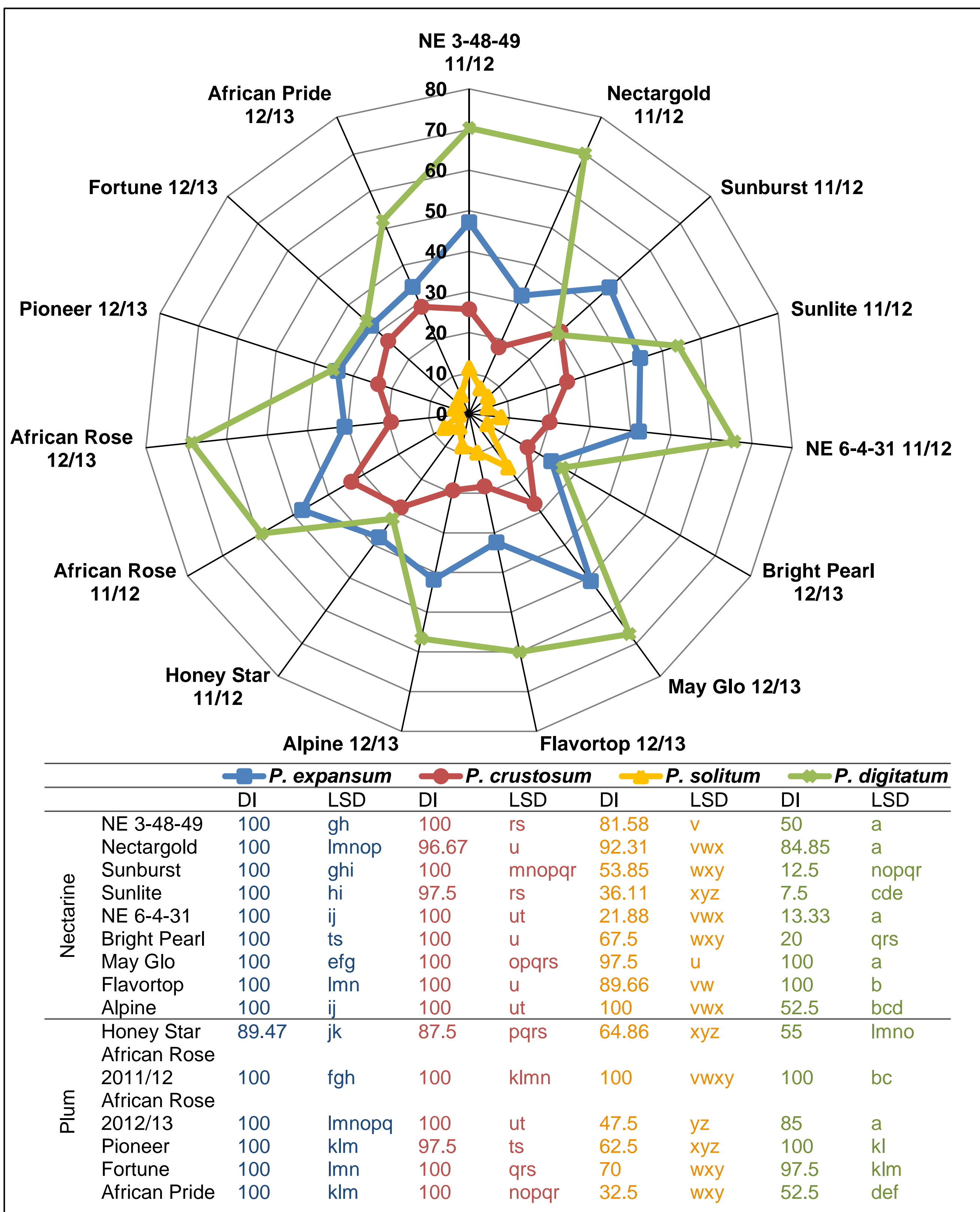


Fig. 3.1 Comparing *Penicillium* citrus -and pear chain isolates in terms of mean lesion sizes (10 fruit) produced on nectarine (cv. Bright Pearl). Mean diameter of control (wound) was subtracted from lesion diameters. Vertical bars represent standard deviation. Different letters are significanly different (P < 0.05) according to Fisher protected Least Significant Difference.

Aggressiveness of *Penicillium* spp. on nectarine and plum cultivars. Independent experiments for the nectarine and plum cultivar trial were not significantly different (P = 0.15), although inconsistencies were noted. Similar to the trial comparing different *Penicillium* spp. isolates, *P. digitatum* produced distinctly different results on NE 3-48-49 and Bright Pearl in the trial repeat. Only 20% of sites inoculated with *P. digitatum* on NE 3-48-49 in the initial experiment yielded results (ld = 77.28±4.73mm) after 7d incubation. Contrary to the first experiment, the second experiment yielded lesions of similar size (ld = 72.18±12.08mm), but with higher disease incidence (80%). *Penicillium digitatum* did not cause lesions on Bright Pearl in the initial experiment, but lesions were observed in the second experiment [ld = 26.60.18±11.07mm (40%)] after 7d incubation.

The different interactions between cultivar and *Penicillium* spp. were significantly different based on lesion size (P < 0.0001). *Penicillium digitatum* caused the largest lesions on most cultivars, however, disease incidence varied (Fig. 3.2). Low incidence was recorded on Sunburst, Sunlite, NE 6-4-31 and Bright Pearl. *Penicillium expansum* and *P. crustosum* caused lesions throughout both cultivar ranges at high incidence. *Penicillium solitum* caused the smallest lesions and disease incidence was low on some cultivars (Sunlite, NE 6-4-31, African Rose 2012/13 and African Pride).



		P. expansum		*P. crustosum*		*P. solitum*		*P. digitatum*	
		DI	LSD	DI	LSD	DI	LSD	DI	LSD
Nectarine	NE 3-48-49	100	gh	100	rs	81.58	v	50	a
	Nectargold	100	lmnop	96.67	u	92.31	vwx	84.85	a
	Sunburst	100	ghi	100	mnopqr	53.85	wxy	12.5	nopqr
	Sunlite	100	hi	97.5	rs	36.11	xyz	7.5	cde
	NE 6-4-31	100	ij	100	ut	21.88	vwx	13.33	a
	Bright Pearl	100	ts	100	u	67.5	wxy	20	qrs
	May Glo	100	efg	100	opqrs	97.5	u	100	a
	Flavortop	100	lmn	100	u	89.66	vw	100	b
	Alpine	100	ij	100	ut	100	vwx	52.5	bcd
Plum	Honey Star	89.47	jk	87.5	pqrs	64.86	xyz	55	lmno
	African Rose 2011/12	100	fgh	100	klmn	100	vwxy	100	bc
	African Rose 2012/13	100	lmnopq	100	ut	47.5	yz	85	a
	Pioneer	100	klm	97.5	ts	62.5	xyz	100	kl
	Fortune	100	lmn	100	qrs	70	wxy	97.5	klm
	African Pride	100	klm	100	nopqr	32.5	wxy	52.5	def

Fig. 3.2 Figure of mean lesion diameter (mm), and table of least significant difference (LSD) and disease incidence (DI) (%) of pathogenic *Penicillium* spp. on nectarine and plum cultivars (20 fruit per cultivar) after 7d incubation (5d incubation for Nectargold) at room conditions. Means of wounds from control fruits were subtracted from means of lesions. Letters that are dissimilar are significantly different ($P < 0.05$) based on mean of lesion diameter according to Fisher protected Least Significant Difference.

Symptom expression of *Penicillium* spp. on nectarine and plum cultivars. Symptom expression was relatively consistent across the cultivars evaluated (Fig. 3.3). Lesions commenced with browning and softening of underlying tissue. Symptoms were more visible on light coloured cultivars. Softening of tissue resulted in a slightly sunken appearance of lesions. White mycelial growth developed from brown infected tissue, subsequently yielding conidiophores producing conidia. In the case of *P. digitatum* infected cultivars, cellular collapse caused a wrinkled appearance of decayed areas. The wrinkling appearance became apparent when fruit surfaces were overlaid with conidia. *Penicillium digitatum* was able to produce copious amounts of lime green conidia within the incubation period. The other *Penicillium* spp. yielded blue to blue-grey conidia. *Penicillium expansum* produced the highest amount of conidia of the blue mould causing *Penicillium* spp. Overall, more conidia were produced on nectarines than on plums.

The first signs of mycelial growth and sporulation were evaluated on the 2012/13 cultivars (Table 3.3). The first visual signs of mycelial growth from *P. expansum* and *P. crustosum* were noted on the 3d of incubation for all cultivars. Sporulation was observed at the earliest, on the 5d of incubation for *P. expansum* and 3d for *P. crustosum* in nectarine cultivars. *Penicillium crustosum* was the first to sporulate. *Penicillium solitum* was the slowest to produce mycelia and conidia on plum the cultivars. *Penicillium digitatum* was slow to produce mycelia in comparison to the size of lesions, however, sporulation followed shortly afterwards.

Table 3.3 Days of incubation for first visible signs of mycelia and conidia

Cultivar	**E**		**C**		**S**		**D**	
	m	**c**	**m**	**c**	**m**	**c**	**m**	**c**
Bright Pearl	3	5	3	3	3	5	3	5
May Glo	3	5	3	5	3	5	5	5
Flavortop	3	5	3	3	3	5	3	5
Alpine	3	5	3	3	3	5	5	5
African Rose	3	7	3	7	5	-	5	5
Pioneer	3	5	3	5	5	7	5	5
Fortune	3	5	3	5	5	-	3	7
African Pride	3	7	3	5	7	7	5	7

40 lesions on 20 fruit evaluated per cultivar-*Penicillium* interaction. **E,** *Penicillium expansum*; **C,** *P. crustosum*; **S,** *P. solitum*; **D,** *P. digitatum*; **m,** mycelial growth; **c,** conidia.

Fig. 3.3 Symptoms caused by *Penicillium* spp. on nectarine and plum cultivars after 7d incubation at room conditions.

Observations from SEM images. All interactions were compatible for germination of conidia and the development of mycelia within 24h (Table 3.4). The degree of germination and mycelial growth depended on the host-*Penicillium* spp. interaction. Only microphotographs revealing new findings are presented in Figure 3.4. No conidiophores were produced at 24h or on pears at 48h. The 48h incubation period showed a significant progression in the life stages of all *Penicillium* spp. on nectarine. *Penicillium expansum* was the only species that did not produce conidia on nectarine within the 48h incubation period, although mycelial growth was abundant and conidiophore development was observed (Table 3.4 + Fig. 3.4D). *Penicillium crustosum* produced the most conidia within 48h on nectarine (Table 3.4 + Fig. 3.4A), followed by *P. digitatum* (Table 3.4 + Fig. 3.4B). Severe twisting and coiling of *P. expansum* mycelia were observed on pears after 48h (Fig. 3.4E). *Penicillium expansum* and *P. crustosum* showed little germination on lemons within 24h (also observed from *P. expansum* on pear), yet *P. expansum* was able to sporulate after 48h (Fig. 3.4C). *Penicillium expansum* was able to produce more conidia on lemons within 48h than *P. digitatum*, although *P. digitatum* produced the most abundant mycelia within the same period. *Penicillium crustosum* was unable to produce conidiophores on lemons within

Table 3.4 Scanning electron microscopy observations of lesions caused by *Penicillium* spp. on fruits after 24 and 48h incubation at room conditions

		P. expansum		***P. crustosum***		***P. digitatum***	
		24h	**48h**	**24h**	**48h**	**24h**	**48h**
Nectarine	G	+	+	+	+	+	+
	M	1	5	3	5	1	5
	C	-	1	-	5	-	4
	S	-	-	-	5	-	2
Pear	G	+	+	+	+	+	+
	M	2	3	1	4	2	2
	C	-	-	-	-	-	-
	S	-	-	-	-	-	-
Lemon	G	+	+	+	+	+	+
	M	1	1	1	2	3	5
	C	-	2	-	-	-	2
	S	-	2	-	-	-	1

Four lesions were evaluated per fruit-*Penicillium* interaction. Values indicate development of the life stage assessed (intensity increase from 1 to 5). **G,** Germination of conidia; **M,** Mycelial growth; **C,** Conidiophore counts (based on presence of metula); **S,** Sporulation.

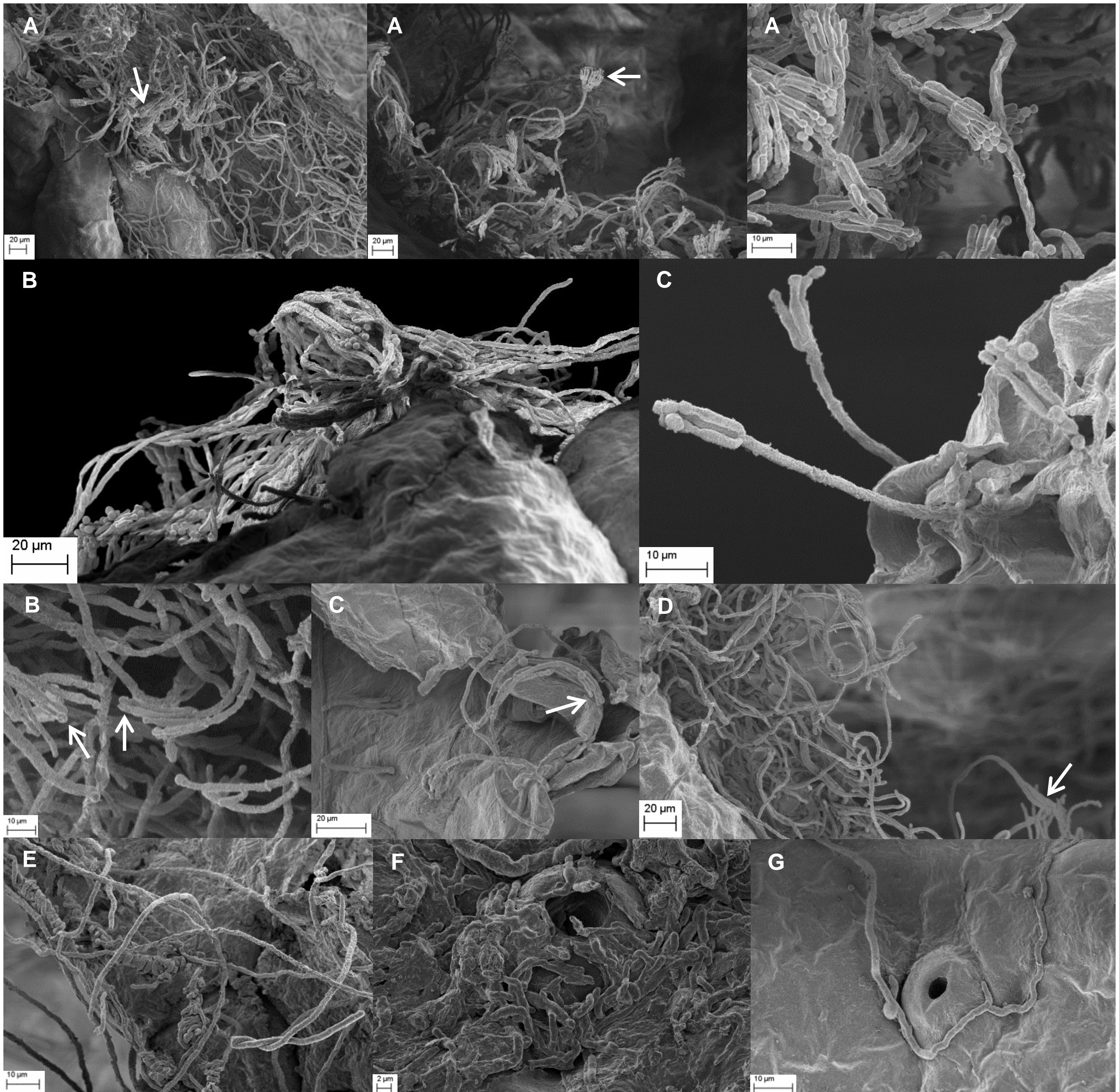


Fig. 3.4 Scanning electron micrographs of *Penicillium* spp. on nectarine, pear and lemon. **A,** *P. crustosum* sporulating on nectarine (48h); **B,** *P. digitatum* sporulating on nectarine (48h); **C,** *P. expansum* sporulating on lemon (48h); **D,** *P. expansum* producing metula on nectarine but no conidia (48h); **E,** Coiling and twisting of *P. expansum* mycelia on pear (48h); **F,** *P. digitatum* not penetrating open stomata of lemon (24h); **G,** *P. digitatum* mycelium growing around open stomata of lemon (48h).

48h (Table 3.4). Observations revealed that *P. digitatum* did not grow towards or penetrate open stomata of lemon fruit (Fig. 3.4F–G). This was also observed from *P. crustosum* on lemons after 48h (mycelium growing over open stomata). Stomatal interactions were not observed on other hosts. No conidia or fungal activity was observed on control fruits.

Reisolation from fruits and species identification. Isolates made from symptomatic fruits were successfully grouped according to PCR-RFLP and positively identified using sequencing (Table 3.5). This confirmed Koch's postulates by identifying the isolated *Penicillium* spp. as the species previously inoculated into the fruits.

Table 3.5 Identity of *β-tubulin* sequences and GenBank accession numbers

Identification	Isolate no.	Host: cultivar	Accession no.
Penicillium expansum	1	N: Sunlite	KF952541
	3	N: Nectargold	KF952542
	8	P: African Rose	KF952543
	9	N: Bright Pearl	KF952544
	13	P: African Rose	KF952545
	16	P: African Pride	KF952546
P. crustosum	19	N: Sunburst	KF952547
	23	N: Bright Pearl	KF952548
	25	P: African Rose	KF952549
	28	N: Alpine	KF952550
	32	P: Pioneer	KF952551
	34	P: Honey Star	KF952552
P. solitum	36	N: Sunburst	KF952553
	42	P: African Rose	KF952554
	43	N: Bright Pearl	KF952555
	46	N: Flavortop	KF952556
	49	P: Pioneer	KF952557
P. digitatum	53	N: NE 3-4-31	KF952558
	56	N: Bright Pearl	KF952559
	58	N: Alpine	KF952560
	60	P: African Rose	KF952561
	62	P: Pioneer	KF952562

N, Nectarine; **P,** Plum.

4. DISCUSSION

All *Penicillium* spp. (*P. expansum*, *P. crustosum*, *P. solitum* and *P. digitatum*) inoculated into nectarine and plum proved to be pathogenic. *Penicillium expansum* is a well-known postharvest pathogen of stone fruits (Ceponis and Friedman, 1957). To our knowledge, this is the first report demonstrating the pathogenicity of *P. crustosum*, *P. solitum* and *P. digitatum* on plum. One study demonstrated *P. crustosum* causing disease on peaches (cv. Late Peach of Leonforte) (Restuccia *et al.*, 2006). They recorded 100% disease incidence

after 15d in a biological control experiment. The size of lesions and symptoms were not provided. The purpose of their study was to investigate the potential use of commercial biocontrol products to inhibit disease caused by *P. crustosum* and *Mucor circinelloides* Tiegh. on peaches. Nectarine is a mutant of peach and thus belongs to the same species but a different variety (Blake, 1932). Pathogenicity of *P. crustosum* on nectarine was thus expected, but our study is the first to specifically provide evidence of *P. crustosum* pathogenicity on nectarine and present new information on decay caused by *P. crustosum* on *P. persica*. *Penicillium solitum* has never been associated with or isolated from stone fruits before. Ma *et al*. (2003) previously made use of *P. digitatum* isolates from nectarine and plum, but no connections were made to decay and no pathogenicity trials were conducted. Navarro *et al*. (2011) report infection volumes of roughly 1300mm^3 and 1500mm^3 caused by *P. digitatum* on nectarine (Flavela and Flanoba). Large infection volumes were not recorded and no symptoms were illustrated or described.

Penicillium digitatum, the most important postharvest pathogen of citrus (Eckert and Eaks, 1989; Marcet-Houben *et al*., 2012), has recently been identified pathogenic on apple and pear (Louw and Korsten, 2014; Vilanova *et al*., 2014). *Penicillium digitatum* caused larger lesions than *P. expansum* on some of the pear cultivars (Louw and Korsten, 2014), even though *P. expansum* is known to be the most important *Penicillium* spp. on pome fruits in terms of decay (Pitt and Hocking, 2009; Snowdon, 2010). Similar to some pome fruit cultivars, *P. digitatum* produced the largest lesions on most of the nectarine and plum cultivars evaluated in this chapter, demonstrating the potential of the species to be the most aggressive *Penicillium* spp. on these fruit types.

Penicillium expansum was observed as a classic postharvest pathogen of nectarine and plum. *Penicillium crustosum* showed similarities. Both species were pathogenic throughout the cultivar ranges resulting in high disease incidence and moderate to high aggressiveness. In general, *P. expansum* was more aggressive than *P. crustosum* and remained the species infecting at the highest disease incidence (99.30%). Both species are able to produce the harmful mycotoxins patulin and penitrem A (Frisvad *et al*., 2004; Frisvad and Samson, 2004; Pitt and Hocking, 2009).

In our study, *P. solitum* was evaluated as the least aggressive species with low disease incidence (67.85%). The significance of the species in the fresh produce market is considered negligible. The species has a very small host range, it is not known to produce

any significant mycotoxins (Frisvad and Samson, 2004; Pitt and Hocking, 2009) and only causes small lesions when pathogenic (apple and pear) (Louw and Korsten, 2014). However, little is still known of the species (Pitt *et al*., 1991; Pitt and Hocking, 2009) and it has the ability to sporulate rapidly from small lesions, which can contribute to higher inoculum loads in the air of fruit storage environments. Higher inoculum loads of pathogens can lead to increased disease incidence and severity (Vilanova *et al*., 2012a; 2012b; 2014).

This is the first study describing symptoms caused by *P. crustosum*, *P. solitum* and *P. digitatum* on nectarine and plum. Similar to *P. expansum*, *P. crustosum* and *P. solitum* caused blue mould on nectarine and plum. It is difficult to distinguish between the blue mould causing *Penicillium* spp. based on symptom expression alone. Visual evaluation of symptoms would result in the causal agent being identified as *P. expansum* based on general perceptions, particularly by market agents or inspectors. *Penicillium solitum* caused similar symptoms on nectarine and plum as on apples and pears (Louw and Korsten, 2014). *Penicillium digitatum* characteristically caused green mould on nectarine and plum. Symptoms on nectarine and plum were similar in colour (shade of green) to those produced on pear (Louw and Korsten, 2014) but not on citrus (Louw and Korsten, 2015). Symptoms on citrus frequently had a darker (bluish -or greyish-green) shade. *Penicillium digitatum* sporulated more profusely on nectarine and plum than on apple and pear. Also, conidia-covered-skin of decayed nectarine and plum fruits frequently had a wrinkled appearance.

Micrographs from SEM reinforced visual findings that *P. digitatum* can successfully infect and colonise nectarine and pear (Louw and Korsten, 2014). *Penicillium digitatum* was the second fastest sporulating species on nectarine and was observed producing mycelia and sporulating faster and more abundantly on nectarine than on citrus. This was not observed during the evaluation of symptomatic fruits (without SEM). Conidia from symptomatic fruit (visible or not) increase inoculum loads and play an important role in cross-contamination and host specificity shifts. These findings highlight the importance of *P. digitatum* in the stone fruit industry and its potential to cross-contaminate and infect different hosts.

Penicillium expansum sporulated first on lemon and last on nectarine when viewing SEM micrographs. Images also supported findings of *P. crustosum* aggressiveness and ability to invade nectarine tissue. *Penicillium crustosum* produced the most abundant conidia within 48h on nectarine. This corresponded with findings where the least number of days (earliest on 3d) were required to visually observe conidia on most cultivars inoculated with *P.*

crustosum. No conidiophores were observed on lemon. *Penicillium crustosum* required similar conditions (older fruit and high inoculum levels) than *P. expansum* to infect and cause symptoms on citrus (Louw and Korsten, 2015; Vilanova *et al*., 2012b). *Penicillium expansum* was able to produce larger lesions on lemon (plug inoculation method) than *P. crustosum* (Louw and Korsten, 2015). This may indicate that *P. expansum* is better adapted to cross-contaminate and cross-infect these hosts. The severe twisting and coiling observed from *P. expansum* on pears might be due to the harsh SEM preparation process, however, these deformations were not observed from other host-pathogen interactions or at 24h. Further research is required to elaborate on or clarify these aspects.

Lesions (size) caused by isolates obtained from the pear chain environment were not different from lesions caused by isolates from the citrus chain environment. This supports the finding that isolates, irrespective of the fruit environment they originate from, can have similar aggressiveness on different hosts. Overlapping fruit chains can thus introduce inoculum from different fruit types into an environment where they are handled and retained together. This can result in potential cross-contamination and subsequent infection as shown in this chapter. The role of inoculum load in such cases is significant, even for non-host pathogens (Vilanova *et al*., 2012a; 2012b; 2014).

The pathogenicity and high aggression of *P. digitatum* on some pome fruit cultivars were linked to old and over-mature fruit (Louw and Korsten, 2014; Vilanova *et al*., 2014). Fruit physiology was not evaluated in this chapter, but it was observed that fruit age played a similar role in the pathogenicity tests of *P. digitatum* on nectarine and plum. This was particularly noted when comparing interactions on freshly picked nectarines (cvs. Sunburst and Sunlite) to older fruits used in trial repeats (long stored) (cvs. NE 3-48-49, Bright Pearl and African Pride). Recently picked nectarines inoculated with *P. digitatum* showed smaller lesions and very low disease incidences. Disease incidence on NE 3-48-49 increased from 20 to 80% due to 1d prolonged cold storage. Bright Pearl used to compare different environmental isolates were stored 4d longer in the trial repeat, resulting in the disease incidence increasing from 0 to 20% (citrus isolate) and 0 to 60% (pear isolate). Likewise, Bright Pearl used to compare aggressiveness on different cultivars caused a disease incidence shift from 0 to 40%. *Penicillium solitum* was also affected by fruit age (cvs. Sunburst, Sunlite and African Pride), but to a lesser extent than *P. digitatum*. The remainder of the *Penicillium* spp. evaluated in this chapter were not affected by fruit age. Future work will focus on the influence of fruit maturity and ripeness on host defence mechanisms and decay

caused by these pathogens. The significance of such research would depict pathogenic profile shifts as fruit mature and ripen.

Fruit can ripen during extended distribution systems since it remains a challenge to ensure and maintain consistent control of temperatures in cold chains (Freiboth *et al*., 2013; Haasbroek, 2013; Maheshwar and Chanakya, 2006). Storage or transport of fruit above their optimal pulp temperature (-0.5°C for most stone fruits) can facilitate ripening and shorten shelf life (Kader, 2011; Kader and Mitchell, 1989; PPECB, 2013). Over-mature and riper fruit will be more susceptible to decay (Kader, 2011; Vilanova *et al*., 2014). This opens an opportunity for postharvest pathogens, especially those that require riper fruit to infect and cause rapid decay (i.e. *P. digitatum*).

Handling of citrus, pome and stone fruits in close proximity anywhere along the fresh produce chain can further contribute to decay caused by *P. digitatum* on these fruit types. The potential of cross-contamination and cross-infection taking place cannot be avoided. In SA, the start of the stone fruit export season overlaps with the end of the citrus export season and intersects with the pome fruit export season (PPECB, 2013). Inoculum loads tend to increase as seasons progress. High inoculum levels of *P. digitatum* can thus be present in the fresh produce chain at the end of the citrus season. Inoculum levels can be even higher if sanitary practices are neglected. These aspects can contribute to decay caused by *P. digitatum* on stone fruits, especially at the end of the export chain when fruits can be riper. Little to nothing is known of *P. digitatum* decay of stone fruits in the fresh produce chain. The causal agents of decay are rarely identified or identified based on symptom expression. Further research is needed to isolate and associate *P. digitatum* with postharvest losses in the fresh produce chains of stone fruits. The next two chapters will deal with host-pathogen interactions of *P. digitatum* and *P. expansum* on a single plum and nectarine cultivar. This will serve as a model system to better understand the infection and colonisation of *P. digitatum* on its newly confirmed hosts.

