

Impact of postharvest storage on the infection and colonization of *Penicillium digitatum* and *Penicillium expansum* on nectarine

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Abstract

Very few studies have investigated the host-pathogen interaction of *Penicillium* spp. on nectarine. *Penicillium digitatum* was recently identified pathogenic and highly aggressive on nectarine. A strong association was made to host age/ripeness. This point to a new mechanism or life strategy used by *P. digitatum* to infect and colonize previously thought non-hosts. The aim of this study was to determine the effect of postharvest storage of nectarine on the infection and colonization of *P. digitatum* and *P. expansum* at a molecular and physical (firmness and pH) 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 longer stored fruit. Cold storage had the largest effect on *P. digitatum*. Inoculum load had a meaningful effect on both *Penicillium* spp. Storage 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 7d postharvest fruit (other genes unaffected). It partly explains the larger lesions caused on older or 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 is largely still unclear (gene expression) what specifically trigger/s the increase in disease incidence (infection) and lesion diameter (colonization) of *P. digitatum* on older or riper fruit. 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 colonize of these pathogens on the same host.

Keywords: host-pathogen interaction; pH modulation; ddPCR; cold storage; inoculum load; stone fruit.

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 postharvest pathogens (i.e. *Monilinia* spp., *Rhizopus* spp., *Mucor* spp. and *Penicillium* spp.) that can cause decay and contribute to losses (Crisosto and Mitchell 2011; Kader 2011). *Monilinia* spp. are the most important postharvest pathogens of apricot, peach and nectarine but *Penicillium expansum* Link is also recognized (losses and mycotoxin production) (Pitt and Hocking 2009; Snowdon 2010). Recent findings have drawn attention to other *Penicillium* spp. that can pose a risk to the fruits (Louw and Korsten 2016). Other pathogenic *Penicillium* spp. of nectarine include *P. crustosum* Thom,

P. digitatum (Pers.) Sacc. and *P. solitum* Westling (Louw and Korsten 2016). 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). *P. digitatum* was able to produce the largest lesions on nectarine and plum. Lesions caused by *P. solitum* were small and decay can be regarded as negligible (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 were less expected. This species, closely associated with citrus (Frisvad and Samson 2004; Stange et al. 2002), was recently identified highly aggressive on pome and stone fruits (Louw and Korsten 2014, 2016). In this case, it was able to cause much larger lesions than *P. expansum* within a shorter period of time. *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, 2016).

Riper fruit will not only be physiologically more favorable (i.e. carbon and nitrogen levels, pH changes) but also more susceptible (weaker host defence) for infection and colonization (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 (i.e. pome and citrus fruits) (Prusky et al. 2004; Sánchez-Torres and González-Candelas 2003; Yao et al. 1996; Zhang et al. 2013).

The production of ethylene or its precursors were shown to play an important role during the infection and colonization 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* (Barad et al. 2016a, 2016b; Bi et al. 2016; Prusky et al. 2004; Marcos et al. 2005; Sánchez-Torres and González-Candelas 2003; Zhang et al. 2013). 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 study is to determine the effect of postharvest storage of nectarine on the infection and colonization of *P. digitatum* and *P. expansum* at a molecular (gene expression) and physical (fruit firmness and pH) level. The impact of environmental conditions (cold storage) and pathogen pressure (inoculum load) will also be investigated.

Materials and methods

Fruit origin and handling. ‘Sunlite’ nectarine (*Prunus persica* (L.) Batsch var. *nucipersica* (Suckow) C. Schneider) was selected based on availability. Fruit of similar size, maturity and quality were used for trials. Fruit originated from a commercial farm in the Waterberg region in the Limpopo Province during the 2016/17 growing season. The fruit was harvest mature [industry guidelines (DAFF 2013)] and untreated (postharvest). Fruit was collected, transported to UP plant pathology laboratories and placed in cold storage ($5.3\pm 0.5^{\circ}\text{C}$; $83.2\pm 2.5\%$ RH). It was removed late afternoon to climatize overnight for trial inoculation the next day. Fruit was stored at ambient conditions ($23.8\pm 0.6^{\circ}\text{C}$; $65.7\pm 9.1\%$ RH) to produce three different postharvest storage periods (1d, 4d and 7d postharvest) prior to inoculation.

***Penicillium* spp. cultures.** Cultures of *P. digitatum* and *P. expansum* originated from 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 sterilized distilled water and stored at ambient temperature. Isolates

were cultured on MEA and incubated at 25°C for 5–7d. Conidia were harvested in sterilized Ringer's solution (Merck) with 0.05% Tween 80 (Associated Chemical Enterprises, Johannesburg). A haemocytometer was used to determine conidial concentrations.

Quality parameters of fruit. Three replicates were used to determine fruit indices. This was done prior to inoculation for each storage period. Weight (g), firmness (N), 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 calculated as °Brix/TA.

Effect of storage on infection and colonization of *Penicillium* spp. Fruit was dipped into 0.5% sodium hypochlorite (NaOCl) for ±5min for surface sterilization. Thereafter, it was rinsed (x2) via dipping into sterilized tap water (5min each) and allowed to air dry on a disinfected table. Inoculation of fruit was as described by Louw and Korsten (2014, 2016). The fruit (10 replicates for each storage period and *Penicillium* spp.) were wound-inoculated via pipetting 20µl conidial suspension (10⁵ 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 (each on a side) of each fruit using a sterilized micropipette (20–100µl). Fruit was randomized 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 (fruit held upright) lesion diameters. Advanced symptom development was recorded from fruit that incubated for longer. The trial was repeated.

Effect of storage and inoculum load on decay. Conidial suspensions, and sterilization 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 storage period, *Penicillium* spp. and conidial concentration (including control). Fruit was randomized and incubated under ambient conditions for 5d. The recording of lesion diameter was as described earlier. The number of days required for mycelia and conidia formation was noted. The trial was repeated.