5. CONCLUSION

All the *Penicillium* spp. included in the study proved pathogenic on all of the nectarine and plum cultivars evaluated. Aggressiveness decreased from *P. digitatum*, *P. expansum*, *P. crustosum* to *P. solitum* for most cultivars. This is the first report demonstrating the pathogenicity of *P. digitatum*, *P. crustosum* and *P. solitum* on plum and *P. solitum* on nectarine. Novel information was added to the aggressiveness and symptom expression of

each species on nectarine and plum. Decay caused by *P. digitatum* was more prominent on older fruit (days postharvest). Higher disease incidence and more severe symptoms were found on fruits stored for longer periods of time. *Penicillium digitatum* was highly aggressive, causing rapid decay and completely covering fruits with green conidia within 5d incubation at ambient conditions. *Penicillium digitatum* can be described as a concern to the stone fruit industry, especially towards the end of the season. Since stone fruits can be handled and stored in the same environment as citrus or pome fruits during fruit exports, cross-contamination can be expected if high levels of decay occur in the same environment. Stone fruits, like nectarine and plum, are also prone to be riper at the end of the export chain. Little information is available on *P. digitatum* in the stone fruit industry and the causal agents of symptomatic fruit are rarely identified in the export market (decay is only noted). This study highlights the importance to isolate and correctly identify pathogens in the postharvest environment or at the market or retail level. There is also a need to evaluate pathogenicity on fruit at different ripeness levels and on a spectrum of cultivars before conclusive statements can be made about postharvest pathogens and losses in fresh produce chains. The significance of such research would depict pathogen profile shifts as fruit move through extended export chains and integrate with other fruit types during storage and distribution where cross-contamination can take place.

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Chapter 4

Impact of ripeness on the infection and colonisation of *Penicillium digitatum* and *P. expansum* on plum

ABSTRACT

Penicillium digitatum was identified a postharvest pathogen of nectarine and plum in the previous chapter. Although little is known of this host-pathogen association, an increase in disease occurrence and severity was noted on riper fruit. This chapter aimed to determine the effect of ripening on the infection and colonisation of *P. digitatum* and *P. expansum* on plum at a physical (disease incidence/severity, pH and firmness) and molecular (gene expression) level. Storage conditions and inoculum loads were also considered in the host-pathogen associations. Disease incidence and severity of *P. digitatum* was significantly affected by ripeness, cold storage and inoculum load. Both species caused acidification of infected tissue and advanced ripening of healthy (uncolonised) tissue of infected plum. Only expression of the polygalacturonase genes varied between the species (*P. digitatum*: increased; *P. expansum*: decreased) based on incubation time (24h vs 48h). Few molecular changes were noted with *P. digitatum* (only *ACCD* decreased) due to ripeness. *Penicillium expansum* expressed higher levels of *pacC* and *creA* on riper fruit. The potential of a dual mechanism of pH modulation was discussed; higher pH at and beyond lesion borders will facilitate invasion, maceration and colonisation (nutrient uptake and growth) by/during acidification. The pH of lesions was comparable to the natural pH of the host. Alkalinisation accomplished via ammonium/ammonia accumulation can also be linked to the pathogen's nitrogen metabolism. Host ripening directly (elicited) or indirectly (ethylene stress) caused by pathogen attack can increase the pH of uncolonised tissue. *Penicillium digitatum* can be considered an important pathogen of riper fruit often found in long or ill-managed distribution chains. It is still unclear what stimulates (molecular) the opportunistic lifestyle expressed by *P. digitatum* on plum. There was little to no correlation between the expression of genes and increased disease incidence and severity on riper fruit. Future work should consider the decline of host resistance during ripening. More *in vivo* research is needed to validate the dual mechanism of pH modulation.

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1. INTRODUCTION

Plum is globally one of the top 10 most produced deciduous fruits. Fresh exports are mainly directed to Russia, Germany and the United Kingdom, and reached 754 234 metric tons in 2016 (HORTGRO, 2017). Plums are climacteric fruit with high ethylene production and relative perishability, wound easily and is susceptible to numerous postharvest pathogens (Crisosto and Mitchell, 2011; Kader, 2011). Several *Penicillium* spp. affect the postharvest quality of plum (Chapter 3: Louw and Korsten, 2016).

Penicillium expansum Link is a well-known pathogen that contributes to postharvest losses of plum (Pitt and Hocking, 2009; Snowdon, 2010). *Penicillium digitatum* (Pers.) Sacc. was previously described as a postharvest pathogen with a narrow host range, mainly restricted to citrus (Frisvad and Samson, 2004; Stange *et al*. 2002). Complex trade systems lead to extensive handling and long storage of multiple fruit types in central facilities, increasing the risk of exposing fruits to high inoculum levels of various pathogens. *Penicillium digitatum* has been identified in the pome and stone fruit environments (Ma *et al.*, 2003; Scholtz and Korsten, 2016) and recently shown to be pathogenic on both fruit types (Louw and Korsten, 2014; Chapter 3: Louw and Korsten, 2016). Although *P. digitatum* has never been described as a concern to either of these fruit export industries it was found highly aggressive on older or riper fruit.

Fruit ripening leads to numerous physiological changes. Changes in sugar, pH and antifungal compounds (decline) have shown to significantly affect host susceptibility and fungal pathogenicity (Prusky, 1996; Prusky *et al.*, 2016). Under these conditions, infecting pathogens are more able to secrete small effector molecules to modulate environmental pH to an optimal state. This will allow upregulation of genes involved in the production of specific pathogenicity factors (Prusky *et al.*, 2016).

Penicillium expansum and *P. digitatum* are acidifying pathogens. They release organic acids and utilise ammonium to modulate environmental pH. Ideal pH conditions will upregulate the production and secretion of mycotoxins and pectolytic enzymes such as polygalacturonase (PG) (Prusky *et al.*, 2004; Sánchez-Torres and González-Candelas, 2003; Yao *et al.*, 1996; Zhang *et al.*, 2013). It was recently suggested that the availability of carbon is a key factor to trigger the production and secretion of small pH-modulating molecules (ammonia and organic acids) (Prusky *et al.*, 2016). A link was also made between the colonisation of *P. digitatum* and *P. expansum* and the ethylene production of their hosts

(Barad *et al.*, 2016b; Chalutz and Lieberman, 1977; Jia *et al.*, 1999). Examining the correlation between physiological changes due to ripening and expression of pathogenicity/virulence genes associated with such changes (i.e. pH, sugar content, ethylene levels) can prove useful to identify and understand the mechanism/s associated with the increased disease incidence and severity of *P. digitatum* on riper fruit.

Much research has been done to study the host-pathogen interaction of *P. expansum* on apple and *P. digitatum* on citrus (Barad *et al.*, 2016b; López-Pérez *et al.*, 2015). Information on the pathogenicity of *P. digitatum* on previously thought non-hosts (pome and stone fruits) is recent and limited. This chapter aims to determine the effect of ripening on the infection and colonisation of *P. digitatum* and *P. expansum* on plum at a physical (disease incidence/severity, pH and firmness) and molecular (gene expression) level. The impact of cold storage and inoculum load on infection and disease development will also be evaluated.

2. MATERIALS AND METHODS

Fruit source and handling. 'Fortune', a Japanese plum (*Prunus salicina* Lindl.) cultivar, was selected based on seasonality and availability. Fruit was collected from an organic farm in the Waterberg region of the Limpopo Province. It was harvested in 2015 and 2016 at a mature stage based on commercial industry guidelines (DAFF, 2015). Fruit was placed in cooler boxes, transported to UP plant pathology laboratories and placed into cold storage (5.26±0.52°C; 83.16±2.51% RH) upon arrival. Fruit was removed from cold storage late afternoon to climatise overnight for inoculation the following day. Four ripeness levels were selected for this chapter (Argenta *et al*., 2003); 1d, 4d, 8d and 12d postharvest. Fruit was ripened by incubation under ambient conditions (25.50±0.67°C; 59.89±2.81% RH) over the number of required days prior to inoculation.

Fungal cultures. *Penicillium digitatum* and *P. expansum* isolates originated from chapter 3 (Louw and Korsten, 2016). They were previously isolated from symptomatic fruit, purified (single-spore isolation) and preserved in sterilised water. Cultures were grown on malt extract agar (MEA) (Merck, Johannesburg, South Africa) at 25°C for 5–7d in darkness prior to conidial harvest. Conidial suspensions were prepared in sterilised Ringer's solution (Merck) amended with 0.05% Tween 80 (Associated Chemical Enterprises, Johannesburg). Conidial concentrations were determined using a haemocytometer.

Fruit physiology. Fruit physiology was determined on each day of inoculation. Three fruit at each ripeness level was used as representatives for measuring fruit physiological indices. Fruit weight (g), firmness (kg), sugar content (°Brix), pH, titratable acidity (TA) (% malic acid) and sugar/acid ratio (°Brix/TA) were determined. Fruit firmness was measured using a Turoni TR 53205 penetrometer (T.R. Turoni S.R.L., Forli, Italy) with a 5mm diameter stainless steel cylindrical probe. The sugar content was determined from fruit juice (blended) using an ATAGO® pocket refractometer (Labex, Johannesburg). The pH was determined from fruit juice using a Hanna® HI1131 electrode connected to a Hanna HI2210 pH meter (Hanna Instruments, Johannesburg). The TA was determined by titrating 10 ml juice with 0.1 mol L^{-1} NaOH. Phenolphthalein was used as indicator.

Effect of fruit ripeness on infection and colonisation of *Penicillium* spp. Fruit (2015) was surface sterilised by dipping into 0.5% sodium hypochlorite (NaOCl) solution for 5min, double rinsed by dipping into sterile tap water (≥5min each) and allowed to air dry. Fruit inoculation was similar to that described by Louw and Korsten (2014; 2016). Thirty fruit from each ripeness level (1d, 4d and 8d postharvest) were wounded (1.5 x 3mm) on opposite sides (two wounds; each on a side) using a sterile micropipette tip (20–200µl). Ten fruit per ripeness level were inoculated (20µl of 10^5 conidia/ml) with *P. digitatum*, *P. expansum* or control solution (sterile Ringer's solution with 0.05% Tween 80) at the wound sites. Fruit was randomised on a disinfected table and incubated at ambient conditions (25.34±0.64°C; 61.31±3.63% RH) for 5d. Horizontal and vertical (calyx axis vertical) lesion diameters were recorded 2d, 4d and 5d post-inoculation. Some fruit (≥3) were incubated longer to study advanced symptom development. The trial was completed in triplicate.

The multifactorial effect of fruit ripeness, inoculum load and cold storage on the infection and colonisation of *Penicillium* spp. Conidial suspensions, fruit sterilisation and wounding were as previously described. Suspensions were diluted to obtain 10^6, 10^5 and 10^4 conidia/ml. Five fruit (2016) from each ripeness level (1d, 4d, 8d and 12d postharvest) were inoculated with each concentration of *Penicillium* spp. or control solution for each storage condition. Fruit for ambient storage was randomised and incubated for 5d as previously described. Fruit for cold storage (5.71±0.90°C; 89.05±2.55% RH) was randomised in single layer open top boxes placed (closed but not sealed) in sterilised semi-transparent white plastic bags and incubated for 26d. Bags were used to prevent/reduce excessive water loss and cross-contamination. Lesion diameters were recorded as described earlier. Symptom development was noted throughout the incubation processes. The trial was repeated.

Firmness and pH of infected sites over time. Fifteen fruit (2015) from each ripeness level (1d, 4d and 8d postharvest) were wounded and inoculated with each *Penicillium* spp. (10^5 conidia/ml) and control solution. Preparation of conidial suspensions, fruit sterilisation, wounding, inoculation, randomisation and incubation (ambient) were as described earlier. The pH and firmness of three inoculated fruit were measured every day over a 5d incubation period for each ripeness level and *Penicillium* spp. The pH was measured by directly placing a Hanna FC200 pH electrode (Hanna Instruments) connected to a Hanna HI2210 pH meter into the inoculated sites. Tissue firmness was measured using a penetrometer by directly pressing the 5mm diameter stainless steel cylindrical probe into inoculated sites (skin not removed). The trial was done in triplicate.

Absolute quantification of regulatory genes. Up to twenty fruit (2016) from each ripeness level (1d, 4d, 8d and 12d postharvest) were wounded and inoculated with each *Penicillium* spp. (10^5 conidia/ml) and control solution. Preparation of conidial suspensions, fruit sterilisation, wounding, inoculation, randomisation and incubation (ambient) were as described earlier. Infected and healthy (control) tissue from inoculated sites were isolated 24h and 48h post-inoculation using a sterilised 8mm diameter cork borer. Samples were snap frozen in liquid nitrogen and stored below -72°C. The trial was repeated to obtain three biological replicates for each *Penicillium* spp. and control for all ripeness levels.

Samples from 1d and 12d postharvest fruit were selected to continue the gene expression analysis. Isolated discs were pooled and ground using a sterilised and liquid nitrogen cooled KCG201S coffee grinder (Kambrook, China). Total RNA was extracted from each replicate using 100–150mg homogenised tissue according to RNeasy® Plant Mini Kit (Qiagen®, Hilden, Germany) specifications. Initial RNA quantity was evaluated using the Qubit® 2.0 Fluorometer (Invitrogen™, Life Technologies™, Carlsbad, CA, USA) with Qubit RNA HS Assay Kit (Invitrogen™, Life Technologies™, USA). RNA analysis was conducted using the Agilent 2100 bioanalyzer (Agilent Technologies, Inc., Palo Alto, CA, USA). 50ng of total RNA was used for cDNA synthesis according to iScript™ Advanced cDNA Synthesis Kit for RT-qPCR (Bio-Rad Laboratories, Inc., Hercules, CA, USA) specifications. Thermocycler conditions for cDNA synthesis were 30min at 42°C for reverse transcription (RT) and 5min at 85°C for RT inactivation.

Although the use of reference genes and normalisation is not a specific requirement for droplet digital PCR (ddPCR) (Zmienko *et al.*, 2015), it was deemed necessary due to the

nature of the experiment. This was accomplished by standardising input RNA (50ng) for cDNA synthesis and normalising data of target genes against a reference gene. Specific pathogen genes were targeted in the study. Gene-specific primers (Table 4.1) for *PG*, 1-aminocyclopropane-1-carboxylic acid deaminase gene (*ACCD*), *pacC*, *creA* and *β-actin* (reference) were designed using Primer 3+ software (Untergasser *et al.*, 2007). Primers were designed for potential multiplexing using EvaGreen technology in a QX200 AutoDG ddPCR system. These genes were selected based on their association with pH modulation (Prusky *et al.*, 2004; Prusky *et al.*, 2016; Sánchez-Torres and González-Candelas, 2003; Yao *et al.*, 1996; Zhang *et al.*, 2013) and fruit ripening (Barad *et al.*, 2016b; Chalutz and Lieberman, 1977; Jia *et al.*, 1999). Additional samples were kept for RNA sequencing (future work). Due to the high sensitivity and specificity of the ddPCR system, only two technical replicates were included for each biological replicate.

Table 4.1 Primer sets used for gene expression analysis of *Penicillium* spp.

Primers	Forward (5'–3')	Reverse (5'–3')	Target
Pg2Pd	agcctgaccaactccaacat	ctccttagcgccatcgatac	*PG* of *P. digitatum*; synthesis of PG
Pg1Pe	aaaggcaggttgctccagta	aggccagaccagtcaaatcc	*PG* of *P. expansum*; synthesis of PG
ACCDPd	cggttcttgtttgtgctgtg	ccttcctcttcgcgtcct	*ACCD* of *P. digitatum*; ethylene biosynthesis
ACCDPe	acggtgcttgtttgtgctgt	gcctcaacagtggcagaag	*ACCD* of *P. expansum*; ethylene biosynthesis
PacCPd	ccggtgagctactgccttg	caggttgaggttgttggtgct	*PacC*: C2H2 transcription factor of *P. digitatum*; pH regulation
PacCPe	ggacatttcccaggatagca	gatagagcggggtcaatcag	*PacC*: C2H2 transcription factor of *P. expansum*; pH regulation
CreAPd	cgcaagtagagcgagacgaccaca	tgcatacgcggaaagcgaagg	*CreA*: C2H2 transcription factor of *P. digitatum*; carbon regulation
CreAPe	cgcattcaaacgatgacgatgatggct	aggaaggagcagtggagttgggtg	*CreA*: C2H2 transcription factor of *P. expansum*; carbon regulation
βaP	cttcccgatggacaggtcat	tggataccgccagactcaag	*β-actin* of *P. digitatum* and *P. expansum*; reference genes

The work-flow for the QX200 AutoDG ddPCR system includes four main steps; PCR set up with supermix, droplets generation, thermal cycling, and droplet reading. PCR reactions were set up using QX200 ddPCR EvaGreen Supermix (Biotium, Inc., Bio-Rad Laboratories, Inc., Hercules, CA, USA) in ddPCR 96-well PCR plates and sealed in a PX1 PCR Plate

Sealer (Bio-Rad Laboratories, Inc., USA) according to manufacturer's instructions. Droplets were generated in the QX200 AutoDG (Bio-Rad Laboratories, Inc., USA). Generated samples were sealed and placed into a C1000 Touch thermal cycler (Bio-Rad Laboratories, Singapore). PCR cycle conditions were one cycle of 5min at 95°C (enzyme activation), 40 cycles of 30sec at 95°C (denaturation) and 1min at 54.5°C (annealing/extension), and one cycle of 5min at 4°C and 5min at 90°C (signal stabilisation). Samples were held at 4°C before being placed into a QX200 Droplet Reader (Bio-Rad Laboratories, Inc., USA). Data were analysed using QuantaSoft™ Software (Bio-Rad Laboratories, Inc., USA).

Reisolation, preservation and identification. Isolations from two symptomatic fruit from each experiment of the ripeness trials were performed for each *Penicillium* sp. Isolates were plated on MEA and incubated as described earlier. Cultures were assessed for morphological similarity once sufficient growth had occurred. Cultures were purified (single spore isolation), preserved (cryo-preservation) and identified via DNA sequencing of *β-tubulin* as described by Louw and Korsten (2015). The identity of cDNA sequences was confirmed prior to downstream application. Sequences analysis was conducted using the ABI3500/3500XL Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). DNA sequences of additional *Penicillium* isolates assist in expanding the culture collection and, if selected, the database of the Microflex™ Matrix-Assisted Laser Desorption/Ionisation Time-of-Flight Mass Spectrometry (MALDI-TOF MS) (Bruker Daltonik GmbH, Bremen, Germany) system used at UP for rapid identification of species.

Statistical design and data analysis. The trial evaluating the multifactorial effect of fruit ripeness, inoculum load and cold storage was conducted twice. The remainder of the trials were conducted three times. Individual fruit were randomised according to a complete randomised design with factorial arrangement. Four measurements were taken per fruit to determine lesion diameter (two inoculation sites, each with horizontal and vertical diameter measurements). These measurements were averaged and regarded as a replicate. Data were subjected to analysis of variance (ANOVA) using Statistical Analysis System (SAS) (version 9.4; SAS Institute Inc., Cary, NC, USA). Bartlett's test for homogeneity was used to disclose similarity among repeat of trials (independent experiments). Trial repeats were pooled if not significantly different ($P > 0.05$). Means were separated using Fisher protected Least Significant Difference.

3. RESULTS

Fruit physiology. Fruit indices of trial repeats were not significantly different (2015: P = 0.08–0.78, except firmness P = 0.001; 2016: P = 0.24–0.92). Only firmness showed fruit from 2015 became significantly riper (P = 0.03). All fruit indices, except sugar/acid ratio (P = 0.13), showed fruit from 2016 became significantly riper (P = 0.0004–0.04). Firmness was the most sensitive method to prove ripening (Fig. 4.1). Firmness decreased, °Brix and pH increased while the remainder of the indices stayed relatively consistent over the incubation period.

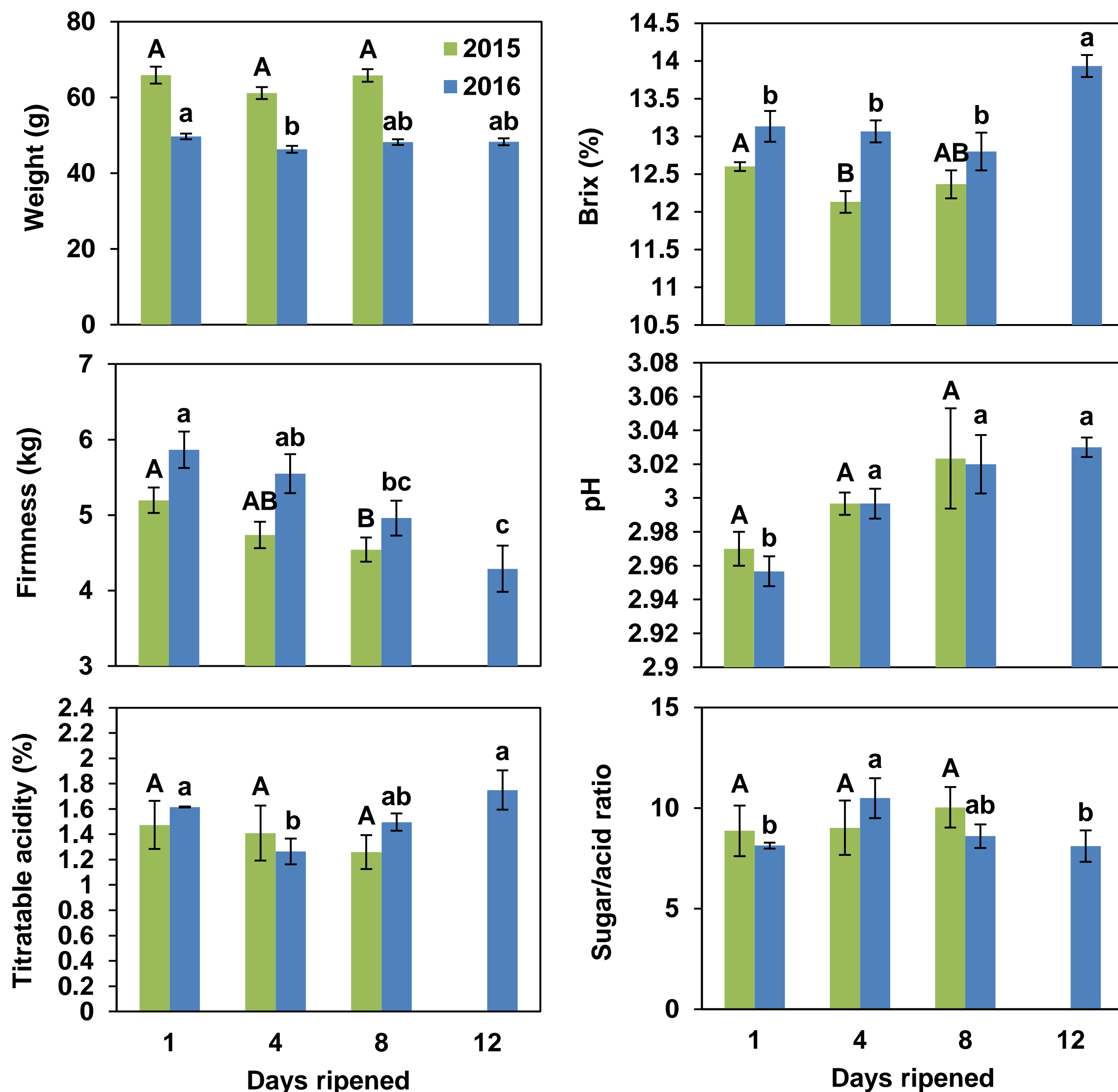

Fig. 4.1 Fruit indices of plum (cv. Fortune) ripened at ambient conditions (**1,** 1d postharvest; **4,** 4d postharvest; **8,** 8d postharvest; **12,** 12d postharvest). Vertical bars indicate standard error. Different letters (only letters of similar case are comparable) indicate treatments that are significantly different ($P < 0.05$) based on Fisher protected Least Significant Difference. Fruit from 2015 were not ripened to 12d postharvest.

Effect of fruit ripeness on infection and colonisation of *Penicillium* spp. Trial repeats were not significantly different ($P > 0.86$). The interaction between ripeness and *Penicillium* spp. was significantly different ($P < 0.0007$). *Penicillium digitatum* was more aggressive than *P. expansum* at all ripeness levels (Fig. 4.2). Decay caused by *P. expansum* was not affected by the ripeness of the fruit, whereas the rate of decay caused by *P. digitatum* significantly increased on riper fruit. Disease incidence of *P. expansum* was 100% for all ripeness levels. Disease incidence for *P. digitatum* increased on riper fruit; 68.33±10.41% (1d postharvest), 88.33±7.64% (4d postharvest) and 77.25±7.83% (8d postharvest). Mycelia of *P. digitatum* on 1d and 4d ripened fruit were first observed 3d post-inoculation but not on 8d ripened fruit within the 5d incubation period. The species produced lime green conidia only after 5d incubation (Fig. 4.3B). Mycelia and conidia of *P. expansum* on 1d, 4d and 8d ripened fruit were observed after 3d incubation. Less mycelia and conidia were however detected on fruit ripened for 8d.

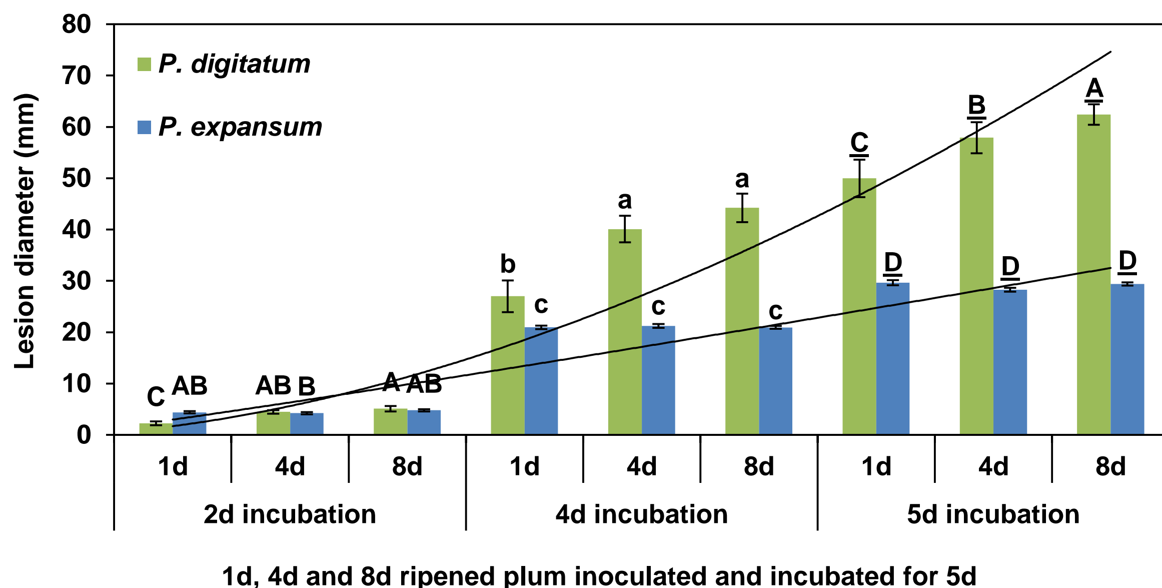

Fig. 4.2 Lesion growth of *Penicillium digitatum* and *P. expansum* on plum (cv. Fortune) of different fruit ripeness levels over 5d ambient incubation. Wounds have been subtracted from lesion diameters. Vertical bars indicate standard error. Different letters (only letters of similar case and underlining are comparable) indicate treatments that are significantly different ($P < 0.05$) based on Fisher protected Least Significant Difference.

Fig. 4.3 Plum (cv. Fortune) inoculated with *Penicillium* spp. and incubated at ambient conditions. **A,** 4d ripened fruit incubated for 5d (left) and 8d (right); **B,** 1d ripened fruit incubated for 10d.

The multifactorial effect of fruit ripeness, inoculum load and cold storage on the infection and colonisation of *Penicillium* spp. Trial repeats were not significantly different ($P > 0.74$). The interaction between ripeness, storage condition, inoculum load and *Penicillium* spp. was significantly different ($P = 0.005$). The largest lesions were caused by *P. digitatum* under ambient conditions and were greatly affected by all variables (Fig. 4.4). Lesion diameter increased as fruit ripened and inoculum load increased but cold storage significantly retarded lesion and symptom development. Optimal conditions (most ripe fruit and highest inoculum load) were needed for *P. digitatum* to cause lesions of similar size to *P. expansum* under cold storage. *Penicillium expansum* was less affected by ripeness and less sensitive to cold storage (largest lesions). The effect of inoculum load on *P. expansum* was more clearly observed under cold storage conditions. Disease incidence for *P. digitatum* varied, increasing as ripeness and inoculum load increased (Table 4.2). Disease incidence was again 100% for *P. expansum* at all variables.

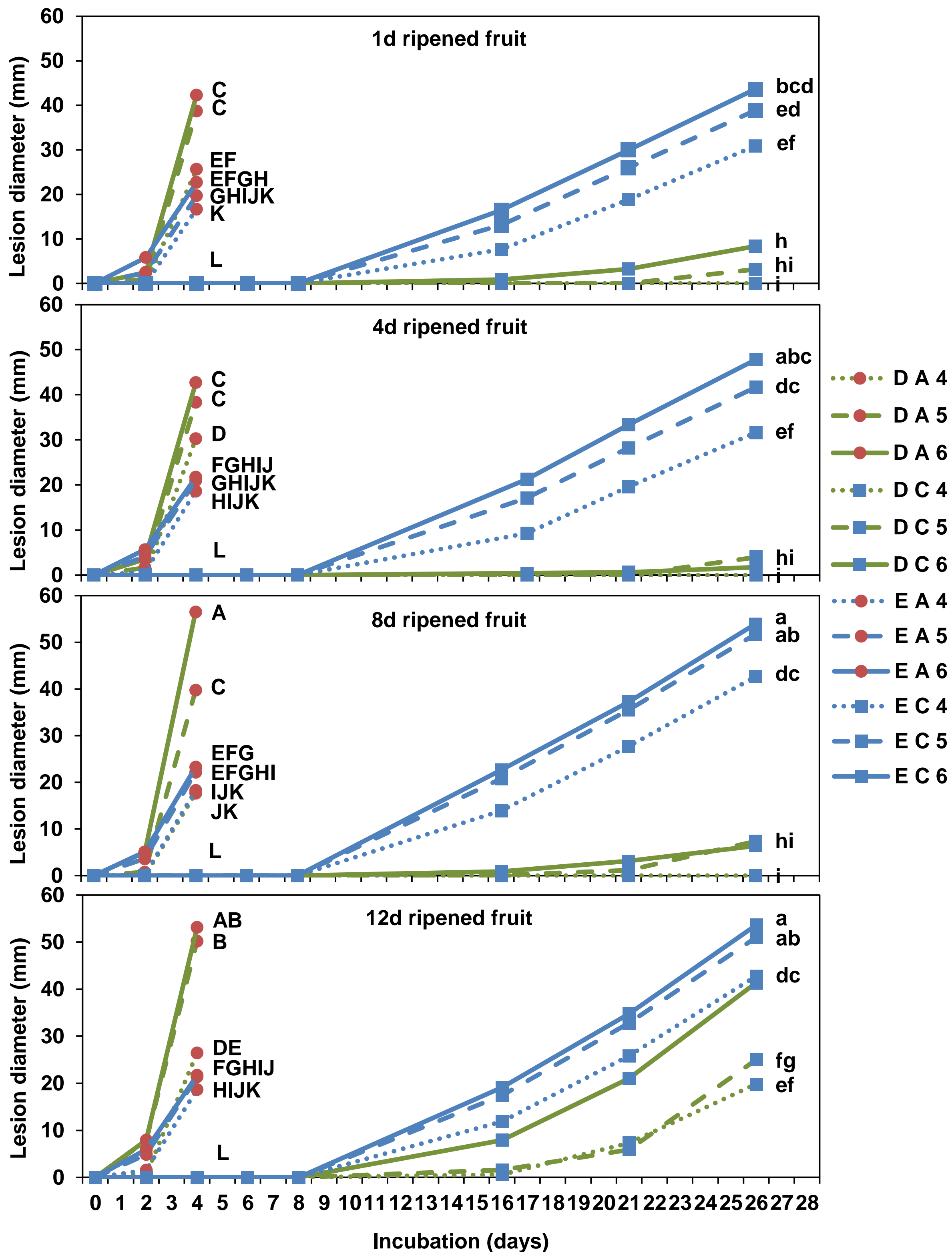

Fig. 4.4 Lesion diameter caused by different concentrations (**4–6**) of *Penicillium digitatum* (**D**) and *P. expansum* (**E**) on plum (cv. Fortune) at ambient (**A**) and cold (**C**) conditions. **4,** 10^4 conidia/ml (200 conidia); **5,** 10^5 conidia/ml (2 000 conidia); **6,** 10^6 conidia/ml (20 000 conidia). Wounds have been subtracted from lesion diameters. Different letters (only letters of similar case and ripeness are comparable) denote treatments that are significantly different (P < 0.05) based on Fisher protected Least Significant Difference.

Table 4.2 Disease incidence (%) of *Penicillium* spp. under different storage conditions, inoculum loads and plum (cv. Fortune) ripeness levels

Penicillium spp.	Storage	Inoculum (conidia/ml)	Ripeness (days postharvest)			
			1d	4d	8d	12d
P. digitatum	Ambient	10^4	20	30	25	65
		10^5	75	80	70	100
		10^6	90	85	90	100
	Cold	10^4	0	0	0	30
		10^5	25	10	35	45
		10^6	75	30	85	90
P. expansum	Ambient/ Cold	$10^4/10^5/10^6$	100	100	100	100

Firmness and pH of infected sites over time. Trial repeats were not significantly different based on firmness measurements from 2–5d incubation (*P* = 0.74–0.99). The interaction between ripeness and *Penicillium* spp. was significantly different based on firmness. The 2d incubation was identified as the best to observe the effect of ripeness on the firmness of infected sites (*P* = 0.03). Firmness of *P. digitatum* infected sites decreased faster on riper fruit (Fig. 4.5). This was less obvious with *P. expansum* infected sites as firmness dropped at a relatively similar rate for all ripeness levels. Firmness of control fruit remained high.

Trial repeats were not significantly different based on pH measurements from 2–5d incubation (*P* = 0.43–0.86). Ripeness significantly affected the pH of infected sites. This was observed after 1d (*P* = 0.0007), 3d (*P* = 0.04) and 5d (*P* = 0.006) incubation. Overall, pH at infection sites of riper fruit increased faster compared to that of less ripe fruit. pH at wound sites of control fruit barely increased over the incubation period.

Infected fruit ripened much quicker than uninfected fruit. The infected fruit was not only darker in colour (Fig. 4.3A), but the pH of uncolonised tissue (no decay) was much higher. The pH of uncolonised tissue was measured a maximum distance away from inoculated sites of 5d incubated fruit. The pH values of uncolonised tissue of fruit inoculated with *P. digitatum* were: 1d ripened = 4.87±0.12; 4d ripened = 3.62±0.29; 8d ripened = 3.24±0.21. For *P. expansum* it was: 1d ripened = 4.72±0.12; 4d ripened = 3.84±0.41; 8d ripened = 3.78±0.47. The pH increased the further measurements were taken away from decayed tissue, thus the larger the lesion the smaller the pH difference between colonised and

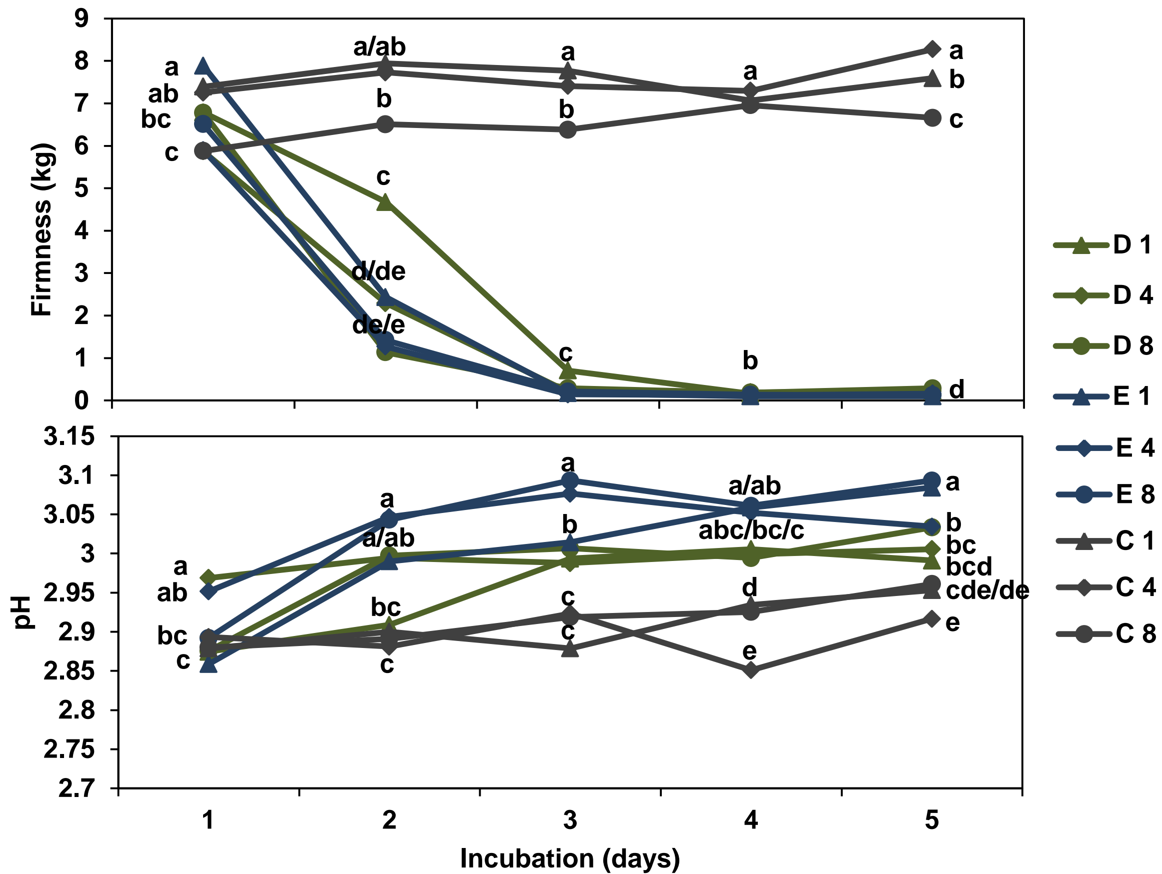

Fig. 4.5 Firmness and pH of lesions caused by *Penicillium digitatum* (**D**) and *P. expansum* (**E**) on plums (cv. Fortune) of different ripeness levels (**1,** 1d ripened; **4,** 4d ripened; **8,** 8d ripened) over 5d incubation. **C,** control. Different letters (only letters in the same day are comparable) show significant differences ($P < 0.05$) for each day based on Fisher protected Least Significant Difference.

uncolonised tissue. In the case of the larger lesions of *P. digitatum,* the difference between colonised and uncolonised tissue became less than what was observed with *P. expansum*. Most *P. digitatum*-inoculated fruit were completely colonised after 5d incubation (max lesion diameter). The pH anywhere within the lesion border remained low and similar to that at the inoculation site; this was particularly observed with *P. expansum* (deviated ±0.01 or ±0.02).

Absolute quantification of regulatory genes. Independent experiments were not significantly different based on the expression of genes ($P = 0.44$–0.90). Expression values for the target genes were high but that of the reference gene was higher, resulting in negative log values of normalised data (Fig. 4.6). From this data, expression of *PG* was significantly higher for *P. digitatum* whereas expression for *ACCD* and *creA* was significantly higher for *P. expansum*. *PacC* was similar ($P = 0.55$). The ripeness of fruit did not have a significant effect on gene expression ($P = 0.26$–0.92). For *P. digitatum*, *PG* and *ACCD* significantly

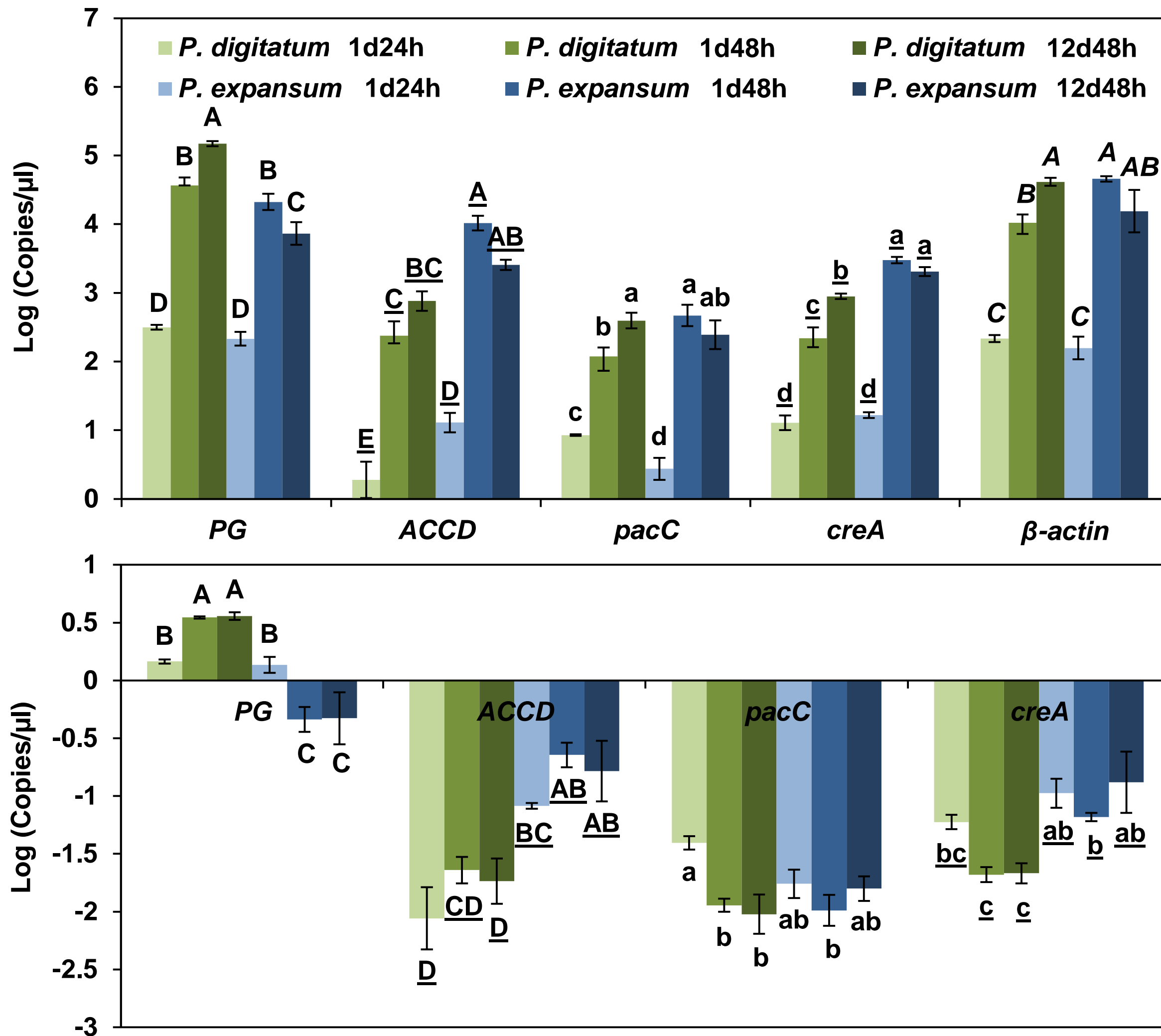

Fig. 4.6 Gene expression of *Penicillium digitatum* and *P. expansum* when inoculated in plum (cv. Fortune) of different ripeness levels (1d and 12d postharvest) after 24h and 48h incubation. Raw data (top) was normalised (bottom) using the reference gene (*β-actin*). Vertical bars indicate standard error. Different letters (only letters of similar case, underlining and italicising are comparable) indicate treatments that are significantly different ($P < 0.05$) based on Fisher protected Least Significant Difference.

increased and *pacC* and *creA* significantly decreased over incubation time. In the case of *P. expansum*, *PG* significantly decreased, *ACCD* significantly increased, and *pacC* and *creA* were relatively unaffected.

Reisolation, preservation and identification. Cultures from symptomatic fruit were successfully grouped. Representative isolates were positively identified as the original species inoculated into fruit using NCBI standard nucleotide BLAST of *β-tubulin*. Similarity (identity) was 99–100% with query cover of 99–100%. Pure cultures were stored in the *Penicillium* culture collection at UP.

4. DISCUSSION

Penicillium digitatum did not only infect but also caused large lesions at all stages of fruit ripeness. The connection between decay caused by *P. digitatum* on older fruit was originally not as prevalent on plum (Chapter 3: Louw and Korsten, 2016). In this chapter, a significant difference in lesion diameter and disease incidence for *P. digitatum*, but not *P. expansum*, was noted due to host ripeness. Chapter 3 was the first report to demonstrate decay caused by *P. digitatum* on plum. There, decay was associated with older or end market fruit but fruit indices were not measured since the focus was to determine pathogenicity and assess the aggressiveness of different *Penicillium* spp. on different plum and nectarine cultivars (Louw and Korsten, 2016). In this chapter, the link between disease incidence and severity, and fruit age was demonstrated.

Expanding on the concept (Louw and Korsten, 2014; 2016), it was hypothesised that the correlation between decay caused by *P. digitatum* on riper fruit would be more obvious on certain plum cultivars as shown here and in chapter 3 or nectarine as revealed in chapter 5. The association was postulated to be linked to the acidity of the host as described by Prusky *et al.* (2004). Plums are generally more acidic than nectarines (pH 2.80–4.45 vs pH 3.92–4.18) (US FDA/CFSAN, 2007) and pH is an important regulatory factor during infection and colonisation of *P. digitatum* and *P. expansum* (Barad *et al.*, 2016b; López-Pérez *et al.*, 2015; Zhang *et al.*, 2013). Prusky *et al.* (2002; 2004) reported enhanced *Penicillium* spp. colonisation in host tissue with low pH and larger lesions caused by *P. expansum* on more acidic apple cultivars. This would also explain why *P. digitatum* was able to cause larger lesions on ‘Granny Smith’ apples [more acidic (Keller *et al.*, 2004)] compared to other apple cultivars (Louw and Korsten, 2014).

Prusky *et al.* (2004) reported *P. expansum* causing larger lesions on Granny Smith (pH 3.45 at the inoculation site) than on ‘Rome’ (pH 3.77) and ‘Fuji’ (pH 4.46), but at the same time *pepg1* (endopolygalacturonase gene) accumulation was the highest in cultures grown on media with a pH 4 and much lower at pH < 3.5. The activity of PG isolated from *P. expansum* ranged from pH ~3 to <6.5 and was determined optimal at pH 4–5.5 (*in vitro*) (Jurick *et al.*, 2010; Yao *et al.*, 1996). Jurick *et al.* (2009) reported the activity for PG isolated from *P. solitum* between pH >3 to >7 but optimal at pH 4–4.5. In fact, a few well-known genes involved in the pathogenicity process of *Penicillium* spp. were overexpressed on alkaline/neutral media (pH 7) compared to acidic media (pH 4) (Barad *et al.*, 2016b).

Based on *in vitro* work, larger lesions on less acidic cultivars or at least on cultivars with pH ≥ 3.5 would be expected. How is this applicable to fruit with pH < 3.5 or as in our case pH 3?

Our study is the first to demonstrate pH 2.9–3.1 for tissue colonised by *P. expansum* and *P. digitatum.* Few reports reveal *P. expansum* lowering the pH of colonised tissue <3.5 and none as low as what has been reported by *P. digitatum* on citrus. The pH of lesions caused by *P. digitatum* on 'Navel' oranges = 3.12±0.07, 'Oro Blanco' grapefruit = 3.10±0.14 (7d incubation) (Prusky *et al.*, 2004) and *Citrus unshiu* = 3.22 ±0.15 (4d incubation) (Zhang *et al.*, 2013). It was confirmed that pH modulation depends on nutritional (*in vitro*) and host (*in vivo*) conditions (Bi *et al.*, 2016).

Transcriptome analysis of pH-regulatory genes from *P. expansum* colonised apple tissue revealed a far different gene expression profile compared to that from cultures (pH 4 and 7). This was expected, but the low expression of fungal genes in clusters 3 and 6 from cultures compared to colonised tissue was not. Cluster 3 included genes involved in patulin biosynthesis and amidase activity (possible means of ammonium production). Cluster 6 included genes associated with host-cell-wall degradation; important for virulence and functionalities associated with pathogenicity. Genes and activities included chitinase-associated genes, aspartic endopeptidase-pep1 encoding genes, and pectin lyase (degrade pectin polymers) and PG (tissue maceration) activities (Barad *et al.*, 2016b). Aspartic endopeptidase is associated with pathogenicity and pH modulation of *P. digitatum* in citrus (López-Pérez *et al.*, 2015). It contributes to fungal colonisation by degrading host cell-wall components (serves as nitrogen source) or inactivates defence proteins (Naumann and Price, 2012). These findings point out the differences between *in vitro* and *in vivo* work in gene expression studies and the incomplete understanding of the mechanisms involved in infection and colonisation of host tissue by *Penicillium* spp.

Barad *et al.* (2016a) indicated that although *pacC* is overexpressed on media pH 7 and not on media pH 4.5, local ammonification at the leading edge of lesions contributed to the activation of *pacC* responsiveness in acidic tissue (pH 4). *PacC* plays a significant role in the activation of D-gluconic acid (pH modulation), and regulation of pathogenicity and secondary metabolic processes (i.e. patulin) (Barad *et al.*, 2016a). Li *et al.* (2015) pointed out that the expression of patulin biosynthesis genes of *P. expansum* also depends on specific nutritional growth conditions. The role of nutritional conditions (carbon and nitrogen source)

and activity of defensive genes on host acidification, and pathogenicity and virulence of *Penicillium* spp. add complexity.

The pH measurements of infected plum tissue in this study revealed similarities with findings by Barad *et al.* (2016a). They inoculated 'Golden Delicious' apples with *P. expansum* and measured pH at the inoculated site, an intermediate site, leading edge of the lesion and healthy tissue after 5d incubation. The pH of healthy tissue was 4 compared to 3.58 at the inoculated site, mainly due to high accumulation of gluconic acid. The pH at the leading edge of lesions was ~3.75 with much higher concentrations of ammonia. Barad *et al.* (2016a) were not specific where healthy tissue was taken from these *P. expansum* colonised apples, but pH 4 is higher than expected from freshly harvest Golden Delicious apples (pH 3.64) (Keller *et al.*, 2004). This corresponded with findings in this chapter. Although the pH of control fruit was low (pH < 3), that of uncolonised tissue of infected plums were much higher (pH of 1d ripened fruit after 5d incubation: *P. digitatum* = 4.87±0.12 and *P. expansum* = 4.72±0.12). Comparing this to pH 3.01 and 3.08 (respectively) at inoculated sites, acidification of colonised plum tissue could be confirmed.

The pH at the infection sites of *P. digitatum* and *P. expansum* were similar to the pH of control (uninfected) plum fruit. This was also observed from Barad *et al.* (2016a) as the pH 3.58 at the inoculation site is similar to that of freshly harvested Golden Delicious apples [pH 3.64 (Keller *et al.*, 2004)] after 5d incubation. Prusky *et al.* (2004) reported on pH values at inoculated sites (*P. expansum*) of various apple cultivars incubated for 7d; Fuji = 3.96, 'Gala' = 3.88, Golden Delicious = 3.88, Granny Smith = 3.64, 'Red Delicious' = 4.07. Unfortunately, they never reported pH values of control fruit (uninfected), only of healthy (uncolonised) tissue from the inoculated fruit. Keller *et al.* (2004) provide pH values for these cultivars (freshly harvested); Fuji = 3.91, Gala = 3.86, Golden Delicious = 3.64, Granny Smith = 3.42 and Red Delicious = 4.10. According to this, *P. expansum* held the pH of colonised tissue close to the initial pH of the cultivar it infected, even 7d after inoculation. This indicates that acidification by *Penicillium* spp. can be used to maintain pH of colonised tissue at a level similar to that of the particular cultivar or host.

Similar to this chapter, the pH measurements of uncolonised tissue of inoculated apples were much higher than that of tissue at the inoculated sites (Δ_{pH} = 0.31–0.88) and freshly harvested fruit (Δ_{pH} = 0.34–0.9) [comparing pH data from Keller *et al.* (2004) with Prusky *et al.* (2004)]. The accumulation of ammonia increased towards the lesion border but not in

healthy uncolonised tissue (Barad *et al.*, 2016a). Ammonium concentrations were higher (x2.7–5.4) in healthy compared to decay tissue of *P. expansum* infected apples. Similar observations were reported for *P. digitatum* on Oro Blanco grapefruit. On the other hand, organic acids (citric and gluconic) were much higher in decayed compared to healthy tissue of *P. expansum* infected apples and *P. digitatum* infected grapefruit (Prusky *et al.*, 2004). This indicates that while the *Penicillium* spp. utilise the ammonia/ammonium (nitrogen metabolism) in colonised tissue, they cause the production of more of it at the point of colonisation (leading edge of the lesion). At the same time, they maintain an acidic environment within colonised tissue while pH of uncolonised tissue increases [Δ_{pH} = 0.31–0.88 on apples (Prusky *et al.*, 2004) and Δ_{pH} = 1.64–1.86 on plums]. The pH of uncolonised tissue of infected apples, citrus (Prusky *et al.*, 2004) and plums were respectively 3.95–4.77, 4.55–4.77 and 4.72–4.87, close to the optimum (pH 5) for the accumulation of organic acids (citric) as revealed from cultures (Prusky *et al.*, 2004).

Barad *et al.* (2016a) observed a dual pattern of pH modulation (alkalinisation and acidification) from *P. expansum* during long periods of culturing (up to 10d) under dynamic nutritional growth conditions. Alkalinisation at lesion boarders was due to ammonia accumulation (Barad *et al.*, 2016a). Increase in pH ahead of colonisation (uncolonised tissue) can be due to enhanced ripening or senescence directly (elicited) or indirectly [host stress (Glick, 2014)] caused by *Penicillium* attack. Fruit ripening will in return affect host physiology (i.e. sugar levels) and susceptibility (Prusky *et al.*, 2016). Ammonia accumulation is affected by carbon source. High amounts of ammonia accumulated when *P. expansum* was grown on secondary medium containing 15mM sucrose but not on medium with 175mM. At the same time, no gluconic acid (pH increased >6) was produced at 15mM but high amounts were found at 175mM (pH decreased to ~4) (Bi *et al.*, 2016). The higher pH at and beyond lesion borders will thus facilitate invasion, maceration and colonisation (nutrient uptake and growth) via acidification. With acidification comes the pathogenicity and virulence enhancing factors related to it. This somewhat depicts a dual mechanism of pH modulation. It is unclear whether the *Penicillium* spp. are purposefully increasing pH by the production (via digestion) of ammonia and increased ripening or whether it is a derivative due to pathogen attack and its metabolism.

Ammonium is a preferred nitrogen source of *Penicillium* spp. (Ross and Luckner, 1984). It enhances *pacC* responsiveness, enables the use of pectolytic enzymes which causes tissue maceration (Barad *et al.*, 2016a) and induces expression of *mepB*, *CuAO* and *ACCD* (Barad

et al., 2016b). MepB is involved in the regulation of nitrogen metabolism. CuAO enhanced accumulation of H_2O_2, contributing to reactive oxygen species (ROS) leading to cell damage and necrosis (Barad *et al.*, 2016b; Song *et al.*, 2014). ACCD is a deaminase that is induced by 1-aminocyclopropane-1-carboxylic acid (ACC) (precursor of ethylene) accumulation. It cleaves ACC, leading to the production of ammonia and α-ketobutyrate (Glick, 2014; Jia *et al.*, 2000).

In our study, *PG* and *ACCD* were upregulated while *pacC* and to a lesser extent *creA* were downregulated over time (24h vs 48h incubation) by *P. digitatum*. Only *ACCD* was upregulated by *P. expansum* (remainder went down). Expression of *PG* was much lower in *P. expansum* compared to *P. digitatum* after 48h. The host environment was already ideal (i.e. acidity, nitrogen and carbon availability) for *P. digitatum* to rapidly transcribe for *PG* and *ACCD*. While *PG* expression increases under acidic conditions (pH 3.5-5.0) (Prusky *et al.*, 2004; Yao *et al.*, 1996), expression of *ACCD* and *pacC* should decrease (*in vitro*) (Barad *et al.*, 2016a). Citrus (*C. unshiu*; peel pH 4.64 ±0.13) inoculated with *P. digitatum* showed an increase in *pacC* and *PG* expression from 24h to 48h. *PacC* expression was low under acidic conditions (pH 3–6) and when glucose was the sole carbon source (*in vitro*) (Zhang *et al.*, 2013).

Expression of *ACCD* and *pacC* can increase when ammonium/ammonia concentrations are high, even under acidic conditions. However, *ACCD* expression is much less affected by pH and more by ammonium/ammonia levels than *pacC* (Barad *et al.*, 2016a; 2016b). The fact that *creA* was downregulated could indicate that sucrose levels were initially low, corresponding to the lower °Brix value of fresh fruit (1d postharvest). This will be more ideal for *Penicillium* to increase ammonia levels (Bi *et al.*, 2016) and thus also *ACCD*. Sucrose levels will increase as plum cultivars ripen (Sudar *et al.*, 2011). ACCD can further increase ammonia levels depending on ACC availability (Glick, 2014; Jia *et al.*, 2000).

Different biosynthetic pathways can be used by *P. expansum* and *P. digitatum* to produce ethylene. Recent *in vitro* work showed that ACC was not linked to ethylene production of *P. digitatum* and *P. expansum* (Yang *et al.*, 2017). There was also no correlation between the presence and production of ethylene and colony diameter on PDA. Conidia formation of *P. expansum* was however affected by ACC. These processes are dependent on growth conditions and although ethylene was revealed unimportant for *Penicillium* growth, it and its precursors can be used to advance host ripening.

The beneficial interaction of plant growth-promoting bacteria that produce ACCD is well reported (Glick, 2014). Some pathogens also produce this enzyme; *P. citrinum* (Jia *et al.*, 2000) and *P. expansum* (Barad *et al.*, 2016b). As mentioned previously ammonia produced by the cleavage of ACC can be used by the pathogen as a nitrogen source to modulate environmental pH and regulate certain genes, pectolytic enzymes and toxins (Barad *et al.*, 2016a; 2016b). The upregulation of *ACCD* in this chapter corresponds with the increased ripening of infected fruit (uncolonised tissue) probably as a result of ACC synthesised by *P. digitatum* and *P. expansum* (Barad *et al.*, 2016b; Yang *et al.*, 2017) and the host due to stress caused by infection (stress ethylene) (Glick, 2014). Under stress conditions, plants synthesise a large amount of ACC which will be converted to ethylene and intensify the stress effect (i.e. senescence, chlorosis and leaf abscission). Decreasing ACC levels with ACCD will thus alleviate the effect of the stress (Glick, 2014) unless used by the pathogen to advance growth and/or attack (nitrogen source). This provides some indication for the increase in pH of infected sites (ammonia accumulation) (Barad *et al.*, 2016a) and uncolonised tissue (ripening) of inoculated plum as revealed in our study. More research is needed to identify the significant shift in lesion size caused by *P. digitatum* on riper fruit. Host defence and measurement of ammonium/ammonia levels should be considered.

Why did *PG* of *P. expansum* decrease over time? Little information is available on the change in *PG* expression of *P. expansum* over time. Sánchez-Torres and González-Candelas (2003) reported differential expression for different PG genes and *in vitro* vs *in vivo* experiments. There was no expression of *pepg1* (similar *PG* to ours) but decreasing expression of *pepg2* (24h, 48h and 72h) under *in vitro* conditions with apple pectin as carbon source. With infected and heat treated apples (Golden Delicious; pH 4.1), *pepg1* was not expressed at 24h and similarly expressed (very low) at 48h and 72h whereas *pepg2* decreased over time. Differential expression of *pepg1* and *pepg2* could have been due to differences in the environmental pH (Sánchez-Torres and González-Candelas, 2003).

Ripeness had little (slight decrease in *ACCD*) to no effect on the expression of *P. digitatum* genes. This is beside the fact that lesions were smaller (38.7mm vs 50.2mm), and disease incidence (75% vs 100%) and the reference gene lower (less biomass) in fresh fruit (1d postharvest) as compared to riper fruit (12d postharvest). °Brix significantly increased, giving the idea that expression of *creA* would increase for both species and not only for *P. expansum* (Bi *et al.*, 2016). The difference between the sugar/acid ratio of 1d and 12d postharvest fruit was nonsignificant and pH of lesions didn't show much movement (slight

increase) while infection and colonisation took place. These findings indicate that although *P. expansum* and *P. digitatum* share similarities when infecting, it is apparent that some mechanisms are vastly different, even on the same host. Also, the mechanism behind the infection and colonisation of *P. digitatum* on very acidic and previously thought non-hosts such as plum is different compared to its less acidic known hosts (i.e. citrus peel) (Zhang *et al.*, 2013).

Ripeness led to higher expressions of *pacC* in *P. expansum* (*PG* and *ACCD* unaffected). The lower expression of *ACCD* from *P. digitatum* (significant) and *P. expansum* (nonsignificant) could indicate a decrease in ammonium/ammonia levels. It also corresponds with the higher sugar levels and upregulation of *creA* as higher sucrose levels can lead to less ammonia produced by *P. expansum* (*in vitro*) (Barad *et al.*, 2016a; Bi *et al.*, 2016). Fortune is a cultivar that produces sucrose and fructose but not glucose or sorbitol (Roussos *et al.*, 2015). Although an increase in sucrose leading to a decrease in ammonia could mean a downregulation of *pacC*, the slightly higher pH could have resulted in it being upregulated as (unlike *ACCD*) it is heavily affected by pH and less by ammonium/ammonia levels (Barad *et al.*, 2016a). The upregulation of *ACCD* on 1d postharvest fruit from 24h to 48h and downregulation on 12d postharvest fruit (48h) indicates that more ACC will be synthesised in fresh fruit and not necessarily in riper fruit. The riper fruit was possibly already at a state where there wasn't a need to produce more ACC (i.e. host environment already favourable).

There was no correlation between lesion diameter and *PG* expression on plum over the 48h incubation period. With *P. expansum*, lesions became larger over time but *PG* was downregulated. Lesions caused by *P. digitatum* on riper fruit were larger but *PG* expression was unchanged. This can be linked to findings that *P. expansum* caused larger lesions on more acidic cultivars (pH 3.45 vs pH 3.77 and pH 4.46) but *PG* (*pepg1*) expression is lower on media with pH < 3.5 compared to pH 4 (Prusky *et al.*, 2004). It is unclear what is causing the larger lesions on riper fruit. Other enzymes, enzyme activity, mycotoxins, pathogen growth stimulation, induced senescence, lack of host resistance are all factors that can contribute.

Organic acids, ammonium/ammonia and carbon source play a significant role in the infection and colonisation of *Penicillium* spp. These and potentially many other factors function in synergy for pathogenesis to result. Host-pathogen interactions are immensely complex and to a great degree still not properly understood. As a result, many studies only

investigated the effect of these factors independently or *in vitro*. Although understandable and very helpful, the findings from *in vitro* experiments can be vastly different from that observed *in vivo* (Barad *et al.*, 2016b; López-Pérez *et al.*, 2015; Sánchez-Torres and González-Candelas, 2003). This makes it difficult to draw parallels when trying to understand the regulation and expression of certain genes. This is particularly true for necrotrophic pathogens as comparing *in vitro* with *in vivo* is like comparing saprophytic with pathogenic life stages. It will lead to difficulties when trying to understand the infection and colonisation of living and changing host cells. This is why Barad *et al.* (2016b) found contradicting results in clusters 3 and 6. These genes (some associated with pathogenicity and virulence) were upregulated in colonised apple tissue but downregulated *in vitro* (pH 4 and pH 7).

Some pathogens become significantly more aggressive on riper fruit, emphasising the risk related to these species late in the fresh produce chain when fruit are prone to be riper. *Penicillium digitatum* has been observed as one such pathogen. As pointed out in this chapter, cold storage and inoculum load (facility hygiene) had a greater impact on *P. digitatum* than on *P. expansum* on plum. If managed correctly, it could prove sufficient to control *Penicillium* green mould of plum. However, the sensitivity of *P. digitatum* towards cold storage when infecting and colonising plum was not shared on citrus (Louw and Korsten, 2015). Low concentrations of *P. digitatum* (6.3 x 10^4 conidia/ml) were still able to infect and cause decay (lesion diameter = 43.8±5.6mm) of 'Eureka' seeded lemons over 26d cold storage (5.0±0.7°C and 86.4±4.5% RH). Exposing these fruit types to one another in the same facilities will thus increase the risk of losses, especially for plum as it receives no control treatment against *P. digitatum*. This is especially a concern in countries where citrus dominates the market. Disease control at the end of long distribution chains or towards the end of the season could be adapted to lower the risk of opportunistic interactions, such as described in this study, taking place. Future studies should therefore determine the presence and impact of *P. digitatum* in fresh produce chains of plum.

5. CONCLUSION

This is the first study demonstrating the effect of ripeness on the infection and colonisation of *P. digitatum* and *P. expansum* on plum. Fruit ripeness only affected the decay potential of *P. digitatum*. Disease incidence and severity significantly increased as plums became riper. Low storage temperature and inoculum loads prevented or greatly reduced disease caused by

P. digitatum. *Penicillium expansum* was the least affected by these factors. Disease incidence was unaffected but disease severity was greater as inoculum concentrations increased. Combining uninterrupted cold storage regimes of freshly harvested plums will effectively control *P. digitatum* if inoculum loads are low. This is however not the case with *P. expansum*, more targeted and effective control measures are required. This is the first study to use ddPCR to quantify the expression of genes in postharvest pathology of fruit. Normalisation was required as the biomass of pathogens isolated from infected tissue will vary depending on host susceptibility (i.e. affected by ripeness) and incubation time. This chapter highlighted the potential of a dual mechanism of pH modulation; higher pH at and beyond lesion borders to facilitate invasion, maceration and colonisation (nutrient uptake and growth) by/during acidification. Acidification primarily via secretion of organic acids and uptake of ammonium/ammonia maintain an acidic environment similar to the initial pH of the host. Alkalinisation, if facilitated by the pathogen, can be accomplished by the accumulation of ammonia. The most probable reason for the increase in pH of uncolonised tissue can be due to advanced host ripening directly (elicited) or indirectly (ethylene stress) caused by the pathogen. Although not tested, the importance of ammonium/ammonia (nitrogen source, pH modulation and gene regulation) for gene expression under acid conditions was highlighted. Ripeness had little to no effect on the expression of *P. digitatum* genes even though lesion size and disease incidence significantly increased as fruit ripened during longer storage (days postharvest). The high expression of *PG* could provide insight into the rapid decay caused by *P. digitatum* compared to *P. expansum*. However, no correlation between lesion size and gene expression indicates that other factors are playing a more important role. Differences in gene expression profiles of *P. digitatum* and *P. expansum* were evident. Difference in expression of *PG* was most significant over time with *pacC* and *creA* being most significant over ripeness level. There is a link between organic acids, and ammonium/ammonia and sugar levels in the infection process of these *Penicillium* spp. During infection pH and sugar is lower (low sugar lead to an increase in ammonia) but as colonisation takes place (acidification) accelerated ripening will follow which can lead to the increase of pH and sugar of uncolonised tissue (less favourable for ammonia production). All indicators point to a different mode of action for *P. digitatum* when infecting and colonising plum. Other host and environmental factors are expected to play a part. Further investigation is needed to understand the opportunistic life strategy utilised by *P. digitatum* on plum.

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Chapter 5

Impact of ripeness on the infection and colonisation of *Penicillium digitatum* and *P. expansum* on nectarine

ABSTRACT

Very few studies have investigated the host-pathogen interaction of *Penicillium* spp. on nectarine. *Penicillium digitatum* was identified as being pathogenic and highly aggressive on nectarine and plum in chapter 3. A strong association was made to host age/ripeness. This points to a new mechanism or life strategy used by *P. digitatum* to infect and colonise previously thought non-hosts. The aim of this chapter was to determine the effect of nectarine ripeness on the infection and colonisation of *P. digitatum* and *P. expansum* at a molecular and physical level. The impact of environmental conditions (cold storage) and pathogen pressure (inoculum load) was also investigated. Although disease incidence was much lower, lesions caused by *P. digitatum* was similar in size to *P. expansum* on freshly harvested nectarine. Disease incidence and lesion diameter significantly increased (larger than *P. expansum*) on riper fruit. Cold storage had the largest effect on *P. digitatum*. Inoculum load had a meaningful effect on both *Penicillium* spp. Ripeness significantly affected pH modulation and gene expression. The pathogens not only decreased but also increased and maintained (similar to initial pH of the host) pH of infected tissue. The polygalacturonase gene (*PG*) and *creA* were upregulated by *P. digitatum* on riper fruit (other genes unaffected). It partly explains the larger lesions caused on riper fruit. A different expression profile was observed from *P. expansum*; strong downregulation in *PG* and slight upregulation in *pacC*. Very different life strategies were used by the two *Penicillium* spp. when infecting nectarine. Unlike what is known on citrus, *P. digitatum* showed an opportunistic lifestyle that takes advantage of specific host and environmental conditions. It was unclear what specifically trigger/s the increase in disease incidence (infection) and lesion diameter (colonisation) of *P. digitatum* on riper fruit. The downregulation of *PG* by *P. expansum* corresponds with other studies but requires further investigation. The role of mycotoxins in *P. expansum* infection and colonisation could provide some answers. The differences between *in vivo* and *in vitro* studies make it difficult to directly correlate results. Further research is still needed to differentiate and understand the infection and colonisation of these pathogens on the same host.

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1. INTRODUCTION

Globally, over 20.73 million metric tons of nectarines and peaches were produced during 2016/17 (USDA, 2017). Nectarines are climacteric fruit with high relative perishability. The fruit wound easily and have a range of pathogens that can infect it and contribute to losses (Crisosto and Mitchell, 2011; Kader, 2011). Pathogenic *Penicillium* spp. of nectarine include *P. expansum* Link, *P. crustosum* Thom, *P. digitatum* (Pers.) Sacc. and *P. solitum* Westling (Chapter 3: Louw and Korsten, 2016). *Penicillium expansum* is a recognised concern to this industry (Pitt and Hocking, 2009; Snowdon, 2010) but little is known of *P. crustosum* and *P. digitatum* even though they pose a realistic threat in the export chain (Louw and Korsten, 2016; Navarro *et al.*, 2011; Restuccia *et al.*, 2006). Lesions caused by *P. solitum* were small (Chapter 3: Louw and Korsten, 2016).

Decay linked to *P. crustosum* is understandable as it is a pathogen with a broad host range (Pitt and Hocking, 2009). Decay caused by *P. digitatum* and the severity thereof was less expected. This species, closely associated with citrus (Frisvad and Samson, 2004; Stange *et al*. 2002), was recently identified as being highly aggressive on pome and stone fruits. In this case, it was able to cause much larger lesions than *P. expansum* within a shorter period of time (Louw and Korsten, 2014; 2016). *Penicillium digitatum* is also known to be present in these fruit environments (Ma *et al.*, 2003; Scholtz and Korsten, 2016). Disease incidence and severity can be higher on older or riper fruit (Louw and Korsten, 2014; Chapter 3: Louw and Korsten, 2016).

Riper fruit will not only be physiologically more favourable (i.e. carbon and nitrogen levels, pH changes) but also more susceptible (weaker host defence) for infection and colonisation (Prusky, 1996; Prusky *et al.*, 2016). These changes will affect host-pathogen interactions (Bi *et al.*, 2016; Prusky *et al.*, 2016). *Penicillium* spp. are described as acidifying pathogens via the release of organic acids and uptake of ammonium. Modulating environmental pH via this manner was connected to the upregulation of pathogenicity and virulence factors. The production and secretion of secondary metabolites such as mycotoxins and pectolytic enzymes lead to cell necrosis and tissue maceration of the host (Prusky *et al.*, 2004; Sánchez-Torres and González-Candelas, 2003; Yao *et al.*, 1996; Zhang *et al.*, 2013).

Host ripeness and the production of ethylene or its precursors were shown to play an important role during the infection and colonisation of *P. digitatum* and *P. expansum* (Barad *et al.*, 2016b; Chalutz and Lieberman, 1977; Jia *et al.*, 1999; Marcos *et al.*, 2005).

Understandably, previous fruit-*Penicillium* interaction research focussed on apple-*P. expansum* and citrus-*P. digitatum.* Very little research has been done on stone fruits. The new host-pathogen association of *P. digitatum* on nectarine gives reason to further investigate the mechanism/s involved in this interaction. The aim of this chapter is to determine the effect of host ripeness (nectarine) on the infection and colonisation of *P. digitatum* and *P. expansum* at a molecular and physical level. The impact of environmental conditions (cold storage) and pathogen pressure (inoculum load) will also be investigated.

2. MATERIALS AND METHODS

Fruit origin and handling. 'Sunlite' nectarine (*Prunus persica* (L.) Batsch var. *nucipersica* (Suckow) C. Schneider) was selected based on availability. Fruit originated from a commercial farm in the Waterberg region in the Limpopo Province. Fruit harvested during the 2016 growing season was harvest mature [industry guidelines (DAFF, 2013)] and untreated (postharvest). Fruit was collected, transported to UP plant pathology laboratories and placed in cold storage (5.26±0.52°C; 83.16±2.51% RH). It was removed late afternoon to climatise overnight for trial inoculation the next day. Fruit was allowed to ripen at ambient conditions (23.79±0.61°C; 65.69±9.14% RH) to produce three different ripeness levels (1d, 4d and 7d postharvest).

***Penicillium* cultures.** Cultures of *P. digitatum* and *P. expansum* originated from chapter 3 (Louw and Korsten, 2016). These isolates were made from symptomatic fruit, plated on malt extract agar (MEA) (Merck, Biolab Diagnostics, Johannesburg, South Arica), single-spore isolated, preserved in sterilised distilled water and stored at ambient temperature. Isolates were cultured on MEA and incubated at 25°C for 5–7d. Conidia were harvested in sterilised Ringer's solution (Merck) with 0.05% Tween 80 (Associated Chemical Enterprises, Johannesburg). A haemocytometer was used to determine conidial concentrations.

Fruit indices. Three replicates were used to determine fruit indices. This was done prior to inoculation for each ripeness level. Weight (g), firmness (kg), sugar content (°Brix), pH, titratable acidity (TA) (%) and sugar/acid ratio were of interest. Firmness was determined using a Turoni TR 53205 penetrometer (T.R. Turoni S.R.L., Forli, Italy) fitted with a 5mm stainless steel cylindrical probe. Sugar content was measured from blended fruit juice (whole) using an ATAGO® pocket refractometer (Labex, Johannesburg). The same juice was used to measure pH with a Hanna® HI1131 electrode coupled to a Hanna HI2210 pH meter (Hanna Instruments, Johannesburg). Ten ml juice with 0.1 mol L^{-1} NaOH and

phenolphthalein as indicator was used to determine TA and expressed in malic acid (%). Sugar/acid ratio was determined by °Brix/TA.

Effect of fruit ripeness on infection and colonisation of *Penicillium* spp. Fruit was dipped into 0.5% sodium hypochlorite (NaOCl) for ±5min for surface sterilisation. Thereafter, it was rinsed (x2) via dipping into sterilised tap water (5min each) and allowed to air dry on a disinfected table. Inoculation of fruit was as described in previous chapters. The fruit (10 replicates for each ripeness level and *Penicillium* spp.) were wound-inoculated via pipetting 20µl conidial suspension (10^5 conidia/ml) or control solution (sterile Ringer's solution with 0.05% Tween 80) into wound sites (1.5 x 3mm). Two wounds for inoculation were made on opposite sides of each fruit using a sterilised micropipette (20–100µl). Fruit was randomised and incubated for 5d at ambient conditions (24.00±0.62°C; 68.32±7.20% RH). Lesion sizes were recorded after 2d, 4d and 5d incubation by measuring the horizontal and vertical (calyx axis vertical) lesion diameters. Advanced symptom development was recorded from fruit that incubated for longer. The trial was conducted three times.

Effect of fruit ripeness and inoculum load on decay. Conidial suspensions, and sterilisation and wounding of fruit were completed as described earlier. Initial suspensions were prepared at 10^6 conidia/ml and diluted to 10^5 and 10^4. Five replicates were used for each combination of ripeness level, *Penicillium* spp. and conidial concentration (control included). Fruit was randomised and incubated under ambient conditions for 5d. The recording of lesion diameters was as described earlier. The number of days required for mycelia and conidia formation was noted. The trial was repeated (conducted twice).

Effect of inoculum load and cold storage on decay. Conidial suspensions, and sterilisation and wounding of fruit were completed as described earlier. Five replicates were used for each combination of storage condition, *Penicillium* spp. and conidial concentration (control included). For ambient storage, fruit was randomised and incubated on a disinfected table for 5d and results were recorded as described earlier. For cold storage, fruit was randomised on a disinfected trolley and incubated in a cold room (5.26±0.52°C; 83.16±2.51% RH). Results were recorded every fifth day from 16–31d post-inoculation. The development of symptoms was noted during the incubation time. The trial was repeated.

Firmness and pH of infected sites. Fifteen fruit for each ripeness level and *Penicillium* spp. were wound-inoculated (10^5 conidia/ml). Control fruit was included. Sterilisation, wounding, randomisation and incubation (ambient) of fruit and preparation of conidial

suspensions were as described earlier. Of the fifteen inoculated fruit for each combination of ripeness level and *Penicillium* spp. or control, three fruit were used for each day over a 5d incubation period to measure firmness and pH at inoculated sites. Firmness was measured with a penetrometer by directly piercing the inoculated site. The skin at inoculated sites was not removed prior to measurements. The pH was measured using a Hanna FC200 pH electrode (Hanna Instruments) fitted to a Hanna HI2210 pH meter by direct placement into an inoculated site. The pH at uncolonised areas (max distance away from inoculated sites) was also measured. The trial was conducted three times.

Absolute quantification of genes. Up to twenty fruit were wound-inoculated with each *Penicillium* spp. (10^5 conidia/ml) or control solution for each ripeness level. Sterilisation, wounding, randomisation and incubation (ambient) of fruit and preparation of conidial suspensions were as described earlier. Healthy (control) and infected tissue at the inoculated sites were sampled after 24h and 48h incubation. A sterilised cork borer (8mm diameter) was used to isolate inoculated sites. Tissue samples were immediately dropped into liquid nitrogen to snap freeze. Samples were placed in sterilised Bijoux or McCartney bottles and stored ≤ -72°C. The trial was repeated three times to produce three biological replicates for each combination of ripeness level and *Penicillium* spp. or control.

Samples from 1d and 7d postharvest fruit were selected for processing. Other and additional samples were kept in storage for possible future work. The selected samples were ground in a sterilised and cooled (liquid nitrogen) KCG201S coffee grinder (Kambrook, China). 100–150mg homogenised tissue was used for total RNA extraction by means of the RNeasy® Plant Mini Kit (Qiagen®, Hilden, Germany). Total RNA quantity was determined with the Qubit® 2.0 Fluorometer (Invitrogen™, Life Technologies™, Carlsbad, CA, USA) using the Qubit RNA HS Assay Kit (Invitrogen™, Life Technologies™, USA). RNA analysis was done using the Agilent 2100 bioanalyzer (Agilent Technologies, Inc., Palo Alto, CA, USA). cDNA synthesis was done with 1µg total RNA using the iScript™ Advanced cDNA Synthesis Kit for RT-qPCR (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Cycle conditions for reverse transcription were 42°C for 30min and 85°C for 5min.

The same primers used in chapter 4 were used for gene expression analysis (Table 4.1). The polygalacturonase gene (*PG*), 1-aminocyclopropane-1-carboxylic acid deaminase (*ACCD*), *pacC* and *creA* were selected based on their association with fruit ripening (Barad *et al.*, 2016b; Chalutz and Lieberman, 1977; Jia *et al.*, 1999) and/or pH modulation (Prusky *et al.*,

2004; Prusky *et al.*, 2016; Sánchez-Torres and González-Candelas, 2003; Yao *et al.*, 1996; Zhang *et al.*, 2013). Although reference genes and normalisation is not mandatory for droplet digital PCR (ddPCR) (Zmienko *et al.*, 2015), the previous chapter deemed it necessary due to the nature of our experiments. *β-actin* was included as reference gene. Only two technical replicates were needed for experiments due to the high sensitivity and low error of the QX200 AutoDG ddPCR system (Bio-Rad Laboratories, Inc., USA).

The QX200 AutoDG ddPCR system includes four main steps: set up of PCR reactions in a ddPCR 96-well PCR plate, generation of droplets in the QX200 AutoDG, thermal cycling, and absolute quantification of samples in the QX200 Droplet Reader. QX200 ddPCR EvaGreen Supermix (Biotium, Inc., Bio-Rad Laboratories, Inc., Hercules, CA, USA) was used to set up PCR reactions. Samples were 10 or 10^2 diluted depending on expression levels. The detection limit of ddPCR depends on the number of negative droplets. A PX1 PCR Plate Sealer (Bio-Rad Laboratories, Inc., USA) was used to seal plates before and after droplet generation. A C1000 Touch thermal cycler (Bio-Rad Laboratories, Singapore) was used for amplification. Cycle conditions included one cycle at 95°C for 5min (enzyme activation), 40 cycles at 95°C for 30sec (denaturation) and 54.5°C for 1min (annealing/extension), and one cycle at 4°C for 5min and 90°C for 5min (signal stabilisation). Samples were held at 4°C. Thereafter, it was transferred to the droplet reader. The QuantaSoft™ Software (Bio-Rad Laboratories, Inc., USA) was used for data analyses.

Reisolation, preservation and identification. Two isolates for both *Penicillium* spp. were made from symptomatic fruit for each experiment from the ripeness and inoculum load trials. The isolates were cultured on MEA, purified (single spore isolation), identified via DNA sequencing (*β-tubulin*) and preserved in sterilised 10% glycerol stored at or below -70°C (cryo-preservation) as described by Louw and Korsten (2015). The identity of the cDNA sequences was also confirmed prior to downstream application. Sequences analysis was conducted with the ABI3500/3500XL Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). The DNA Sequencing Facility of the Faculty of Natural and Agricultural Sciences at UP was used to confirm species identity. The preserved isolates with DNA sequence identity were added to the *Penicillium* culture collection at UP.

Statistical design and data analysis. Trials where different inoculum concentrations were used, were completed twice. All other trials were repeated three times. Randomisation was done according to the complete randomised design. Each inoculated fruit produced four

pseudoreplicates (horizontal and vertical lesion diameter measurements from two inoculated sites) which were averaged into a single rep. Statistical Analysis System (SAS) (version 9.4; SAS Institute Inc., Cary, NC, USA) was used to determine the analysis of variance (ANOVA). The similarity between trial repeats was determined using Bartlett's test for homogeneity. In the case of nonsignificant difference ($P > 0.05$), trial repeats were pooled. Fisher protected Least Significant Difference was used to separated means.

3. RESULTS

Fruit indices. Fruit indices for trial repeats were not significantly different ($P = 0.22$–0.85). All, except sugar ($P = 0.37$), indicated fruit became significantly riper ($P < 0.003$). Weight, firmness and TA decreased while pH and sugar/acid ratio increased (Fig. 5.1).

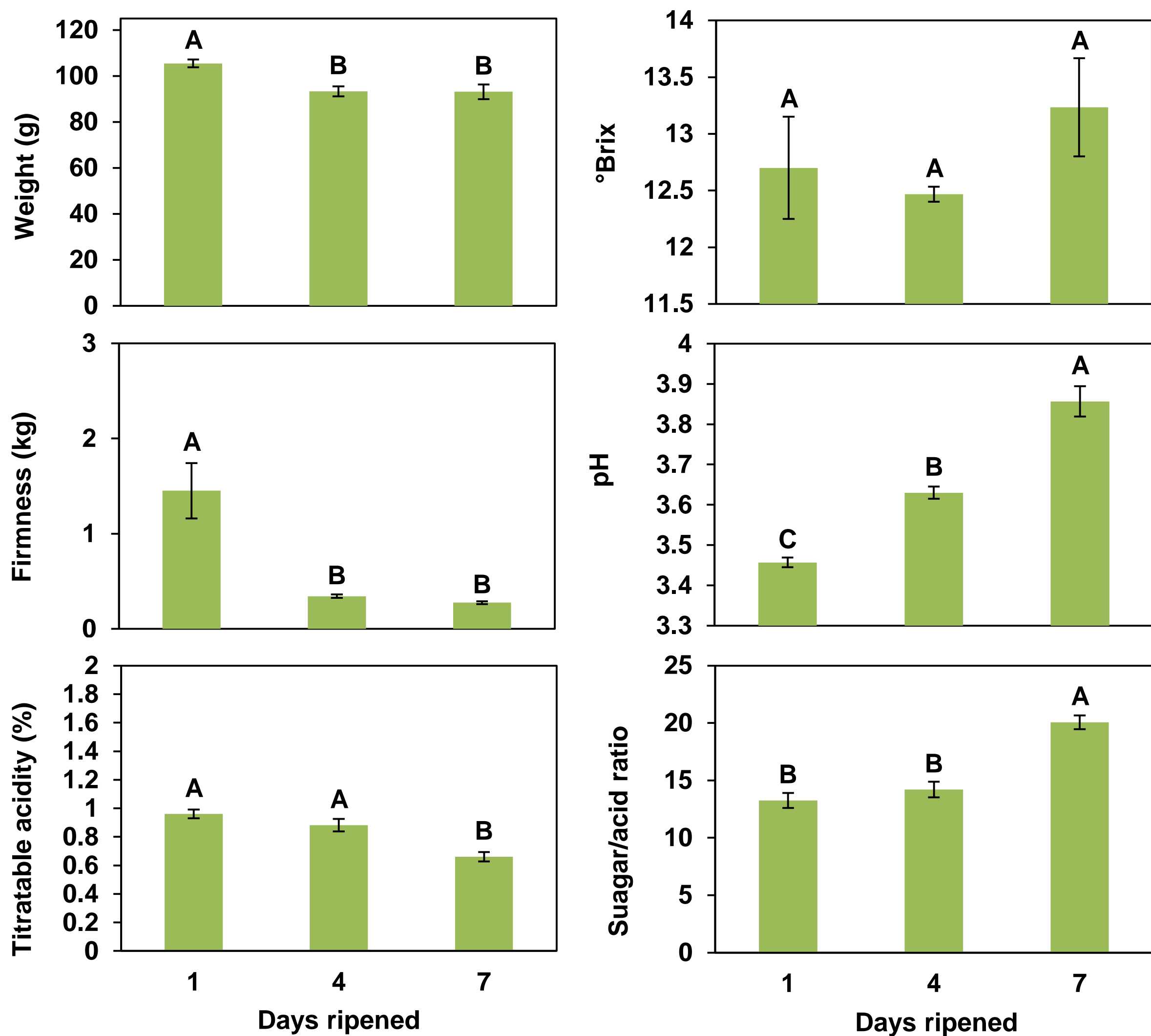

Fig. 5.1 Fruit indices of nectarine (cv. Sunlite) ripened at ambient conditions (**1,** 1d postharvest; **4,** 4d postharvest; **7,** 7d postharvest). Vertical bars indicate standard error. Different letters (only letters of similar case are comparable) indicate treatments that are significantly different ($P < 0.05$) based on Fisher protected Least Significant Difference.

Effect of fruit ripeness on infection and colonisation of *Penicillium* spp. Trial repeats were not significantly different ($P > 0.76$). The interaction effect between ripeness and *Penicillium* spp. was significantly different ($P < 0.0001$). Lesions caused by *P. digitatum* were larger on riper fruit, quickly surpassing that of *P. expansum* in size on 4d and 7d postharvest fruit (Fig. 5.2). The 7d postharvest fruit terminated after 4d incubation due to complete or near complete decay of fruit. Disease incidence for *P. expansum* was 100% at al ripeness levels while that of *P. digitatum* increased as fruit became riper; 20±10% (1d postharvest), 81.48±11.84% (4d postharvest) and 100% (7d postharvest). Blue conidia from *P. expansum* were observed as early as 3d after incubation whereas the lime green conidia produced by *P. digitatum* were first observed after 4d (7d postharvest fruit) or 5d (1d and 4d postharvest fruit) incubation (Fig. 5.3). Once sporulation started, copious amounts of conidia were produced within a short period of time.

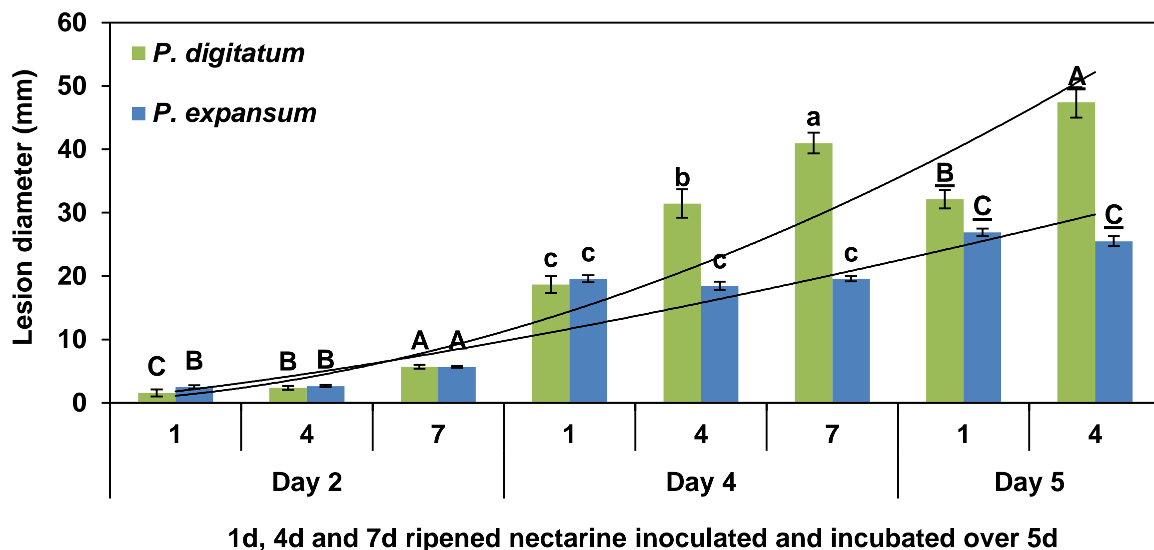

Fig. 5.2 Lesion development of *Penicillium* spp. on nectarine (cv. Sunlite) of different ripeness levels over 5d ambient incubation. Control (wound size) was subtracted from lesion diameters. Vertical bars indicate standard error. Different letters (only letters of similar case and underlining are comparable) indicate treatments that are significantly different ($P < 0.05$) based on Fisher protected Least Significant Difference.

Effect of fruit ripeness and inoculum load on decay. Trial repeats were not significantly different after 4d incubation ($P = 0.96$). There was a significant interaction between *Penicillium* spp., ripeness and inoculum load after 2d ($P = 0.04$) and 4d ($P = 0.004$) incubation. *Penicillium digitatum* caused larger lesions than *P. expansum* for all concentrations on 7d postharvest fruit but not necessarily on 1d postharvest fruit (Fig. 5.4). Lesions were larger for both species when fruit were riper and inoculum loads higher.

Disease incidence for *P. digitatum* was low on 1d postharvest fruit (10–45%) but high on 7d postharvest fruit (100%). *Penicillium expansum* repeatedly expressed a 100% disease incidence for all conditions.

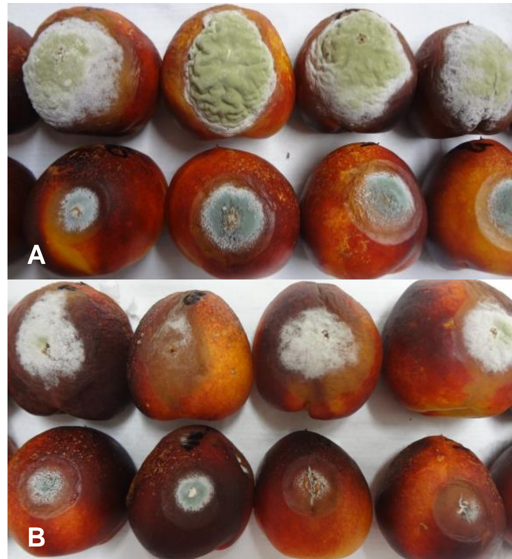

Fig. 5.3 Nectarine (cv. Sunlite) inoculated with *Penicillium digitatum* (top) and *P. expansum* (bottom) and incubated at ambient conditions. **A,** 4d ripened fruit incubated for 5d; **B,** 7d ripened fruit incubated for 4d.

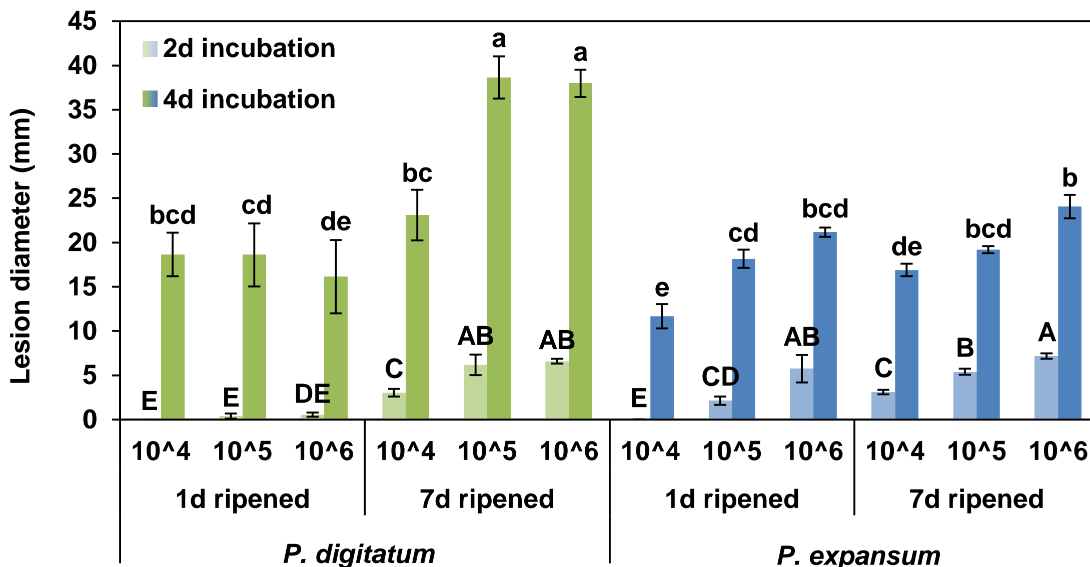

Fig. 5.4 Lesion diameters caused by different concentrations (10^4–10^6 conidia/ml; 200–20 000 conidia) of *Penicillium* spp. in nectarine (cv. Sunlite) of different ripeness levels (**1d,** 1d postharvest; **7d,** 7d postharvest). Wounds have been subtracted from lesion diameters. Vertical bars indicate standard error. Different letters (only letters of similar case are comparable) indicate treatments that are significantly different ($P < 0.05$) based on Fisher protected Least Significant Difference.

Effect of inoculum load and cold storage on decay. Trial repeats were not significantly different after 5d (P = 0.65) and 31d (P = 0.47) incubation. The interaction of inoculum load and storage condition had a significant effect on lesions caused by the *Penicillium* spp. (Fig. 5.5). *Penicillium digitatum* was unable to cause lesions under cold storage. Cold storage significantly reduced lesion development (size) and disease incidence of *P. expansum*. Disease incidence decreased from the usual 100% (ambient and 10^6) to 95% (10^5) and 65% (10^4) due to cold storage.

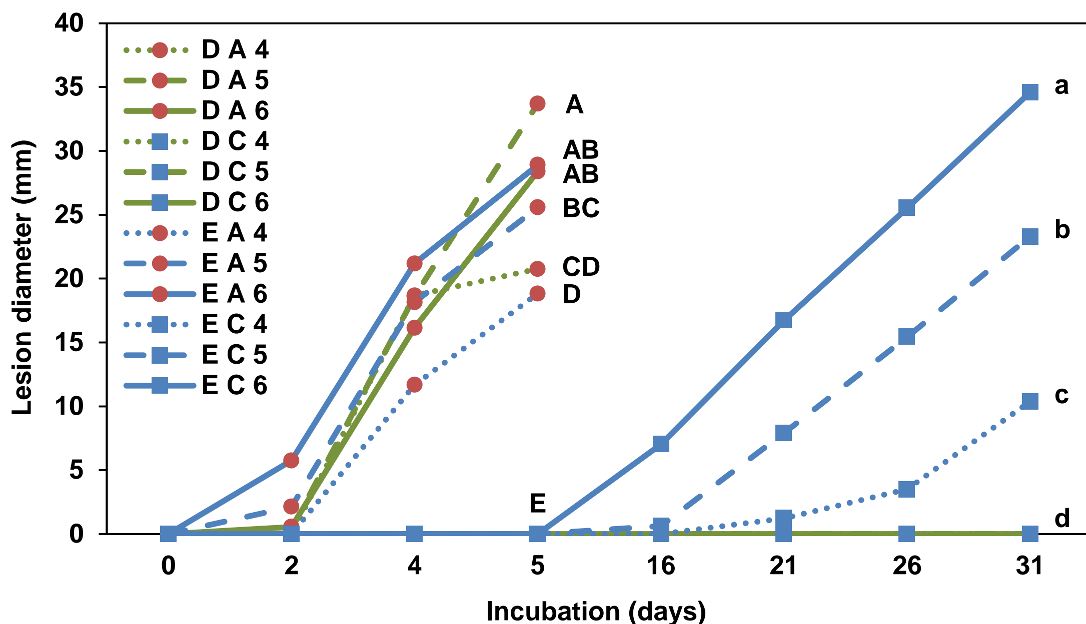

Fig. 5.5 Lesion diameter caused by different concentrations (**4–6**) of *Penicillium digitatum* (**D**) and *P. expansum* (**E**) on nectarine (cv. Sunlite) (1d postharvest) at ambient (**A**) and cold storage (**C**) conditions. **4,** 10^4 conidia/ml (200 conidia); **5,** 10^5 conidia/ml (2 000 conidia); **6,** 10^6 conidia/ml (20 000 conidia). Wounds have been subtracted from lesion diameters. Different letters (only letters of similar case are comparable) indicate treatments that are significantly different (P < 0.05) based on Fisher protected Least Significant Difference.

Firmness and pH of infected sites. Trial repeats were not significantly different based on the firmness of lesions (P = 0.32–0.43). Measurements at 4d and 5d incubation were disregarded. Firmness increased from 4d onwards due to large mycelial masses developing (growth) under inoculated sites. In the case of control fruit, longer incubated/stored fruit was dryer and skin more elastic (skin not removed for these experiments). Ripeness significantly affected lesion firmness (P < 0.0001). The effect was also different depending on *Penicillium* spp. (P < 0.0001). Lesion firmness of *P. digitatum* deteriorated slower in 1d postharvest fruit but not necessarily in 4d or 7d postharvest fruit when comparing it to that of *P. expansum* (Fig. 5.6).

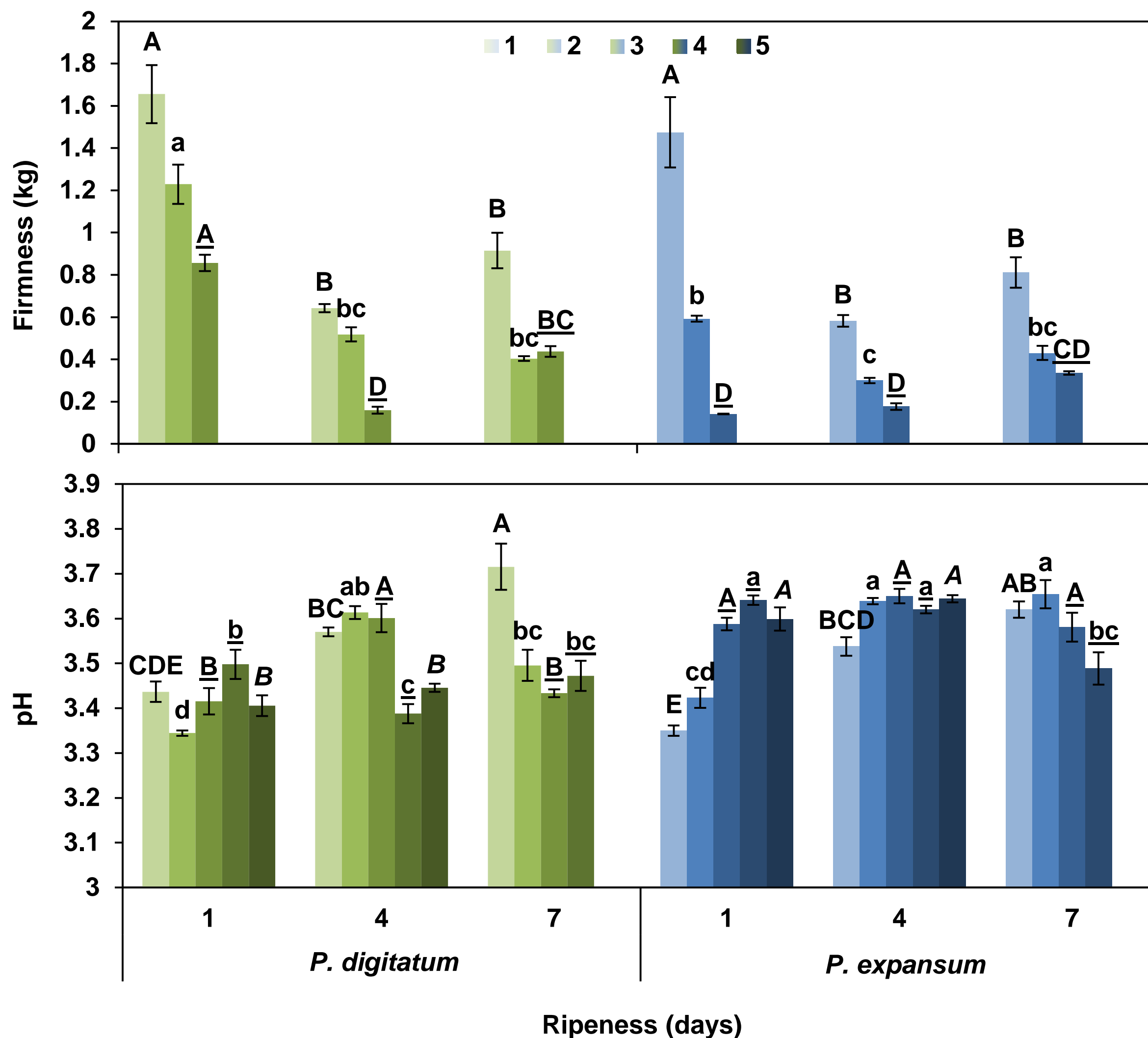

Fig. 5.6 Firmness and pH of lesions caused by *Penicillium* spp. on nectarine (cv. Sunlite) of different ripeness levels (**1,** 1d postharvest; **4,** 4d postharvest; **7,** 7d postharvest) over 5d incubation (**1–5**). Vertical bars indicate standard error. Different letters (only letters of similar case and underlining are comparable) indicate treatments that are significantly different ($P < 0.05$) based on Fisher protected Least Significant Difference.

Trial repeats were not significantly different based on pH measurements from 2–5d incubation (P = 0.30–0.97). Similar to firmness, ripeness significantly affected the pH of infected sites ($P < 0.03$) and the effect was *Penicillium* spp. dependent ($P < 0.01$). The pH of *P. digitatum* lesions remained relatively consistent on 1d postharvest fruit but decreased on riper fruit (Fig. 5.6). Equilibrium was eventually reached at roughly 3.4. For *P. expansum* infected fruit, pH of lesions increased on 1d and 4d postharvest fruit but decreased on 7d postharvest fruit. Here equalisation took place at 3.6 but decreased to a near similar pH than *P. digitatum* with 7d postharvest fruit. The pH of control fruit continually increased from 3.4 (1d postharvest fruit at 1d incubation) to 4 (7d postharvest at 4d incubation). The pH of

colonised tissue (lesions) was generally lower than that of uncolonised (infected fruit) and healthy tissue (control fruit). There were some exceptions with 1d postharvest fruit (Table 5.1).

Table 5.1 pH of uncolonised and colonised tissue of *Penicillium*-inoculated nectarine (cv. Sunlite) at different ripeness levels

Incubation (days)	***Penicillium* spp.**	**Tissue**	**Ripeness (days postharvest)**		
			1d	**4d**	**7d**
4	*P. digitatum*	Uncolonised	3.56±0.1**DEFG**	3.60±0.09**DEFG**	4.00±0.20**AB**
		Colonised	3.50±0.1**EFG**	3.39±0.07**G**	3.47±0.1**FG**
	P. expansum	Uncolonised	3.75±0.13**BCDE**	3.78±0.21**BCD**	3.81±0.17**BCD**
		Colonised	3.64±0.03**DEFG**	3.62±0.03**DEFG**	3.49±0.11**EFG**
	Control*	Unwounded	3.55±0.08**DEFG**	4.23±1.1**A**	3.96±0.20**BC**
		Wounded	3.49±0.13**EFG**	3.70±0.12**CDEF**	3.98±0.15**AB**
5	*P. digitatum*	Uncolonised	3.45±0.14**cde**	3.77±0.33**a**	.
		Colonised	3.41±0.07**e**	3.45±0.03**cde**	.
	P. expansum	Uncolonised	3.43±0.05**de**	3.63±0.14**abc**	.
		Colonised	3.60±0.08**abcd**	3.64±0.02**ab**	.
	Control*	Unwounded	3.62±0.06**abcd**	3.69±0.25**ab**	.
		Wounded	3.52±0.17**bcde**	3.69±0.18**ab**	.

*Control wounded: site where fruit was inoculated with sterile Ringer's solution with 0.05% Tween 80; Control unwounded: sites max distance away from the inoculation sites. Data is displayed with standard deviation. Different letters (case dependent) are significantly different ($P < 0.05$) based on Fisher protected Least Significant Difference.

Absolute quantification of genes. Trial repeats were not significantly different based on gene expression profiles (P = 0.51–0.94). Absolute and normalised data (reference gene) are displayed (Fig. 5.7). The high expression of the reference gene led to negative log values when normalisation was completed. Only normalised data will be discussed. Expression of *ACCD* increased and *pacC* decreased for *P. digitatum* over incubation time. Expression of *PG* and *pacC* decreased while *ACCD* increased for *P. expansum* over incubation time. The interaction of *Penicillium* spp. and ripeness was significant for the expression of *PG* ($P < 0.001$) and *creA* (P = 0.04). It was higher on 7d postharvest fruit infected with *P. digitatum* (remainder unaffected). In the case of *P. expansum*, *PG* was significantly lower and *pacC* slightly higher.

Reisolation, preservation and identification. Cultures of the *Penicillium* spp. isolated from symptomatic fruit were grouped. Representative cultures were confirmed as the inoculated species via NCBI standard nucleotide BLAST of *β-tubulin*. cDNA of

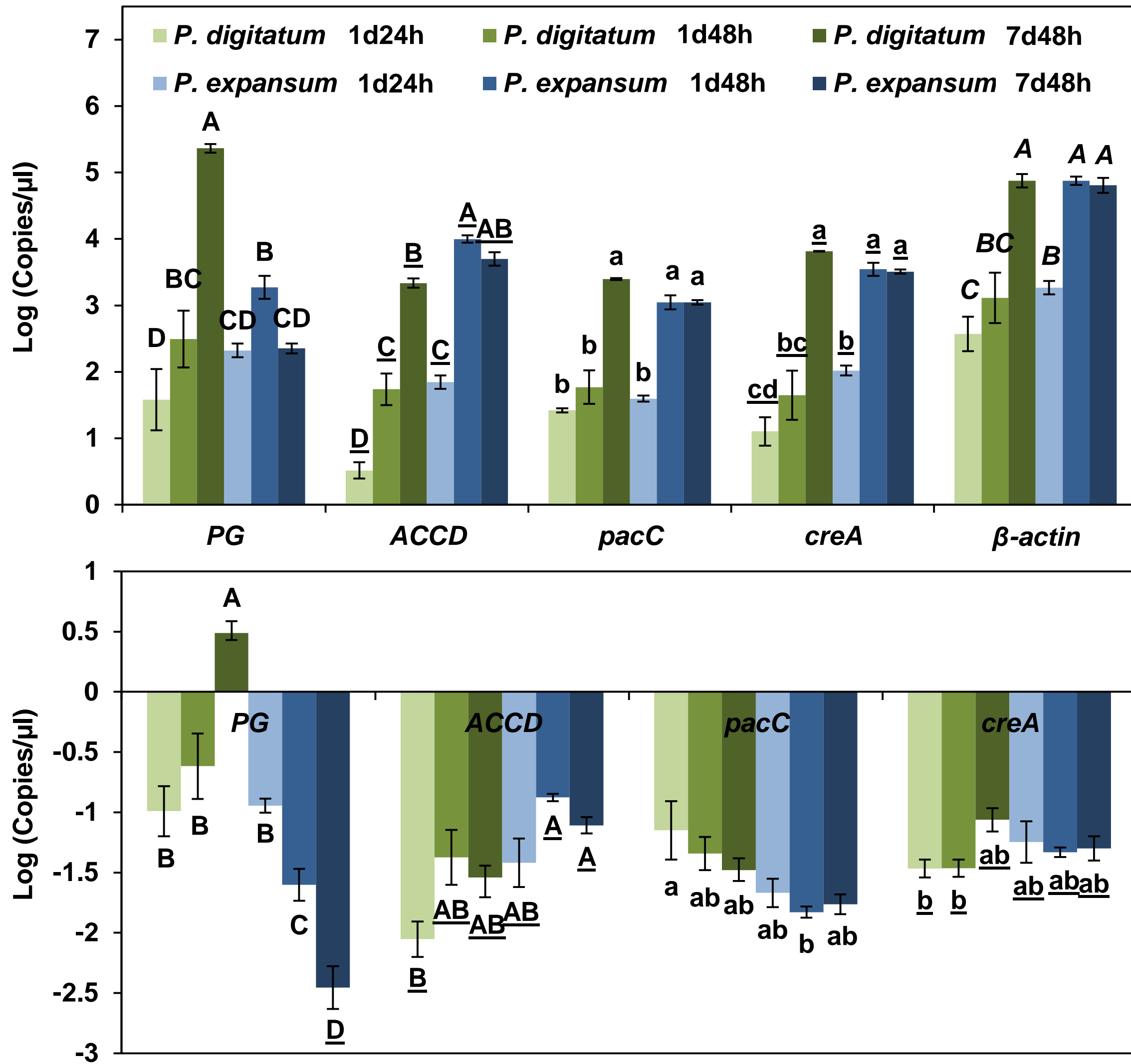

Fig. 5.7 Gene expression of *Penicillium digitatum* and *P. expansum* when inoculated in nectarine (cv. Sunlite) of different ripeness levels (1d and 7d postharvest) after 24h and 48h incubation. Raw data (top) was normalised (bottom) using the reference gene (*β-actin*). Vertical bars indicate standard error. Different letters (only letters of similar case, underlining and italicising are comparable) indicate treatments that are significantly different (P < 0.05) based on Fisher protected Least Significant Difference.

extracted RNA from tissue samples were submitted for identification and confirmed as the target sequences. Identity of sequences had 99–100% similarity with 99–100% query cover. Cultures were purified, preserved and stored in the culture collection at UP.

4. DISCUSSION

Nectarines became riper as fruit were stored for longer periods (days postharvest). The increase in sugar/acid ration should be noted. The pH and sugar are important regulatory factors impacting on infection and colonisation of *Penicillium* spp. (Bi *et al*. 2016; Prusky *et al*. 2004). The large differences in fruit physiology of 1d postharvest vs 7d postharvest fruit

would provide different host environments which should affect infection and colonisation of the *Penicillium* spp. This was clearly the case with *P. digitatum* but not necessarily with *P. expansum*. The effect of ripeness on *P. digitatum* was not only observed by the increase in disease incidence and lesion diameter but also confirmed by the increase in the reference gene (increased biomass). That of *P. expansum* was unchanged by ripeness.

Penicillium digitatum had smaller lesions and low disease incidence on fresh fruit compared to riper fruit. Lesion diameter was even similar in size at 1d postharvest to *P. expansum* but quickly surpassed it on riper fruit. Work from chapter 3 showed low disease incidence (7.5%) for *P. digitatum* on Sunlite but lesions were able to reach ~55mm in diameter after 7d ambient incubation (Louw and Korsten, 2016). This is particularly because fruit was 1d/2d postharvest. In this chapter, disease incidence on 1d postharvest fruit was higher (20%) and lesion diameter showing similarities (32.13mm after 5d incubation). Together with chapter 4, this is the first study demonstrating a significant shift in lesion diameter and disease incidence for *P. digitatum* due to fruit ripeness. Vilanova *et al.* (2014) reported *P. digitatum* able of causing lesions of ±45mm in diameter on over-mature but not immature or commercially mature 'Golden Smoothee' apples. *Penicillium expansum*, similar to what was observed in chapter 3 and 4, was relatively unaffected by fruit age/ripeness. This indicates that these species utilise very different strategies during infection and colonisation.

Scanning electron microscopy from chapter 3 can, to an extent, confirm the above statement. The micrographs revealed large amounts of mycelia produced by *P. digitatum* and *P. expansum* on retail bought 'Crimson Glo' nectarine within 48h. *Penicillium digitatum* however also produced large amounts of conidiophores and sporulated within the same period as compared to very few conidiophores and no conidia produced by *P. expansum*. More differences were observed when both species were inoculated into 'Eureka seeded' lemons. *Penicillium digitatum* produced large amounts of mycelia but fewer conidiophores and conidia as observed on nectarine while *P expansum* produced very little mycelia but conidiophores with longer chains of conidia (Chapter 3: Louw and Korsten, 2016).

Prusky *et al.* (2002; 2004) reported *P. expansum* causing larger lesions on more acidic apple cultivars; 'Granny Smith' (pH 3.45) vs 'Rome' (pH 3.77) and 'Fuji' (pH 4.46). This was also observed for *P. digitatum* when it was screened for pathogenicity on apples (Louw and Korsten, 2014). Comparing work on plum in chapter 4 with that of nectarine further confirmed this. Plum had pH values of 2.97 (1d postharvest) and 3 (4d postharvest) while

that of nectarine was 3.46 and 3.63 (1d and 4d postharvest). Lesions caused by *P. digitatum* on plum averaged at 49.97mm and 57.89mm in diameter (respectively) compared to 32.13mm and 47.40mm on nectarine after 5d ambient incubation. Lesions caused by *P. expansum* on plum were 29.39–29.65mm vs 25.50–26.26.88 on nectarine. Even though larger lesions were caused by *P. expansum* on more acidic fruits/cultivars, the change in pH due to fruit ripeness had no effect on lesion diameter. Other factors are interacting in the response.

Modulation of pH was affected by ripeness. This can primarily be ascribed to the varying pH values of the different ripeness levels. When pH was at its lowest (1d postharvest), *P. digitatum* maintained and *P. expansum* increased pH. When pH was higher (4d and 7d postharvest), *P. digitatum* decreased while *P. expansum* maintained or decreased pH. This confirms that these species will not only acidify host tissue but can also increase pH depending on the host environment. Colonised tissue of an acidic host can be maintained or further acidified by acidification. Acidification can take place by the production of organic acids and uptake of ammonium/ammonia. Alternatively, pH can increase by inhibiting this process and causing the accumulation of ammonium/ammonia (Barad *et al.*, 2016a; Bi *et al.*, 2016; Prusky *et al.*, 2004). As extensively discussed in the previous chapter, Barad *et al.* (2016a), Bi *et al.* (2016) and Sánchez-Torres and González-Candelas (2003) revealed a dual pattern of pH modulation from *P. expansum*. Alkalinisation and acidification were dependent on nutritional conditions.

It was noted in our study that the *Penicillium* spp. didn't modulate pH to a state that would be more favourable for *PG* expression or PG activity. The pH at infected sites of nectarine equalised at 3.4~3.5 for *P. digitatum* and increased to (1d postharvest), remained at (4d postharvest) or dropped below (7d postharvest) 3.6 for *P. expansum*. This corresponded with results of *P. expansum* infected apple cultivars and *P. digitatum* infected citrus fruits. Below optimum pH values of decayed tissue of Granny Smith = 3.64±0.01, 'Gala' = 3.88±0.03 (Prusky *et al.*, 2004) and Golden Delicious = 3.6 (Sánchez-Torres and González-Candelas, 2003) were reported. The pH of decayed citrus peel was 3.12±0.07, 3.10±0.14 ('Navel' and 'Oro Blanco' after 7d incubation) (Prusky *et al.*, 2004) and 3.22±0.15 (*Citrus unshiu* after 4d incubation) (Zhang *et al.*, 2013). This is a point where the expression of *PG* of *P. expansum* (Prusky *et al.*, 2004) and activity of PG of *P. expansum* and *P. solitum* were reported to be very low (Jurick *et al.*, 2009; Jurick *et al.*, 2010; Yao *et al.*, 1996). *In vitro* studies revealed that PG isolated from *P. expansum* is active over pH 3~6.5 and optimal at pH 4–5.5 (Jurick *et*

al., 2010; Yao *et al.*, 1996) depending on nutritional conditions (Bi *et al.*, 2016). Prusky *et al*. (2004) reported expression of *pepg1* (endopolygalacturonase) was the highest when *P. expansum* was grown on media with pH 4 compared to pH < 3.5.

It is unclear why pH of infected tissue dropped to a level where *PG* expression and PG activity is low. However, it is known that *PG* expression differs depending on *in vivo* and *in vitro* studies (Barad *et al.*, 2016b; López-Pérez *et al.*, 2015; Sánchez-Torres and González-Candelas 2003) and PG activity will vary depending on the *Penicillium* spp. The pH of *P. digitatum* colonised tissue of nectarine, plum (chapter 4) and citrus (Prusky *et al.*, 2004; Zhang *et al.*, 2013) was lower than that of *P. expansum* on nectarine, plum (chapter 4) and apple (Prusky *et al.*, 2004). The activity of PG (fractionation, temperature and pH) extracted from *P. expansum* was very different as compared to PG extracted from *P. solitum* (Jurick *et al.*, 2009; Jurick *et al.*, 2010). It can thus be expected that the PG activity of *P. digitatum* is different, possibly having a higher activity at lower pH levels as compared to that of *P. expansum* and *P. solitum*. Future studies should determine this.

The pH values of colonised sites were similar to the initial pH of fresh nectarine (1d postharvest). The pH value prior to inoculation was 3.46 compared to 3.50 (*P. digitatum*) and 3.64 (*P. expansum*) after 4d incubation. The differences remained relatively small for 4d postharvest fruit but increased considerably for 7d postharvest fruit. For *P. digitatum* it was Δ_{pH} = 0.24 and Δ_{pH} = 0.39, and for *P. expansum* it was Δ_{pH} = 0.01 and Δ_{pH} = 0.38 (respectively). Similarities can be drawn to apple cultivars. Barad *et al.* (2016a) reported pH 3.58 at the inoculation site (*P. expansum*) of Golden Delicious (5d incubation). Prusky *et al.* (2004) provided pH values after 7d incubation: Fuji = 3.96, Gala = 3.88, Golden Delicious = 3.88, Granny Smith = 3.64 and 'Red Delicious' = 4.07. It was unclear how fresh and/or what the pH values of uninfected apples were in Barad *et al.* (2016a) and Prusky *et al.* (2004). This can be obtained from Keller *et al.* (2004): Fuji = 3.91, Gala = 3.86, Golden Delicious = 3.64, Granny Smith = 3.42 and Red Delicious = 4.10. Although the pH values fluctuate depending on the incubation period, these results show that *P. digitatum* and *P. expansum* can (to some degree) maintain a pH similar to the initial pH of the host. In cases where fruit tend to be riper or possess very high pH values, a definite lowering in pH can be observed. The pH of decayed tissue seems to be host (Bi *et al.*, 2016; Prusky *et al.*, 2004) and species specific.

Differences in pH between colonised (*P. digitatum*) and uncolonised tissue after 4d incubation were $\Delta_{pH} = 0.06$ (1d postharvest), $\Delta_{pH} = 0.21$ (4d postharvest) and $\Delta_{pH} = 0.53$ (7d postharvest). For *P. expansum* it was $\Delta_{pH} = 0.11$, $\Delta_{pH} = 0.16$ and $\Delta_{pH} = 0.32$ (respectively). Differences between Δ_{pH} of colonised vs uncolonised (infected) and colonised vs control (uninfected) fruit were small. For *P. digitatum* it was $\Delta\Delta_{pH} = 0.05$–0.1, and for *P. expansum* it was $\Delta\Delta_{pH} = 0.04$–0.17. This doesn't correspond with results of *P. expansum* on apple or with the plum work in chapter 4. Colonised vs uncolonised tissue of infected apples were $\Delta_{pH} = 0.31$–0.88 (Prusky *et al.*, 2004) whereas colonised tissue vs tissue of fresh uninfected apples will be $\Delta_{pH} = 0.02$–0.24 [combining data from Keller *et al.* (2004) and Prusky *et al.* (2004)], thus $\Delta\Delta_{pH} = 0.29$–0.64. With plum, *P. digitatum* $\Delta\Delta_{pH} = 0.16$–1.8 and *P. expansum* $\Delta\Delta_{pH} = 0.6$–1.55. The primary reason for the large difference observed with plum was the rapid ripening of infected fruit as compared to slower (natural) ripening of uninfected fruit.

Expression of *P. digitatum* genes showed little change at the different incubation periods (24h and 48h). There was an increase in *ACCD* and decrease in *pacC*. The same cannot be said about *P. expansum* with a decrease in *PG* and *pacC*, and increase in *ACCD*. Results from *P. digitatum* infected 'Navelina' oranges (*Citrus sinensis* L. Osbeck) corresponded with ours. Expression of *PG* (*pg1* and *pg2*) increased and/or remained constant over a 4d incubation period (López-Pérez *et al.*, 2015). The downregulation of *PG* of *P. expansum* was observed in chapter 4 and by Sánchez-Torres and González-Candelas (2003). Sánchez-Torres and González-Candelas (2003) determined the expression of two different PG genes (*pepg1* and *pepg2*) of *P. expansum* from infected Golden Delicious apples (heat treated) and cultures (minimal media with apple pectin). One gene was similar to *pepg1* of Yao *et al.* (1996) and the other highly similar to *PG* of *P. digitatum*. The genes were differentially expressed. There was no expression of *pepg1* but decreased expression of *pepg2* (24h vs 48h) from their *in vitro* work. *In vivo*, there was no expression of *pepg1* at 24h and similar (very low) expression at 48h and 72h while expression of *pepg2* decreased over the 72h period. They commented that differential expression of *pepg1* and *pepg2* could have been due to differences in environmental pH.

Expression of *ACCD* and *pacC* should decrease in an acidic environment (*in vitro*) but can increase when ammonium/ammonia concentrations are high, even under acidic conditions. They are although not similarly affected by these factors. *ACCD* expression is more affected by ammonium/ammonia levels and less by pH whereas *pacC* showed a stronger connection to pH and less to ammonium/ammonia (Barad *et al.*, 2016a; 2016b). The low pH of fruit

could be sufficient to describe the downregulation of *pacC* but it is difficult to draw a proper correlation since the pH of infected sites showed gradual changes from 24h to 48h (Δ_{pH} = 0.07–0.1).

It is possible that an increase in ammonium levels could have contributed to the upregulation of *ACCD*. Ammonium is an important nitrogen source of *Penicillium* (Ross and Luckner, 1984). It can enhance *pacC* responsiveness, modulate environmental pH and induce the expression of a few genes associated with the nitrogen metabolism and cell damage (pectolytic enzymes and toxins) (Barad *et al.*, 2016a; 2016b; Song *et al.*, 2014). One of these is ACC, a precursor of ethylene (Glick, 2014). Accumulation of ACC induces expression of ACCD (Jia *et al.*, 2000) by which it is cleaved to produce more ammonia and α-ketobutyrate (Glick, 2014; Jia *et al.*, 2000). Pathogen attack can also result in stress ethylene, causing a host plant to accumulate ACC and undergo advanced ripening and senescence (Glick, 2014).

Unlike what was observed on plum (chapter 4), the definite connection between infection and increased ripening could not be made since control fruit had similar pH values to uncolonised tissue of infected fruit. The upregulation of *ACCD* (24h vs 48h) was the only indicator of increased ripening. Ripening could have been due to ACC synthesised by the *Penicillium* spp. (Barad *et al.*, 2016b; Yang *et al.*, 2017) and/or the infection itself (stress ethylene) (Glick, 2014). The higher expression of *ACCD* on fresh fruit (1d postharvest) from 24h to 48h but downregulation on riper fruit (7d postharvest) at 48h indicates synthesis of more ACC on fresh fruit but not on riper fruit. It is possible that the host environment at 7d postharvest was already at a favourable state (ripe enough). Testing the nutritional composition of infected fruit (colonised and uncolonised tissue) should be considered in future work.

Fruit ripeness had a large effect on the expression of *PG* and a small to no effect on the expression of *ACCD* and *pacC* for both *Penicillium* spp. The expression of *creA* increased for *P. digitatum* but was unaffected for *P. expansum* due to ripeness. Total sugar content of peach can remain constant or slightly decrease during postharvest storage (Borsani et al. 2009). Nectarine and peach cultivars contain high levels of sucrose but fructose, glucose and to a lesser extent sorbitol also make up for the total sugar content (Colarič *et al.*, 2004). Some of these sugars can significantly decrease (i.e. sucrose and sorbitol) while other increase (i.e. fructose and glucose) during storage (Borsani et al. 2009). The upregulation of

creA indicates that there was possibly more glucose available in the 7d postharvest fruit (Bi et al. 2016; Borsani et al. 2009). Although not significant, it also corresponds with the higher °Brix value of the 7d postharvest fruit.

Little is still known about the effect varying carbon levels of fruit at different maturity and ripeness levels will have on pH modulation (Bi *et al.*, 2016). CreA regulates the carbon catabolite repression. It is a mechanism that ensures preferential utilisation of certain carbon sources (i.e. glucose) (Fernandez *et al.*, 2012; 2014) and prevents expression of genes that would require the metabolism of others. It was also shown to be a mechanism controlling factors that activates acidification or alkalinisation processes. Acidification can be induced under excess sugar, even for pathogens classified with alkalinising lifestyles. Conversely, alkalinisation can be induced under carbon deprived conditions, even by acidifying pathogens (Alkan *et al.*, 2013; Bi *et al.*, 2016; Ment *et al.*, 2015).

From *in vitro* work, Bi *et al.* (2016) showed higher sucrose levels will cause *P. expansum* to produce less ammonia and more gluconic acid. This environment will cause a decrease in expression of *ACCD* and *pacC* (Barad *et al.*, 2016a; 2016b). The decrease (not significant) in the expression of both these genes from *P. digitatum* and the rapid drop in pH of lesions of 7d postharvest fruit corresponded with this. In the case of *P. expansum*, *creA* and pH of lesions of 7d postharvest fruit was unaffected. The increase in expression of *pacC* corresponds with these findings from *P. expansum*. It is, however, unclear why *P. expansum* showed a strong reaction to high (excess) sucrose levels *in vitro* (Bi *et al.*, 2016) but was unaffected by the higher sugar content of 7d postharvest fruit. It is possible that the difference in sugar content was too small for *P. expansum* or the interaction of other factors (i.e. pH, ammonium) play a larger part in the interaction of *P. expansum* on nectarine. The effect varying pH, nitrogen and carbon levels has on the different *Penicillium* spp. is more diverse than originally expected.

The upregulation of *PG* by *P. digitatum* and downregulation by *P. expansum* further enforces the above statement. The higher initial pH of 7d postharvest fruit (3.86) as compared to 1d postharvest fruit (3.46) would be closer to the optimal pH for *PG* expression and PG activity (*in vitro*) (Jurick *et al.*, 2009; Jurick *et al.*, 2010; Prusky *et al.*, 2004). This can be presumed as the cause (in terms of host physiology) for the larger lesions caused by *P. digitatum*. However, this was not observed with *P. expansum* and both species lowered the pH (*P. digitatum* = 3.47; *P. expansum* = 3.49) as incubation continued. *Penicillium digitatum*

lowered the pH of colonised sites faster and lower than *P. expansum*. Other unrevealed factors are playing a major role in this newly discovered disease interaction with its drastic increase in disease incidence and lesion diameter on riper fruit.

Although similarly observed from Sánchez-Torres and González-Candelas (2003), it was unexpected to see a lower expression of *PG* from *P. expansum* over time while lesion diameter continued to increase. *Penicillium expansum* very possibly has a stronger dependence on another mechanism to increase lesion size; other pectolytic enzyme or toxins. *Penicillium expansum* is a known producer of multiple mycotoxins (i.e. citrinin, patulin, roquefortine C) (Frisvad and Samson, 2004; Pitt and Hocking, 2009). The upregulation of *pacC* support this as this gene plays a significant role not only in the activation of D-gluconic acid (pH modulation) but also regulation of pathogenicity and secondary metabolites (i.e. patulin) (Barad *et al.*, 2016a). Expression of genes involved in patulin biosynthesis of *P. expansum* also depends on specific nutritional growth conditions (Li *et al.*, 2015). Damoglou and Campbell (1986) reported an optimal pH range of 3.2–3.8 for the production of patulin by *P. expansum* in apple juice. Tannous *et al.* (2016) confirmed a higher patulin production at pH 4 as compared to 2.5 and 7 on Czapek glucose agar. This is in accordance with the pH of colonised tissue at lesions on nectarine.

Ripeness not only affected lesion diameter but also disease incidence of *P. digitatum*. Many host factors (physical, biochemical and molecular) change during the ripening of fruit. It is currently unclear which of these factors trigger the increase in lesion diameter and disease incidence of this species. The sharp decline in fruit firmness of riper fruit could suggest advancement in fruit senescence, thus deterioration in host resistance and an increase in infection and colonisation of *P. digitatum*. It was originally thought that host physiology played the most significant role but studying host resistance [decline as fruit ripens (Prusky *et al.*, 2016)] might reveal the true cause for the opportunistic lifestyle expressed by *P. digitatum* on nectarine.

This opportunistic lifestyle of *P. digitatum* on nectarine makes it less of a concern early in a fresh produce chain but more so at the end when the fruit is riper. *Penicillium expansum* does not follow this lifestyle on nectarine, constantly causing lesions of similar size with perfect disease incidence (100%) in nearly all cases. It was noticed that the fan in the cold room accelerated moisture loss of exposed fruit in the cold storage trials. This would have affected results. Nonetheless, the sensitivity of *P. digitatum* to cold storage and its

opportunistic lifestyle on nectarine was confirmed. This is however not the case when *P. digitatum* infect and colonise citrus (Eureka seeded lemons). Louw and Korsten (2015) revealed that even with low concentrations (6.3 x 10^4 conidia/ml) *P. digitatum* could cause lesion of 43.8±5.6mm in diameter after 26d cold storage (5.0±0.7°C and 86.4±4.5% RH). The host and environment *P. digitatum* is exposed to thus determines its opportunistic nature.

This report is one of the first to demonstrate and compare significant disease development (high disease incidence and large lesions) of *P. digitatum* and *P. expansum* on the same host. This is primarily due to the recent discoveries demonstrating *P. digitatum* pathogenic and highly aggressive on apples, pears, plums and nectarines (Louw and Korsten, 2014; 2016). It has become clear that the mechanisms used by these two pathogens to infect and colonise their hosts are quite different. The conditions (physical and host environment) these pathogens tolerate vary, thus affecting decay development. This study also allows the comparison of infection and colonisation of *P. digitatum* on nectarine (more acidic new host) to what is already known on citrus [i.e. citrus peel (Zhang *et al.*, 2013)].

5. CONCLUSION

This is the first study to demonstrate the effect of host ripeness on infection and colonisation of *P. digitatum* and *P. expansum* on nectarine. Ripeness significantly affected *P. digitatum* in terms of lesion diameter and disease incidence. The importance of cooling and hygiene to aid disease control was highlighted. *Penicillium expansum* was the least affected by cold storage and inoculum load greatly affected both species. This chapter and chapter 4 are the first studies to use ddPCR to quantify the expression of genes in postharvest pathology of fruit. pH modulation by the *Penicillium* spp. was affected by host ripeness. The potential of increasing, maintaining and decreasing pH was demonstrated. Environmental pH was not modulated to a state where *PG* expression and PG activity are expected to be optimal (*in vitro*). The modulation of pH was described as being host and species specific. Larger lesions are caused on more acidic hosts. *Penicillium digitatum* was able to lower pH faster and further than *P. expansum*. Ripeness had a significant effect on the expression of *PG*. The increased expression of *PG* of *P. digitatum* could explain the rapid decay of riper fruit. The increase in expression of *creA* highlighted the importance of sugar in this new host-pathogen interaction. Although host physiology (i.e. firmness, sugar content, pH, nitrogen levels) could provide some explanation for the increased disease incidence and lesion diameters, it is still not clear what specific factors trigger/s the increase on the riper fruit.

Studying the host response (defence genes) can help to identify the factors leading to the opportunistic lifestyle expressed by *P. digitatum* on nectarine. This was not the case with *P. expansum*. It consistently produced lesions of similar size at 100% disease incidence in nearly all cases (exception with the combination of low inoculum loads and cold storage). The expression of *PG* for *P. expansum* sharply decreased as incubation continued and fruit ripened. Other pathogenicity or virulence factors are playing a more important role in the interaction of *P. expansum*. The increase of *pacC* could indicate the importance of mycotoxins with this species. There is a strong connection between the carbon and nitrogen source, and pH of the host environment for the infection and colonisation of these *Penicillium* spp. These pathogens expressed quite different lifestyles on nectarine. Comparing *in vivo* and *in vitro* studies exhibited a great deal of variances. More research is required to differentiate the interaction of these pathogens on the same host.

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Chapter 6

General discussion

All *Penicillium* spp. inoculated into various nectarine and plum cultivars were pathogenic. Decay of stone fruits caused by *P. expansum* is well-known (Ceponis and Friedman 1957; Snowdon, 2010). To our knowledge, this is the first report of *P. digitatum*, *P. crustosum* and *P. solitum* being pathogenic on plum, and *P. crustosum* and *P. solitum* pathogenic on nectarine. Decay of peaches ('Late Peach of Leonforte') caused by *P. crustosum* was reported by Restuccia *et al*. (2006). The latter study only reported disease incidence (100% after 15d incubation) and did not provide results for lesion diameter or symptom expression. Nectarine and peach are very similar (Blake, 1932). The present study was, however, the first to specifically verify *P. crustosum* pathogenic on nectarine and provided information on lesion development and symptom expression on *Prunus persica* (L.) Batsch. Navarro *et al.* (2011) were the first to report decay caused by *P. digitatum* on nectarine ('Flavela' and 'Flanoba'). Lesions were small (±1300mm^3 and ±1500mm^3) and symptoms were not described. The present study confirmed pathogenicity and provided results on symptom development.

Penicillium digitatum was originally described as a specialised pathogen (narrow host range). The close association with citrus is regarded as a distinctive feature of the species (Pitt and Hocking, 2009). Recent findings have now revealed *P. digitatum* having a broader host range. This pathogen can cross-contaminate and infect stone, citrus and pome fruits (Louw and Korsten, 2014; 2015; 2016). These fruit types often have overlapping export seasons. In essence, an alternative host for *P. digitatum* will be available year round in the major distribution centres which will serve as a source to increase inoculum load and subsequently the risk for cross-contamination and infection. In South Africa, the stone, citrus and pome fruit export seasons specifically overlap (PPECB, 2013). *Penicillium digitatum* was more aggressive than *P. expansum* on all of the hosts. Industry should consider ways to prevent cross-contamination between these hosts.

Heightened infection (disease incidence) and aggression (lesion diameter) were observed with *P. digitatum* infection studies on older stone fruits. This was not the case with any of the other *Penicillium* spp. *Penicillium expansum* and *P. crustosum* consistently (100%) caused large lesions on all cultivars tested with the former being more aggressive. Both species are linked to mycotoxin production; citrinin, communesin B, patulin, penitrem A and roquefortine C among others (Frisvad and Samson, 2004; Frisvad *et al.*, 2004; Pitt and Hocking, 2009). Fruit ripeness was not established with initial findings (Chapter 3: Louw

and Korsten, 2016) thus giving reason to further investigate the matter within the new host-pathogen interaction and compare it to *P. expansum*.

The present study includes the most comprehensive description of symptoms caused by these *Penicillium* spp. on nectarine and plum. It is the first to report and describe green mould symptoms on stone fruits. It was difficult to distinguish between blue mould symptoms caused by *P. expansum*, *P. crustosum* and *P. solitum* if incubation periods were unknown. Symptoms caused by *Penicillium* spp. were similar to that observed on apple and pear (Louw and Korsten, 2014) but not necessarily on citrus (Louw and Korsten, 2015).

Scanning electron microscopy (SEM) provided additional evidence regarding symptom development. *Penicillium digitatum* was identified as the second fastest (after *P. crustosum*) sporulating species on nectarine. It produced more mycelia and conidia on nectarine than on citrus. With SEM we were able to observe conidiation at an earlier stage. All evaluated species (*P. digitatum*, *P. expansum* and *P. crustosum*) were able to produce conidia on nectarine and lemon within 48h incubation. This highlights the potential of cross-contamination and infection between these hosts. This is further supported by different *Penicillium* spp. isolates (environmental isolates from citrus and pear chain) being able to cause lesions of similar size on nectarine ('Bright Pearl'), citrus ('Nules Clementine') and apple ('Golden Delicious') (Louw and Korsten, 2014; 2015; 2016).

Penicillium digitatum can be described as a pathogen of concern to the stone fruit industry. As indicated, effective management of the cold chain and proper cleaning of facilities and containers to reduce inoculum buildup can provide a more effective management strategy to control *Penicillium* green mould on nectarine and plum. However, it remains a challenge to control and manage cold chains (Freiboth *et al.*, 2013; Haasbroek, 2013; Maheshwar and Chanakya, 2006). Suboptimal storage and transport conditions will facilitate fruit ripening (Kader, 2011; Kader and Mitchell, 1989; PPECB, 2013), resulting in more susceptible fruit (Kader, 2011; Prusky *et al.*, 2016; Vilanova *et al.*, 2014). Inoculum loads also tend to increase as the fruit season progresses. Furthermore, mixing different fruit types and fruit from different countries (i.e. distribution or repack facilities) not only influences inoculum buildup but also the survivability and genetic diversity of pathogens in fruit storage and handling environments. *Penicillium digitatum* can better tolerate (growth and reproduction) the cold chain on citrus (lemon) than on stone fruits (nectarine and plum) (Louw and Korsten, 2015; 2016). Higher inoculum loads (Vilanova *et al.*, 2012a; 2012b; 2014) with higher

genetic diversity (different fruits from different countries) and weakened host resistance (i.e. riper) (Prusky *et al.*, 2016) increase the potential of pathogens jumping to new hosts. Opportunistic pathogens will especially take advantage of these conditions (i.e. *P. digitatum*).

To our knowledge, no information is available linking *P. digitatum* to losses of stone or pome fruits in fresh produce chains (local or export). This is largely due to the lack of transparency and expertise during assessments and the time and cost implications involved in accurate identification of the causal agents. Further research is required to identify and associate *P. digitatum* with losses in these fresh produce chains.

Fruit ripeness significantly affected disease incidence and lesion diameters caused by *P. digitatum* on nectarine and plum. The effect of ripeness was more pertinent on nectarine than on plum. This was also noted in chapter 3 (Louw and Korsten, 2016). The differences in fruit physiology of freshly harvested fruits (1d postharvest) as compared to riper fruits (7d/12d postharvest) provided host environments that differentially affected infection and colonisation of *P. digitatum*. This was not the case with *P. expansum*.

Although disease incidence was lower, *P. digitatum* was able to produce larger lesions than *P. expansum* at all ripeness levels of plum. With nectarine, this was only possible when fruit were riper (4d and 7d postharvest). Vilanova *et al.* (2014) demonstrated lesions of ±45mm in diameter caused by *P. digitatum* on over-mature but not commercially mature or immature 'Golden Smoothee' apples. These findings indicate that there are differences between *P. digitatum* and *P. expansum* when infecting and colonising the same host. Moreover, *P. digitatum* revealed lifestyle changes depending on the host it was exposed to. This was somewhat confirmed with SEM. Micrographs showed an abundant amount of mycelia from *P. digitatum* and *P. expansum* on 'Crimson Glo' nectarine (retail bought) after 48h incubation. *Penicillium digitatum* produce large amounts of conidiophores and some conidia, whereas *P. expansum* produce very few conidiophores and no conidia within the same period. On 'Eureka seeded' lemon, *P. digitatum* produced a lot of mycelia but few conidiophores and less conidia within 48h. Very little mycelia were observed from *P expansum* on lemon but conidia chains were longer than that of *P. digitatum* (Louw and Korsten, 2016).

The initial pH and sugar content of fruit should be noted when dealing with *Penicillium* host-pathogen interactions. They are important regulatory factors that affect the infection and colonisation of *Penicillium* spp. (Prusky *et al*., 2004; Bi *et al*., 2016). Plum was more acidic

(pH 2.96 vs 3.46) and contained higher sugar levels (13.13 vs 12.7 °Brix) than nectarine. For *P. digitatum* on 1d postharvest fruits, disease incidence was higher (68.33% vs 20%) and lesion diameter larger (49.97mm vs 32.13mm) on plum than on nectarine after 5d incubation. Lesions caused by *P. expansum* were also larger on plum than on nectarine (29.39mm vs 25.5mm). Disease incidence was 100%. This could indicate the importance of high sugar and low pH values needed for *P. digitatum* to infect and colonise stone fruits early after harvest. This can also be observed from Prusky *et al.* (2002; 2004) and Louw and Korsten (2014) on apples. Larger lesions were caused by *P. expansum* on 'Granny Smith' as compared to 'Rome' and 'Fuji' (Prusky *et al.*, 2002; 2004). *Penicillium digitatum*, *P. expansum* and *P. crustosum* caused larger lesions on Granny Smith than on other apple cultivars (Louw and Korsten, 2014). Granny Smith is one of the most acidic apple cultivars with moderate levels of sugar (Keller *et al.*, 2004).

It seems that the sugar/acid ratio became less important for infection and colonisation as fruit ripened. With 4d postharvest nectarine and plum, the pH significantly increased but °Brix stayed similar while disease incidence and lesion diameter increased for *P. digitatum* but remained the same for *P. expansum*. The acidity of cultivars/fruits thus affected lesion sizes (Louw and Korsten, 2014; Prusky *et al.*, 2002; 2004) but the change in TA/pH due to ripeness either had the opposite effect (*P. digitatum*) or no effect (*P. expansum*) on lesion diameter. Other host factors are affecting the interactions.

There was a drastic increase (more than plum) in lesion diameter and disease incidence of *P. digitatum* on riper nectarine (4d postharvest) after 5d incubation; $\Delta_{\text{lesion diameter}} = 15.27$mm and $\Delta_{\text{disease incidence}} = 61.48\%$. That of plum; $\Delta_{\text{lesion diameter}} = 7.92$mm and $\Delta_{\text{disease incidence}} = 20\%$. The pH, °Brix and firmness of plum showed smaller changes in comparison to that of nectarine. It is possible that the sharp decline in fruit firmness of riper nectarine could indicate a drastic advancement in fruit senescence and thus a possible decline in host resistance. This could be the reason for the greater increase in disease incidence and lesion diameter of *P. digitatum* on riper nectarine compared to plum. Investigating the host response in these interactions could provide useful information in this regard.

pH modulation was affected by host ripeness. This was specifically observed from nectarine. When pH was low (1d postharvest pH = 3.46), *P. digitatum* maintained pH while *P. expansum* increased it. With higher pH (4d postharvest pH = 3.63 and 7d postharvest pH = 3.86), *P. digitatum* lowered whereas *P. expansum* decreased or maintained pH. The pH at

infected sites later equalised at 3.4~3.5 for *P. digitatum*. For *P. expansum*, it increased (1d postharvest), remained at (4d postharvest) or dropped below (7d postharvest) pH 3.6. On plum, pH at infected sites slightly increased and later equalised at pH = 2.99–3.03 for *P. digitatum* and pH = 3.04–3.09 for *P. expansum*. These species will thus not only acidify but can also increase or maintain pH depending on the pH of the host. Environmental pH can be modulated or maintained via acidification. This takes place via the secretion of organic acids and uptake or utilisation of ammonium/ammonia. This mechanism can also be reverted by inhibiting the release of organic acids and causing the accumulation of ammonium/ammonia to increase environmental pH (Barad *et al.*, 2016a; Bi *et al.*, 2016; Prusky *et al.*, 2004).

Barad *et al*. (2016a) reported a dual pattern of pH modulation depending on growth conditions. This was first observed when *P. expansum* was grown on media with dynamic nutritional conditions over a period of roughly 10d. From *in vivo* work on Golden Delicious apples, acidification was reported in the middle of lesions but alkalinisation at lesion borders. The accumulation of ammonia was the potential reason for the increase in pH. Bi *et al.* (2016) reported the effect of carbon source on pH modulation via the accumulation of ammonia. *Penicillium expansum* grown on secondary medium with 15mM sucrose resulted in an increase in ammonia whereas nothing was detected on medium with 175mM sucrose. Gluconic acid contradicted this with high amounts produced at 175mM decreasing pH ≈ 4 and nothing produced at 15mM (pH increased >6). Sánchez-Torres and González-Candelas (2003) demonstrated an increase in pH (5.6) for *P. expansum* grown on minimal media containing apple pectin but a decrease in pH (3.6) of colonised apple tissue (72h incubation).

The pectolytic enzyme polygalacturonase (PG) plays a significant role in tissue maceration (Prusky *et al.*, 2004; Yao *et al.*, 1996). When isolated from *P. expansum*, PG was reported active over pH 3~6.5 but optimal between pH 4–5.5 (Jurick *et al.*, 2010; Yao *et al.*, 1996). This is also linked to nutritional conditions (Bi *et al.*, 2016). Expression of *pepg1* (endopolygalacturonase) from *P. expansum* grown on media with different pH levels revealed high expression levels at pH 4 and low levels at pH < 3.5 (Prusky *et al.*, 2004). With this considered, it was surprising to find neither of the *Penicillium* spp. modulating pH to a level where *PG* expression and PG activity was reported optimal. This was also the case in numerous other studies. The pH of Granny Smith, 'Gala' and Golden Delicious tissue colonised by *P. expansum* were respectively 3.64±0.01, 3.88±0.03 (Prusky *et al.*, 2004) and 3.6 (Sánchez-Torres and González-Candelas, 2003). That of Navel, Oro Blanco and *Citrus unshiu* colonised by *P. digitatum* were respectively 3.12±0.07, 3.10±0.14 (Prusky *et al.*,

2004) and 3.22±0.15 (Zhang *et al.*, 2013). Our study was the first to demonstrate pH 2.9–3.1 for tissue colonised by *P. digitatum* and *P. expansum*.

It is unclear why pH of infected tissue is so dissimilar to what is optimal for *PG* expression and PG activity. However, it has become known that expression of *PG* differs from *in vivo* to *in vitro* studies. In addition, PG activity depends on the *Penicillium* spp. it was isolated from (Jurick *et al.*, 2009; Jurick *et al.*, 2010). Differential expression for different PG genes was reported from *in vivo* and *in vitro* work for *P. digitatum* (López-Pérez *et al.*, 2015) and *P. expansum* (Sánchez-Torres and González-Candelas, 2003). *In vivo* (Golden Delicious) work from Sánchez-Torres and González-Candelas (2003) revealed no (24h) and very low expression (48h and 72h) of *pepg1* but decreased expression of *pepg2* while pH decreased (4 to 3.6). *In vitro*, *pepg1* was not expressed and *pepg2* decreased while pH increased (4.2 to 5.6). *In vivo* ('Navelina' oranges) work by López-Pérez *et al.* (2015) revealed expression of *pg1* increasing and fluctuating over 4d incubation. It later decreased at 7d incubation. For *pg2*, the expression increased and later equalised. From their *in vitro* work, *pg1* was highly expressed at 1d incubation and low at 2–4d incubation while *pg2* showed an increased over 4d incubation. More discrepancies between *in vitro* and *in vivo* work was observed from Barad *et al.* (2016b).

It was noted that *P. digitatum* lowered the pH of colonised tissue lower than *P. expansum* when considering results from infected nectarine, plum (both species), citrus (*P. digitatum*) and apples (*P. expansum*) (Prusky *et al.*, 2004; Zhang *et al.*, 2013). The activity of PG extracted from *P. expansum* was also very different from that of *P. solitum* (Jurick *et al.*, 2009; Jurick *et al.*, 2010). This indicates that the activity of PG isolated from *P. digitatum* will possibly be higher at lower pH levels as compared to PG from *P. expansum* and *P. solitum*.

The pH of colonised plum tissue was very similar to the pH of control fruit. The combined pH of 1d, 4d and 8d postharvest fruit prior to inoculation was 3.00±0.03. The combined pH of colonised tissue after 4d incubation was 3.00±0.01 (*P. digitatum*) and 3.06±0.01 (*P. expansum*). This was not the case with nectarine. The Δ_{pH} was similar to relatively similar for 1d (Δ_{pH} = 0.04 for *P. digitatum* and 0.18 for *P. expansum*) and 4d (Δ_{pH} = 0.24 and 0.01 respectively) postharvest fruit but not for 7d (Δ_{pH} = 0.39 and 0.38 respectively) postharvest fruit. Similarities were observed on apple cultivars. The pH values of decayed tissue from *P. expansum* infected apples after 7d incubation for Fuji = 3.96, Gala = 3.88,

Golden Delicious = 3.88, Granny Smith = 3.64 and 'Red Delicious' = 4.07 (Prusky *et al.*, 2004). Barad *et al.* (2016a) reported pH = 3.58 for Golden Delicious apples infected with *P. expansum* after 5d incubation. The authors did not specify the pH of control fruit (uninfected) and/or how fresh apples were prior to inoculation. The pH values from the freshly harvested apple cultivars: Fuji = 3.91, Gala = 3.86, Golden Delicious = 3.64, Granny Smith = 3.42 and Red Delicious = 4.10 (Keller *et al.*, 2004). Thus Δ_{pH} by combining results from Prusky *et al.* (2004) and Keller *et al.* (2004): Fuji = 0.05, Gala = 0.02, Golden Delicious = 0.24, Granny Smith = 0.22 and Red Delicious = 0.03. Even though the pH varies depending on incubation periods, the ability of *P. digitatum* and *P. expansum* to maintain the pH of colonised tissue similar/close to the initial pH prior to infection was displayed. The pH was lowered in cases where it was too high either naturally (citrus peel) or due to ripening. pH modulation was host (Bi *et al.*, 2016; Prusky *et al.*, 2004) and *Penicillium* spp. specific.

Differences between pH of uncolonised tissue of infected fruit and control fruit were host dependent. On nectarine, the differences between *P. digitatum* colonised and uncolonised tissue (4d incubation) increased as fruit ripened: 1d postharvest Δ_{pH} = 0.06, 4d postharvest Δ_{pH} = 0.21 and 7d postharvest Δ_{pH} = 0.53. With *P. expansum*: Δ_{pH} = 0.11, Δ_{pH} = 0.16 and Δ_{pH} = 0.32 (respectively). Here differences between Δ_{pH} colonised vs uncolonised and Δ_{pH} colonised vs control fruit were small: $\Delta\Delta_{pH}$ for *P. digitatum* = 0.05–0.1 and $\Delta\Delta_{pH}$ for *P. expansum* = 0.04–0.17 (all ripeness levels considered). This was not the case with plum: *P. digitatum* $\Delta\Delta_{pH}$ = 0.16–1.8 and *P. expansum* $\Delta\Delta_{pH}$ = 0.6–1.55. The reason was that infected plum underwent rapid ripening as compared to slower ripening (natural) of control fruit (uninfected plum). The increase in pH of uncolonised tissue due to ripening and the maintaining of an acidic environment of colonised tissue resulted in an increased Δ_{pH}. Combining data from Prusky *et al.* (2004) and Keller *et al.* (2004) revealed similarities on apples infected by *P. expansum*. The Δ_{pH} = 0.31–0.88 for colonised vs uncolonised tissue (Prusky *et al.*, 2004) and Δ_{pH} = 0.02–0.24 for colonised tissue vs uninfected fruit (Keller *et al.*, 2004; Prusky *et al.*, 2004), thus $\Delta\Delta_{pH}$ = 0.29–0.64.

Fresh plum was a more suitable environment than fresh nectarine (1d postharvest) for infection and colonisation. Although similarities could be drawn between the hosts, lesions were larger, and disease incidence and gene expression activities higher on plum at the different incubation periods (24h and 48h). The expression profile of *P. digitatum* was different from *P. expansum*. On plum, *P. digitatum* upregulated *PG* and the 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase gene (*ACCD*) and downregulated

pacC and *creA* while *P. expansum* only upregulated *ACCD* (remainder of genes were downregulated). On nectarine, *P. digitatum* upregulated *ACCD* and downregulated *pacC* while *P. expansum* upregulated *ACCD* and downregulated *PG* and *pacC*.

Downregulation of *PG* observed from *P. expansum* was also reported on Golden Delicious apples. Few reports have investigated the expressional changes of *PG* over time. No expression of *pepg1*, a *PG* similar to Yao *et al.* (1996) and ours, and a decreased expression of *pepg2* were reported from 24h to 48h from *in vitro* (minimal media with apple pectin) work conducted by Sánchez-Torres and González-Candelas (2003). They also reported no expression of *pepg1* 24h after heat treatment and similar (very low) expression 48h and 72h after heat treatment of apples. *Pepg2* was downregulated over the 72h period. Differential expression of the PG genes was suspected due to environmental pH. In the case of *P. digitatum*, results correspond with López-Pérez *et al.* (2015) who inoculated Navelina oranges and observed expression profiles over a 4d incubation period. They found two PG genes (*pg1* and *pg2*) increasing and/or remaining constant over the incubation period.

The low pH of the host would cause a decrease in *pacC* and *ACCD* expression based on *in vitro* studies (Barad *et al.*, 2016a; 2016b; Zhang *et al.*, 2013). However, new reports revealed the importance of ammonium/ammonia as regulator of these genes under acidic conditions. The genes were not similarly affected by the accumulation of ammonium/ammonia. *PacC* was more affected by pH whereas *ACCD* had a stronger reaction to ammonium/ammonia (Barad *et al.*, 2016a; 2016b). The low pH of nectarine and plum could be the reason for a decrease in the expression of *pacC*. Ammonium concentrations were unfortunately not tested in this study but the upregulation of *ACCD* could be an indication of increased ammonium levels.

Ammonium is used as a nitrogen source by *Penicillium* spp. (Ross and Luckner, 1984). It has also been associated with enhanced responsiveness of *pacC*, environmental pH modulation and induced expression of few genes (i.e. *PG* and *ACCD*) linked to host cell damage (pectolytic enzymes and toxins) (Barad *et al.*, 2016a; 2016b; Song *et al.*, 2014). ACC is a precursor of ethylene (Glick, 2014). ACCD regulates ACC levels and is thus also induced by its accumulation. Overexpression of ACC will eventually lead to overproduction of ethylene, resulting in host stress (i.e. advanced ripening, senescence, chlorosis and leaf abscission) (Glick, 2014; Jia *et al.*, 2000). ACCD will cleave ACC, in the process producing more ammonia and α-ketobutyrate (Glick, 2014; Jia *et al.*, 2000).

Ethylene can be produced via a few biosynthetic pathways. *Penicillium digitatum* and *P. expansum* can synthesise ethylene via ACC, 2-keto-4-methylthiobutyric acid and 2-oxoglutarate. Recent findings have shown that neither the precursors themselves nor the ethylene produced by them affected the growth of *P. digitatum* or *P. expansum* on PDA. Although ACC affected conidiation by *P. expansum*, it was not associated with ethylene production by the *Penicillium* spp. (Yang *et al.*, 2017). Accumulation of ACC can, however, be used to manipulate host responses (i.e. advance host ripening) (Glick, 2014).

Physical, microbial or environmental stress inflicted on the host also affect synthesis of ACC. The stress effect caused by the excess of ethylene is called stress ethylene. ACCD produced by plant growth-promoting bacteria can help to overcome this stress effect (Glick, 2014). In the case of pathogens [*P. expansum* (Barad *et al.*, 2016b) and *P. citrinum* (Jia *et al.*, 2000)], ACCD leading to the production of ammonia feeds the pathogen's nitrogen metabolism, can be used to modulate environmental pH and regulate certain genes (Barad *et al.*, 2016a; 2016b). This can be used to facilitate pathogen infection, invasion and colonisation.

The increased ripening of fruit due to infection was more obvious on plum than on nectarine. This is because, unlike plum, uncolonised tissue (infected nectarine) exhibited similar pH values to control fruit (uninfected nectarine). However, the upregulation of *ACCD* in infected nectarine and plum could be an indication of increased ripening as a result of the infection itself (stress ethylene) (Glick, 2014) and/or ACC synthesised by the *Penicillium* spp. (Barad *et al.*, 2016b; Yang *et al.*, 2017). Looking at other fruit indices could have proven useful here. Future work should also consider viewing ammonium/ammonia levels in infected fruit (colonised and uncolonised tissue).

The reason for the downregulation of *creA* in plum and upregulation in nectarine is somewhat unclear. The °Brix of 1d postharvest plum was 13.13 as compared to 12.7 for nectarine. It is possible that the sugar composition played a role here. Nectarine has a higher sucrose level (g/kg) (Colarič *et al.*, 2004) than plum (Roussos *et al*., 2015). 'Fortune' specifically has low concentrations of glucose (Roussos *et al*., 2015). Expression of *creA* increased under higher sucrose and glucose conditions (*in vitro*) (Bi *et al.*, 2016; Fernandez *et al.*, 2012; 2014).

Ripeness had a large effect on the gene expression of the *Penicillium* spp. on nectarine but not plum. This can primarily be ascribed to 1d postharvest plum already being more

appropriate for infection and colonisation than 1d postharvest nectarine. In terms of gene expression, the difference in expression of *PG* on plum vs nectarine was the strongest indicator for this. On plum, *P. digitatum* only downregulated *ACCD* whereas *P. expansum* upregulated *pacC* and *creA*. On nectarine, *P. digitatum* upregulated *PG* and *creA* while *P. expansum* downregulated *PG* and upregulated *pacC*.

Riper fruits had higher sugar content which led to the upregulation of *creA* by *P. expansum* on plum and *P. digitatum* on nectarine. The higher sugar content will lower ammonium/ammonia production by the *Penicillium* spp. (Bi *et al.*, 2016) and thus possibly the downregulation of *ACCD* as seen from *P. digitatum* on plum (in all other cases *ACCD* was downregulated but nonsignificantly). It was interesting to note the upregulation of *ACCD* from 24h to 48h on 1d postharvest fruits but downregulation when comparing 1d postharvest to 7d/12d postharvest fruits. This indicates the need for the *Penicillium* spp. to only synthesise more ACC when fruits are fresh (1d postharvest). Riper nectarine and plum were possibly already at a state that didn't require the upregulation of *ACC*.

The upregulation of *pacC* by *P. expansum* on nectarine and plum may indicate an alternative mechanism used by this species to cause cell degradation (i.e. mycotoxins). *PacC* plays a significant role in pH modulation (D-gluconic acid) and regulation of secondary metabolites such as mycotoxins (i.e. patulin) (Barad *et al.*, 2016a). Li *et al.* (2015) revealed that patulin biosynthesis of *P. expansum* also depends on certain nutritional conditions. The optimal pH range for patulin production as reported in apple juice was 3.2–3.8 (Damoglou and Campbell, 1986). *Penicillium expansum* modulated pH close to or within this range.

The downregulation of *pacC* from *P. expansum* from 24h to 48h (1d postharvest) and upregulation when fruits were riper can be linked to host acidity. At 1d postharvest, nectarine and plum were more acidic and thus the downregulation of *pacC* (also observed with *P. digitatum*) but riper fruits with higher initial pH led to it being upregulated. *Penicillium digitatum* decreased and maintained a more acidic environment than *P. expansum*, the reason for the downregulation of *pacC* even on riper fruits.

Research on the effect of different carbon levels at different maturity or ripeness levels on pH modulation is limited (Bi *et al.*, 2016). The carbon catabolite repression is regulated by creA which ensures that certain carbon sources like glucose (Fernandez *et al.*, 2012; 2014) or sucrose (Bi *et al.*, 2016) are preferably utilised over other carbon sources. The mechanism also plays a role in pH modulation. Bi *et al.* (2016) showed excess sucrose will result in *P.*

expansum producing less ammonia and more gluconic acid (acidification). This environment (*in vitro*) will cause a decrease in expression of *ACCD* and *pacC* (Barad *et al.*, 2016a; 2016b). Excess carbon (i.e. sucrose) can even lead to acidification by alkalinising pathogens and alkalinisation by acidifying pathogens (Alkan *et al.*, 2013; Bi *et al.*, 2016; Ment *et al.*, 2015). The synergetic effect observed between pH, carbon and nitrogen sources complicate *in vivo* studies. This makes host-pathogen interactions more specific and with it more complex than originally thought.

More research is needed to identify the significant shift in lesion size caused by *P. digitatum* on riper stone fruits. Although upregulation of *PG* can be linked to the increase in lesion size on nectarine, it was not the case with plum. In addition, *PG* was downregulated by *P. expansum* on both hosts from 24h to 48h incubation while lesions continued to increase over the same period. Other factors, undetermined in this study, are contributing to the significant shift in lesion diameter and disease incidence of the newly discovered disease interaction between *P. digitatum* and stone fruits (nectarine and plum). Neither can we say enough is known of the interaction between *P. expansum* and stone fruits as very few host-pathogen interaction studies have focused or reported on it.

Since fruit ripeness played a large role in decay caused by *P. digitatum* on nectarine and plum, host physiology was considered the most important factor/s to investigate. Future work should now focus on determining the decline in host resistance as fruit ripen (Prusky *et al.*, 2016) to possibly reveal the true cause/s or trigger/s for the opportunistic lifestyle observed from *P. digitatum*. This lifestyle strategy can specifically be linked to stone fruits. This is confirmed by comparing decay caused by *P. digitatum* on nectarine and plum to lemon (Louw and Korsten, 2015) when under cold storage. Even with low concentrations (6.3×10^4 conidia/ml), *P. digitatum* was able to cause large lesion (43.8±5.6mm) on Eureka seeded lemons after 26 days refrigeration (5.0±0.7°C and 86.4±4.5% RH). The environment and host thus determine the opportunistic nature of *P. digitatum*. This lifestyle can also be expected on pome fruits (Louw and Korsten, 2014; Vilanova *et al.*, 2014).

This is one of the first studies to compare substantial decay (disease incidence and lesion diameter) caused by *P. digitatum* and *P. expansum* on the same host. This is because *P. digitatum* was only recently described as highly aggressive on pome and stone fruits (Louw and Korsten, 2014; 2016). The mechanisms used by these pathogens to infect and colonise are quite different. Host and environmental conditions have a dissimilar effect on these

pathogens, in return affecting infection, colonisation and symptom development. Future research should further investigate and compare the host-pathogen interactions of these pathogens on other stone fruit types and cultivars.

A new approach is needed to investigate pathogenesis. The manipulation of the disease pyramid (chapter 2) by humans, intentionally or not, has led to the selection of new pathogens or host-pathogen interactions. This has specifically been observed from opportunistic pathogens in situations where the host is impaired and environmental conditions favour the pathogen (Brown *et al*., 2012; Casadevall *et al*., 2011; Shapiro-Ilan *et al*., 2005). The more boundaries are pushed to extend the storage life of fresh produce, the more likely the chances might be for new host-pathogen interactions to result. With the discovery of one such pathogen, this study was able to question and contribute to current understanding of the concept of pathogenicity and how disease is perceived. More studies need to investigate such cases by identifying and studying the causal agents of losses in conditions where the disease pyramid is influenced in favour of the pathogen to increase disease occurrence and development.

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Appendices

APPENDIX A

Table 2.1 Definitions of biological terms applied in or applicable to pathogen and pathogenicity

Term	Definition	Source
Pathogenicity	*Pathogenic - Able to cause or produce disease (Collins Dictionary, 2015).	D(E)
	The ability of a parasite to damage the host and to cause disease. Pathogenic - Causing disease (Lawrence, 2011).	D(B)
	*Pathogenic - (Capable of) causing or producing disease (Cammack *et al*., 2006).	D(BM)
	*Pathogenic - Causing or capable of causing disease (Merriam-Webster, 2015).	D(M)
	The ability to cause disease (D'Arcy *et al*., 2001; Rudolph, 1995; Sharma, 2004).	D(P)+T
	The quality or state of being pathogenic; the potential ability to produce disease; the disease-producing ability of a microorganism. Applied to groups or species of microorganisms, whereas virulence is used in the sense of the degree of pathogenicity within the group or species. Some regard pathogenicity as the genetically determined ability to produce disease and virulence as the non-genetically determined ability to produce disease (Lacey and Brooks, 1997; Onstad *et al*., 2006; Shapiro-Ilan *et al*., 2005; Steinhaus and Martignoni, 1970; Tanada and Kaya, 1993). Pathogenicity is qualitative, an all-or-none concept (Onstad *et al*., 2006; Shapiro-Ilan *et al*., 2005).	D(I)+G+ R+T
	The capability to cause disease. Virulence preferred to indicate the degree to which a pathogen is able to induce disease (Dunster and Dunster, 1996).	D(N)
	The capacity to produce disease. A level of pathogenicity can be specified based on the number of infected individuals showing symptoms. Pathogenicity is however not related to disease severity (virulence). Alternatively, pathogenicity can thus be defined as the number of persons who develop disease (symptoms) in proportion to those infected. Pathogenicity usually lacks actual measurements based on the difficulty to specify what constitutes as clinical disease and the difficulty to determine the infected in the absence of clinical disease. Pathogenicity can thus be related to the frequency of occurrence of asymptomatic infections. This is of great importance to epidemiologists (Barr, 1978).	R
	*Pathogenic - Ability to incite disease (Shaw III and Loopstra, 1988).	R
	The capability of a pathogen to cause disease (Agrios, 2005).	T
	The capability of an infectious agent to induce pathology or disease in a host (Schmidt-Posthaus and Wahli, 2015).	T
	Potential of a strain to induce disease or hypersensitive response in a plant species (Singh *et al*., 1995).	T
	The intrinsic capability of a microorganism to penetrate the host defences and cause disease (Siegel, 2012).	T
	The qualitative ability of a pathogen to cause disease (Whipps and Lumsden, 2001).	T

Term	Definition	Source
Pathogen	Any agent that can cause disease (Collins Dictionary, 2015).	D(E)
	Any disease-causing organism (Lawrence, 2011).	D(B)
	Any disease-causing microorganism. Pathogens include viruses and many bacteria, fungi, and protozoans (Martin and Hine, 2015).	D(B)
	Any agent, especially any living organism that can cause disease (Cammack *et al*., 2006).	D(BM)
	A specific causative agent (as a bacterium or virus) of disease (Merriam-Webster, 2015).	D(M)
	A disease-producing organism or biotic agent (D'Arcy *et al*., 2001).	D(P)
	A specific cause of disease. A microorganism capable of producing disease under normal conditions of host resistance and rarely living in close association with the host without producing disease. Any microorganism, virus, substance, or factor causing disease (Onstad *et al*., 2006).	D(I)
	A disease-inducing organism or abiotic agent (Dunster and Dunster, 1996).	D(N)
	An agent (biotic or abiotic) that causes plant disease (Arneson, unknown date).	G
	*Nonpathogen - An organism not inducing disease when challenging another (Andrivon, 1993).	R
	An agency which incites disease (Walker, 1957).	T
	Agent (living or inanimate) that interferes with the physiological process of a plant (McNew, 1960).	T
	An entity that can incite disease (Agrios, 2005).	T
	An inducer of disease (Cowling and Horsfall, 1979).	T
Disease	1. Any impairment of normal physiological function affecting all or part of an organism, especially a specific pathological change caused by infection, stress, etc., producing characteristic symptoms; illness or sickness in general. 2. A corresponding condition in plants. 3. Any situation or condition likened to this (Collins Dictionary, 2015).	D(E)
	A condition in which the normal function of some part of the body (cells, tissues, or organs) is disturbed. A variety of microorganisms and environmental agents are capable of causing disease. The functional disturbances are often accompanied by structural changes in tissue (Martin and Hine, 2015).	D(B)
	Any anatomical abnormality or impairment of the normal functioning of an organism or of any of its parts other than one arising directly from physical injury. It may be caused by environmental factors (e.g. malnutrition, toxic agents, etc.), infective agents (bacteria, viruses, etc.), inherent defects in the organism (e.g. genetic disease), or any combination of these factors (Cammack *et al*., 2006).	D(BM)
	An impairment of the normal state of the living animal or plant body or one of its parts that interrupts or modifies the performance of the vital functions, is typically manifested by distinguishing signs and symptoms, and is a response to environmental factors (as malnutrition, industrial hazards, or climate), to specific infective agents (as worms, bacteria, or viruses), to inherent defects of the organism (as genetic anomalies), or to combinations of these factors (Merriam-Webster,	D(M)

	2015).	
	The abnormal functioning of an organism (D'Arcy *et al*., 2001).	D(P)
	Lack of ease. Departure from the state of health or normality. Condition or process (not a thing) that represents the response of an animal's body to injury or insult. A disturbance of function or structure of a tissue or organ of the body, or of the body in general (Lacey and Brooks, 1997; Onstad *et al*., 2006).	D(I)+T
	An abnormal condition of a plant in which its physiology, morphology, and/or development is altered under the continuous influence of a pathogen (Arneson, unknown date).	G
	Injurious physiological activity, caused by the continued irritation of a primary causal factor, exhibited through abnormal cellular activity and expressed in characteristic pathological condition called symptoms (Whetzel, 1935).	T
	Disease is not a condition… Disease is not the pathogen… Disease is not the same as injury… Disease results from continuous irritation… Disease is a malfunctioning process… (Horsfall and Dimond, 1959).	T
	Disease is an abnormal physiology process in plants. The efficiency of a plant is so reduced that it cannot make maximum use of the factors of its environment for growth and reproduction (McNew, 1960).	T
	Disease is a malfunctioning process that is caused by continuous irritation. This process results in suffering. Hence, disease can be defined as a pathological process (Sharma, 2004).	T
	Any malfunctioning of host cells and tissues that result from continuous irritation by a pathogenic agent or environmental factor and leads to the development of symptoms (Agrios, 2005).	T
Parasite	An animal or plant that lives in or on another (the host) from which it obtains nourishment. The host does not benefit from the association and is often harmed by it (Collins Dictionary, 2015).	D(E) - biological definition
	An organism that for all or some part of its life derives its food from a living organism of another species (the host). It usually lives in or on the body or cells of the host, which is usually harmed to some extent by the association (Lawrence, 2011).	D(B)
	Any organism that spends all or part of its life cycle in (endoparasite) or on (ectoparasite) another living organism of a different species (its host), from which it obtains nourishment and/or protection, and to which it is usually detrimental (Cammack *et al*., 2006).	D(BM)
	An organism living in, with, or on another organism in parasitism (Merriam-Webster, 2015).	D(M)
	In intimate association with another organism on which it depends for its nutrition; not necessarily a pathogen (contrasts with saprophyte) (D'Arcy *et al*., 2001).	D(P)
	An organism that lives at its host's expense, obtaining nutrient from the living substance of the latter, depriving it of useful substance, or exerting other harmful influence upon it (Lacey and Brooks, 1997; Onstad *et al*., 2006).	D(I)+T
	An organism living in or on another living organism (host) from which it extracts nutrients (Arneson, unknown date).	G

	An organism living on or in another living organism (host) and obtaining its food from the latter (Agrios, 2005).	T
Parasitism	1. The relationship between a parasite and its host. 2. The state of being infested with parasites. 3. The state of being a parasite (Collins Dictionary, 2015).	D(E)
	A special case of symbiosis in which one partner (the parasite) receives advantage to the detriment of the other (the host) (Lawrence, 2011).	D(B)
	An association in which one organism (the parasite) lives on (ectoparasitism) or in (endoparasitism) the body of another (the host), from which it obtains its nutrients. Some parasites inflict comparatively little damage on their host, but many cause characteristic diseases (these are, however, never immediately fatal, as killing the host would destroy the parasite’s source of food; compare parasitoid). Parasites are usually highly specialised for their way of life, which may involve one host or several (if the life cycle requires it). They typically produce vast numbers of eggs, very few of which survive to find their way to another suitable host. Obligate parasites can only survive and reproduce as parasites; facultative parasites can also live as saprotrophs. The parasites of humans include fleas and lice (which are ectoparasites), various bacteria, protozoans, and fungi (endoparasites causing characteristic diseases), and tapeworms (e.g. *Taenia solium*, which lives in the gut) (Martin and Hine, 2015).	D(B)
	An intimate association between organisms of two or more kinds; especially one in which a parasite obtains benefits from a host which it usually injures (Merriam-Webster, 2015).	D(M)
	A symbiotic relationship between individuals of two different species in which the host is harmed and the parasite benefits (Onstad *et al*., 2006).	D(I)
	*Parasitic - Having the characteristics of a parasite (Mai and Mullin, 1996).	T
	A symbiotic relationship between two different species in which one (the parasite) benefits at the expense of the other (the host) (Lacey and Brooks, 1997).	T
	The relationship or association between organisms, usually belonging to different species, in which one party, the parasite, benefits from the other, the host. It is often assumed, sometimes incorrectly, that the parasitic relationship is necessarily harmful to the host. Parasitism and disease are distinct biological phenomena that should not be confused (Bateman, 1978).	T
	The removal of food by a parasite from its host (Agrios, 2005).	T
Host	1. An animal or plant that nourishes and supports a parasite. 2. An animal, especially an embryo, into which tissue is experimentally grafted (Collins Dictionary, 2015).	D(E) - biological definition
	1. Any organism on which another organism spends part or all of its life, and from which it derives nourishment or gets protection. 2. The recipient of grafted or transplanted tissue (Lawrence, 2011).	D(B)
	1. An organism whose body provides nourishment and shelter for a parasite or a parasitoid. A definitive (or primary) host is one in which an animal parasite becomes sexually mature; an intermediate (or secondary) host is one in which the parasite passes the larval or asexual	D(B)

	stages of its life cycle. 2. An organism that lives in close association with an inquiline. 3. A cell or organism into which foreign DNA is introduced during gene cloning (Martin and Hine, 2015).	
	1. Any organism in which another organism, especially a parasite or symbiont, spends part or all of its life cycle and from which it obtains nourishment and/or protection. 2. Any organism that harbours a pathogenic or nonpathogenic infectious agent. 3. The recipient of a transplanted tissue or organ graft. 4. A cell or organism that contains recombinant DNA (Cammack *et al*., 2006).	D(BM)
	1. A living animal or plant on or in which a parasite lives. 2. The larger, stronger, or dominant one of a commensal or symbiotic pair. 3a. An individual into which a tissue or part is transplanted from another. 3b. An individual in whom an abnormal growth (as cancer) is proliferating (Merriam-Webster, 2015).	D(M)
	Host plant: a living plant attacked by or harbouring a parasite or pathogen and from which the invader obtains part or all of its nourishment (D'Arcy *et al*., 2001).	D(P)
	An invertebrate that harbours or nourishes another organism. See Accidental host, Alternate host, Definitive host, Intermediate host, Natural host, Normal host, Host of choice, Primary host, Secondary host, Substitute host, Transport host, Typical host (Onstad *et al*., 2006).	D(I)
	A plant that supports the growth and development of the parasite that has infected it (Arneson, unknown date).	G
	A host in which the pathogenic microorganism (or parasite) is commonly found and in which the pathogen can complete its development. The term 'natural host' implies that the host is the usual one and is synonymous with 'typical host' (Lacey and Brooks, 1997).	T
	A plant that is invaded by a parasite and from which the parasite obtains its nutrients (Agrios, 2005).	T
Symbiont	An organism living in a state of symbiosis (Collins Dictionary, 2015).	D(E)
	One of the partners in a symbiosis (Lawrence, 2011).	D(B)
	An organism that is a partner in a symbiotic relationship (Martin and Hine, 2015).	D(B)
	An organism that lives as a partner in a symbiosis (Cammack *et al*., 2006).	D(BM)
	An organism living in symbiosis; especially the smaller member of a symbiotic pair. Also called symbiote (Merriam-Webster, 2015).	D(M)
	An organism living in symbiosis. Usually the smaller member of a symbiotic pair of dissimilar size (also called Microsymbiont). Frequently, those microorganisms associated in a regular mutualistic manner with insects and other invertebrates (Lacey and Brooks, 1997; Onstad *et al*., 2006).	D(I)+T
	One member of a symbiotic relationship (Roberts and Boothroyd, 1984).	T

	1. A close and usually obligatory association of two organisms of different species that live together, often to their mutual benefit. 2. A similar relationship between interdependent persons or groups (Collins Dictionary, 2015).	D(E)
	1. Close and usually obligatory association of two organisms of different species living together, not necessarily to their mutual benefit. 2. Often used exclusively for an association in which both partners benefit, which is more properly called mutualism (Lawrence, 2011).	D(B)
	An interaction between individuals of different species (symbionts). The term symbiosis is usually restricted to interactions in which both species benefit, but it may be used for other close associations, such as commensalism, inquilinism, and parasitism. Many symbioses are obligatory (i.e. the participants cannot survive without the interaction); for example, a lichen is an obligatory symbiotic relationship between an alga or a cyanobacterium and a fungus (Martin and Hine, 2015).	D(B)
	A long-term association between individuals belonging to two different species. The term is often used in a restricted sense to denote associations that are beneficial to one or both partners, although strictly it refers equally to neutral or harmful associations (Cammack *et al.*, 2006).	D(BM)
	1. The living together of two dissimilar organisms in more or less intimate association or close union. 2. The intimate living together of two dissimilar organisms in a mutually beneficial relationship; especially mutualism (Merriam-Webster, 2015).	D(M)
	The living together of two different kinds of organisms that may, but does not necessarily, benefit each organism (D'Arcy *et al.*, 2001).	D(P)
	The living together of individuals of two different species. Especially the living together of dissimilar organisms in a more or less intimate association (as in Mutualism, Commensalism and Parasitism) (Lacey and Brooks, 1997; Onstad *et al.*, 2006).	D(I)+T
Symbiosis	A mutually beneficial association of two or more different kinds of organisms (Agrios, 2005).	T
	1. Any of the taxonomic groups into which a genus is divided, the members of which are capable of interbreeding: often containing subspecies, varieties, or races. A species is designated in italics by the genus name followed by the specific name, for example, *Felis domesticus* (domestic cat). 2. The animals of such a group. 3. Any group of related animals or plants not necessarily of this taxonomic rank (Collins Dictionary, 2015).	D(E) - biological definition
	In sexually reproducing organisms, a group of interbreeding individuals not normally able to interbreed with other such groups. A species is given two names in binomial nomenclature (e.g. *Homo sapiens*), the generic name and specific epithet (italicised in the scientific literature), similar and related species being grouped into genera. Species can be subdivided into subspecies, geographic races, and varieties (Lawrence, 2011).	D(B)
Species	1. A group of organisms that resemble each other more than they resemble members of other groups and cannot be subdivided into two	D(B)

	or more species. The precise definition of what constitutes a species differs depending on which species concept is applied. According to the biological species concept, a species comprises a group of individuals that can usually breed among themselves and produce fertile offspring. However, many other species concepts have been proposed, including the phylogenetic species concept and various typological species concepts. Typically, a species consists of numerous local populations distributed over a geographical range. Within a species, groups of individuals become reproductively isolated because of geographical or behavioural factors, and over time may evolve different characteristics and form a new and distinct species. 2. A rank, or category, used in the classification of organisms. Similar species are grouped into a genus, and a single species may be subdivided into subspecies or races (Martin and Hine, 2015).	
	A fundamental taxonomic category ranking below a genus and consisting of a group of closely related individuals that can interbreed freely to produce fertile offspring (Cammack *et al*., 2006).	D(BM)
	The basic category of biological classification, displaying a high degree of mutual similarity determined by a consensus of informed opinion; a subcategory of genus (Singleton and Sainsbury, 1987).	D(BM)
	1a. A category of biological classification ranking immediately below the genus or subgenus, comprising related organisms or populations potentially capable of interbreeding, and being designated by a binomial that consists of the name of the genus followed by a Latin or latinised uncapitalised noun or adjective agreeing grammatically with the genus name. 1b. An individual or kind belonging to a biological species. 2. A particular kind of atomic nucleus, atom, molecule, or ion (Merriam-Webster, 2015).	D(M)
	Any one kind of life subordinate to a genus but above a race; a group of closely related individuals of the same ancestry, resembling one another in certain inherited characteristics of structure and behaviour and relative stability in nature; the individuals of a species ordinarily interbreed freely and maintain themselves and their characteristics in nature (D'Arcy *et al*., 2001).	D(P)
Infectious agent	An agent capable of producing infection (Heikens, 2003; Khan, 2014).	T
	An agent capable of causing infection (Weber and Rutala, 2001).	T

D, Dictionary of English **(E)**, biology **(B)**, biochemistry, molecular biology or microbiology **(BM)**, medicine **(M)**, plant pathology, **(N)** natural resource management, **(P)** or invertebrate pathology **(I)**; **G,** Glossary (online or printed); **R,** Review/article; **T,** Textbook.
*Definition for the specific term not provided.

APPENDIX B

Table 2.2 Definitions of common terms applied in or applicable to pathogen and pathogenicity

Term	Definition	Source
Ability	1. Possession of the qualities required to do something; necessary skill, competence, or power. 2. Considerable proficiency; natural capability. 3. Special talents (plural) (Collins Dictionary, 2015).	D(E)
	The power or skill to do something 1a. The quality or state of being able; especially physical, mental, or legal power to perform. 1b. Competence in doing. 2. Natural aptitude or acquired proficiency (Merriam-Webster, 2015).	D(E) - full definition
	1. To do something. The fact that somebody/something is able to do something. 2. A level of skill or intelligence (Turnbull *et al*., 2010).	D(A)
	1. Possession of the means or skill to do something. 2. Skill or talent (Stevenson and Waite, 2011).	D(C)
	It primarily denotes the quality or character of being able (as to do or perform) and is applied chiefly to human beings (Merriam-Webster Inc., 1984).	D(S)
	A person's power of body or mind (Garner, 2009).	D(U)
Capability	1. The quality of being capable; ability. 2. The quality of being susceptible to the use or treatment indicated. 3. A characteristic that may be developed; potential aptitude (usually plural) (Collins Dictionary, 2015).	D(E)
	The ability to do something 1. The quality or state of being capable. 2. A feature or faculty capable of development. 3. The facility or potential for an indicated use or deployment (Merriam-Webster, 2015).	D(E) - full definition
	1. The ability or quality necessary to do something. 2. The power or weapons that a country has for war or for military action (Turnbull *et al*., 2010).	D(A)
	Power or ability to do something (an undeveloped or unused faculty) (Stevenson and Waite, 2011).	D(C)
	It is the character in a person (less often, a thing) arising from the possession of the qualities or qualifications necessary to the performance of a certain kind of work or the achievement of a given end (Merriam-Webster Inc., 1984).	D(S)
	1. Power or ability in general, whether physical or mental. 2. The quality of being able to use or be used in a specific way (Garner, 2009).	D(U)
Capacity	1. The ability or power to contain, absorb, or hold. 2. The amount that can be contained; volume. 3a. The maximum amount something can contain or absorb (especially in the phrase filled to capacity). 3b. A capacity crowd (as modifier).	D(E)

	4. The ability to understand or learn; aptitude; capability. 5. The ability to do or produce (often in the phrase at capacity). 6. A specified position or function. 7. A measure of the electrical output of a piece of apparatus such as a motor, generator, or accumulator. 8. A former name for capacitance (electronics). 9a. The number of words or characters that can be stored in a particular storage device. 9b. The range of numbers that can be processed in a register (computing). 10. The bit rate that a communication channel or other system can carry. 11. Legal competence (Collins Dictionary, 2015).	
	The ability to hold or contain people or things; the largest amount or number that can be held or contained; the ability to do something: a mental, emotional, or physical ability 1. Legal competency or fitness. 2a. The potential or suitability for holding, storing, or accommodating. 2b. The maximum amount or number that can be contained or accommodated. 3a. An individual's mental or physical ability. 3b. The faculty or potential for treating, experiencing, or appreciating. 4. Duty, position, role. 5. The facility or power to produce, perform or deploy. Capability; also maximum output. 6a. Capacitance. 6b. The quantity of electricity that a battery can deliver under specified conditions (Merriam-Webster, 2015).	D(E) - full definition
	1. The number of things or people that a container or space can hold. 2. The ability to understand or to do something. 3. The official position or function that somebody has. 4. The quantity that a factory, machine, etc. can produce. 5. The size or power of a piece of equipment, especially the engine of a vehicle (Turnbull *et al.*, 2010).	D(A)
	1. The maximum amount that something can contain or produce (fully occupying the available space). 2. The ability or power to do something (a person's legal competence). 3. A specified role or position. 4. Electrical capacitance (Stevenson and Waite, 2011).	D(C)
	The power or more especially potentiality of receiving, holding, absorbing, or accomplishing something expressed or understood and is said of persons or thing (Merriam-Webster Inc., 1984).	D(S)
	Literally "roomy, spacious" refers figuratively to a person's physical or mental power to receive. The power or ability to receive, hold or contain. In law, it is frequently used in the sense "legal competency or qualification" (Garner, 2009).	D(U)
Cause	To be the cause of; bring about; precipitate; be the reason for (Collins Dictionary, 2015).	D(E) - verb definition
	To make (something) happen or exist: to be the cause of (something);	D(E) -

	to make (someone) feel, have, or do something 1. To serve as a cause or occasion of. 2. To compel by command, authority, or force (Merriam-Webster, 2015).	verb full definition
	The person or thing that makes something happen (Turnbull *et al*., 2010).	D(A)
	Make (something, especially something bad) happen, be the cause of (Stevenson and Waite, 2011).	D(C)
	It is applicable to an agent (as a circumstance, condition, event, or force) that contributes to the production of an effect or to any combination (as of circumstance, condition, or events) that inevitably or necessarily brings about a result (Merriam-Webster Inc., 1984).	D(S)
	*Causal - 1. Of or relating to causes; involving causation; 2. Arising from a cause. *Causative - 1. Operating as a cause; effective as a cause; 2. Expressing a cause. *Causality - The principle of causal relationship' the relation of cause and effect. *Causation - 1. The causing or producing of an effect; 2. The relation of cause and effect (Garner, 2009).	D(U)
Incite	To stir up or provoke to action (Collins Dictionary, 2015).	D(E)
	To cause (someone) to act in an angry, harmful, or violent way; to cause (an angry, harmful, or violent action or feeling). To move to action: stir up, spur on, urge on (Merriam-Webster, 2015).	D(E) - verb full definition
	To encourage somebody to do something violent, illegal or unpleasant, especially by making them angry or excited (Turnbull *et al*., 2010).	D(A)
	Encourage or stir up (violent or unlawful behaviour). Urge or persuade to act in a violent or unlawful way (Stevenson and Waite, 2011).	D(C)
	It stresses stirring up and urging on; frequently it implies active prompting (Merriam-Webster Inc., 1984).	D(S)
	*Incitant - An activating agent. A rare word pertains to physical rather than emotional causation, for example, things that trigger disease or chemical reaction (Garner, 2009).	D(U)
Produce	1. To bring (something) into existence; yield. 2. To bring forth (a product) by mental or physical effort; make. 3. To give birth to. 4. To manufacture (a commodity). 5. To give rise to. 6. To present to view. 7. To bring before the public. 8. To conceive and create the overall sound of (a record) and supervise its arrangement, recording, and mixing. 9. To extend (a line) (Collins Dictionary, 2015).	D(E) - verb definition
	To make (something) especially by using machines; to make or create (something) by a natural process; to cause (something) to exist or happen: to cause (a particular result or effect) 1. To offer to view or notice. 2. To give birth or rise to. 3. To extend in length, area, or volume.	D(E) - verb full definition

	4. To make available for public exhibition or dissemination as to; (a) provide funding for; (b) *t*o oversee the making of. 5a. To cause to have existence or to happen 5b. To give being, form, or shape to 6. To compose, create, or bring out by intellectual or physical effort 7. To cause to accrue. 8. To bear, make, or yield something (Merriam-Webster, 2015).	
	1. To make things to be sold, especially in large quantities. 2. To grow or make something as part of a natural process; to have a baby or young animal. 3. To create something, especially when skill is needed. 4. To cause a particular result or effect. 5. To show something or make something appear from somewhere. 6. If a town, country, etc. produces somebody with a particular skill or quality, the person comes from that town, country etc. 7. To be in charge of preparing a film/movie, play, etc. for the public to see (Turnbull *et al*., 2010).	D(A)
	1. Make, manufacture, or create. 2. Cause to happen or exist. 3. Provide for consideration, inspection, or use. 4. Administer the financial and managerial aspects of (a film or broadcast) or the staging of (a play). 5. Extend or continue (a line) (Stevenson and Waite, 2011).	D(C)
	Bear, yield, turn out (Merriam-Webster Inc., 1984).	D(S)
Induce	1. To persuade or use influence on. 2. To cause or bring about. 3. To initiate or hasten (labour), as by administering a drug to stimulate uterine contractions. 4. To assert or establish (a general proposition, hypothesis, etc.) by induction. 5. To produce (an electromotive force or electrical current) by induction. 6. To transmit (magnetism) by induction (Collins Dictionary, 2015).	D(E) - verb definition
	1a. To move by persuasion or influence. 1b. To call forth or bring about by influence or stimulation. 2a. Effect, cause. 2b. To cause the formation of. 2c. To produce (as an electric current) by induction. 3. To determine by induction; specifically: to infer from particulars. 4a. To cause the embryological formation of; to cause to form through embryonic induction. 4b. To cause or initiate by artificial means. 5. To produce anaesthesia in (Merriam-Webster, 2015).	D(E)
	1. Induce somebody to do something to persuade or influence somebody to do something. 2. Induce something to cause something. 3. Induce somebody/something to make a woman start giving birth to her baby by giving her special drugs (Turnbull *et al*., 2010).	D(A)
	1. Succeed in persuading or leading (someone) to do something. 2. Bring about or give rise to.	D(C)

	3. Produce (an electric charge or current or a magnetic state) by induction. 4. Bring on (childbirth or abortion) artificially, typically by the use of drugs. 5. Derive by inductive reasoning (Stevenson and Waite, 2011).	
	To move another by argument, entreaties, or promises to do or agree to something or to follow a recommended course. Induce usually implies overcoming indifference, hesitation, or opposition especially by offering for consideration persuasive advantages or gains that depend upon the desired decision being made; the term usually suggests that the decision is outwardly at least made by the one induced rather than forced upon him by the one that induces (Merriam-Webster Inc., 1984).	D(S)
	To reason from many specific observations to a general principle. To cause (a result); sense one being the one at issue in discussion of logic (Garner, 2009).	D(U)
Infectious + Agent	<u>Infectious</u> 1. A disease capable of being transmitted. 2. A disease caused by microorganisms, such as bacteria, viruses, or protozoa. 3. Causing or transmitting infection. 4. Tending or apt to spread, as from one person to another. <u>Agent</u> 1. A person who acts on behalf of another person, group, business, government etc. 2. A person or thing that acts or has the power to act. 3. A phenomenon, substance, or organism that exerts some force or effect. 4. The means by which something occurs or is achieved (instrument). 5. A person representing a business concern, especially a travelling salesman (Collins Dictionary, 2015).	D(E)
	<u>Infectious</u> 1a. capable of causing infection (viruses and other infectious agents). 1b. communicable by infection (an infectious disease). 2. That corrupts or contaminates. 3. Spreading or capable of spreading rapidly to others. <u>Agent</u> 1. One that acts or exerts power. 2a. Something that produces or is capable of producing an effect (an active or efficient cause). 2b. A chemical, physical, or biological active principle. 3. A means or instrument by which a guiding intelligence achieves a result. 4. One who is authorised to act for or in the place of another as: - A representative, emissary, official of a government. - One engaged in undercover activities (espionage). - A business representative. 5. A computer application designed to automate certain tasks (Merriam-Webster, 2015).	D(E)
	<u>Infectious</u> 1. An infectious disease can be passed easily from one person to	D(A)

another, especially through the air they breathe. 2. If a person or an animal is infectious, they have a disease that can be spread to others. Agent 1. A person whose job is to act for, or manage the affairs of, other people in business, politics, etc. 2. A person whose job is to arrange work for an actor, musician, sports player, etc. or to find somebody who will publish a writer's work. 3. Secret/double/special agent. 4. A person or thing that has an important effect on a situation. 5. A chemical or a substance that produces an effect or a change or is used for a particular purpose**.** 6. The person or thing that does an action (expressed as the subject of an active verb, or in a 'by' phrase with a passive verb) (Turnbull *et al.*, 2010).	
Infectious 1. A disease or disease-causing organism: - Liable to be transmitted through the environment. - Liable to spread infection. 2. Likely to spread to or influence others. Agent 1. A person that provides a particular service, typically one organising transactions between two other parties. 2. A person who manages financial or contractual matters for a performer, writer, or sportsperson. 3. A person who works in secret to obtain information for a government. 4. A person or thing that takes an active role or produces a specified effect. 5. An independently operating internet program, typically one that performs background tasks such as information retrieval or processing on behalf of a user or other program (Stevenson and Waite, 2011).	D(C)
Infectious 1. Infectious designates a disease resulting from the invasion of and multiplication in the body by germs (as bacteria, protozoa, or viruses) that produce toxin or destroy or injure tissue. 2. As applied to agents related to the causing of disease may be interchangeable and then mean capable of infecting or tending to infect. Agent 1. One who performs the duties of or transacts business for another, but differ in specific application. 2. Agent is very general and may be used to express this idea in any context where a specific term is not required; distinctively, however, it often implies the activity of a go-between (Merriam-Webster Inc., 1984).	D(S)
Infectious Germs and viruses that cause contagious disease, such as influenza and head colds, are easily transmitted from person to person (or animal to animal, as with foot-and-mouth disease). Those that cause infectious	D(U)

	diseases, such as cholera and typhoid, are usually spread through the environment (e.g. contaminated food or water). Some infectious diseases, such as sexually transmitted ones, can be passed from person to person through certain types of direct contact, but not through indirect or casual contact (Garner, 2009).

D, Dictionary of English **(E)**, advance learning **(A)**, concise English **(C)**, synonyms, antonyms, analogous and contrast words **(S)** or modern American usage **(U)**.
***Definition for the specific term not provided.

APPENDIX C

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Postharvest decay of nectarine and plum caused by *Penicillium* spp.

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Abstract Stone fruit are highly perishable and susceptible to numerous postharvest pathogens. *P. expansum* is a well-known pathogen of stone fruit but little is known about other *Penicillium* spp. that could potentially cause decay. This study aims to determine pathogenicity profiles of *P. expansum*, *P. crustosum*, *P. solitum* and *P. digitatum* on selected nectarine and plum cultivars, and in part examine the disease cycle within new fruit-*Penicillium* interactions to observe the potential of the pathogens to cross-infect. Lesions caused by *Penicillium* spp. isolated from the pear and citrus handling chain environments were not different on nectarine. *P. digitatum* was the most aggressive species on most nectarines and plums evaluated. Decay was associated with older fruit (long stored). The highest aggressiveness was observed on Nectargold, May Glo and African Rose. *P. expansum* and *P. crustosum* had the highest disease incidences and were the second and third most aggressive species respectively. *P. solitum* caused small lesions and its role in the fresh produce market can be negligible. Scanning electron microscopy confirmed infection and provided new information on the growth and reproduction of *P. expansum*, *P. crustosum* and *P. digitatum* on infected nectarine, pear and lemon. Pear and lemon can serve as cross-infection sources for stone fruit in the fresh produce chain. To our knowledge this is the most complete description of disease caused by *P. digitatum*, *P. crustosum* and *P. solitum* on nectarine and plum. Rapid decay caused by *P. digitatum* highlighted the potential of the species to contribute to losses in the stone fruit industry. Future research should investigate the presence and impact of *P. digitatum* in the stone fruit supply chain. The role of fruit maturity in fruit-*Penicillium* interactions requires further investigation.

Keywords *Penicillium* · Stone fruit · Green mould · Blue mould · Virulence · SEM

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