Effect of inoculum load and cold storage on decay. Conidial suspensions, and sterilization and wounding of fruit were completed as described earlier. Five replicates were used for each combination of storage condition, *Penicillium* spp. and conidial concentration (including control). For ambient storage, fruit was randomized and incubated on a disinfected table for 5d and results were recorded as described earlier. For cold storage, fruit was randomized on a disinfected trolley and incubated in a cold room ($5.26 \pm 0.52^\circ\text{C}$; $83.16 \pm 2.51\%$ 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 storage period and *Penicillium* spp. were wound-inoculated (10^5 conidia/ml). Control fruit was included. Sterilization, wounding, randomization and incubation (ambient) of fruit and preparation of conidial suspensions were as described earlier. Of the fifteen inoculated fruit for each combination of storage period 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 into 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 uncolonized areas (max distance away from inoculated sites) was also measured. The trial was repeated.

Absolute quantification of genes. Up to twenty fruit were wound-inoculated with each *Penicillium* spp. (10^5 conidia/ml) or control solution for each storage period. Sterilization, wounding, randomization and incubation (ambient) of fruit and preparation of conidial suspensions were as described earlier. Healthy (from control fruit) and infected tissue at the inoculated sites were sampled after 24h and 48h incubation. A sterilized 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 sterilized Bijoux or McCartney bottles and stored $\leq -72^\circ\text{C}$. The trial was repeated to produce three biological replicates for each combination of storage period 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 sterilized and cooled (liquid nitrogen) KCG201S coffee grinder (Kambrook, China). 100–150mg homogenized 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 1 μg 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.

Gene-specific primers (Table 1) were designed using Primer 3+ software (Untergasser et al. 2007). The polygalacturonase gene (*PG*), 1-aminocyclopropane-1-carboxylic acid deaminase gene (*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 normalization is not mandatory for droplet digital PCR (ddPCR) (Zmienko et al. 2015), it was found to be 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).

Table 1 Primers used for gene expression analysis of the *Penicillium* spp.

Primers	Forward (5'–3')	Reverse (5'–3')	Target ^z
Pg2Pd	agcctgaccaactccaacat	ctccttagcgccatcgatac	<i>PG</i> of <i>P. digitatum</i> ; synthesis of PG
Pg1Pe	aaaggcaggttgctccagta	aggccagaccagctcaaatcc	<i>PG</i> of <i>P. expansum</i> ; synthesis of PG
ACCDPd	cggttctgtttgtgctgtg	ccttctcttcgcgtct	<i>ACCD</i> of <i>P. digitatum</i> ; ethylene biosynthesis
ACCDPe	acgggtgcttgtttgtgctgt	gcctcaacagtggcagaag	<i>ACCD</i> of <i>P. expansum</i> ; ethylene biosynthesis
PacCPd	ccgggtgagctactgccttg	caggttgaggttgttgggtct	<i>PacC</i> : C2H2 transcription factor of <i>P. digitatum</i> ; pH regulation
PacCPe	ggacatttcccaggatagca	gatagagcgggggtcaatcag	<i>PacC</i> : C2H2 transcription factor of <i>P. expansum</i> ; pH regulation
CreAPd	cgcaagtagagcagacgaccac a	tgcatacgcggaaagcgaag g	<i>CreA</i> : C2H2 transcription factor of <i>P. digitatum</i> ; carbon regulation
CreAPe	cgcattcaaacgatgacgatgatg gct	aggaaggagcagtgaggattg gggtg	<i>CreA</i> : C2H2 transcription factor of <i>P. expansum</i> ; carbon regulation
βaP	ctccccgatggacaggtcat	tggataccgccagactcaag	<i>β-actin</i> of <i>P. digitatum</i> and <i>P. expansum</i> ; reference

^z*ACCD*, 1-aminocyclopropane-1-carboxylic acid deaminase; *PG*, polygalacturonase.

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 10² 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 stabilization). Samples were held at 4°C. Thereafter, samples were 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 postharvest storage and inoculum load trials. The isolates were cultured on MEA, purified (single spore isolation), identified via DNA sequencing (*β-tubulin*) and preserved in sterilized 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. Sequence 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. The trials determining the effect of different inoculum concentrations, and storage periods and conditions were completed twice. The remainder of the trials were completed three times. Randomization was done according to the complete randomized design. Each inoculated fruit produced four pseudoreplicates (horizontal and vertical lesion diameter measurements from two inoculated sites, each at an opposite sides of a fruit) 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). Data was evaluated for normal distribution. 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.

Results

Quality parameters of fruit. The farmer should have determined harvest dates based on industry guidelines (DAFF 2013). The fruit were, however, observed to be at an advance mature stage after it was harvested. It would have been helpful to measure ethylene production and/or index of absorbance difference measurements for the different storage periods but limitations prevented it. Fruit indices for trial repeats were not significantly different ($P = 0.22\text{--}0.85$). All, except °Brix ($P = 0.37$), indicated the fruit became riper when stored at ambient conditions ($P < 0.003$). Weight, firmness and TA decreased while pH and sugar/acid ratio increased (Fig. 1).

Effect of storage on infection and colonization of *Penicillium* spp. Trial repeats were not significantly different ($P > 0.76$). The interaction effect between storage period and *Penicillium* spp. was significant ($P < 0.0001$). Lesions caused by *P. digitatum* were larger on longer stored fruit, quickly surpassing that of *P. expansum* in size on 4d and 7d postharvest fruit (Fig. 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% for all storage periods while that of *P. digitatum* increased (Fig. 2). 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. 3). Once sporulation started, copious amounts of conidia were produced within a short period of time.

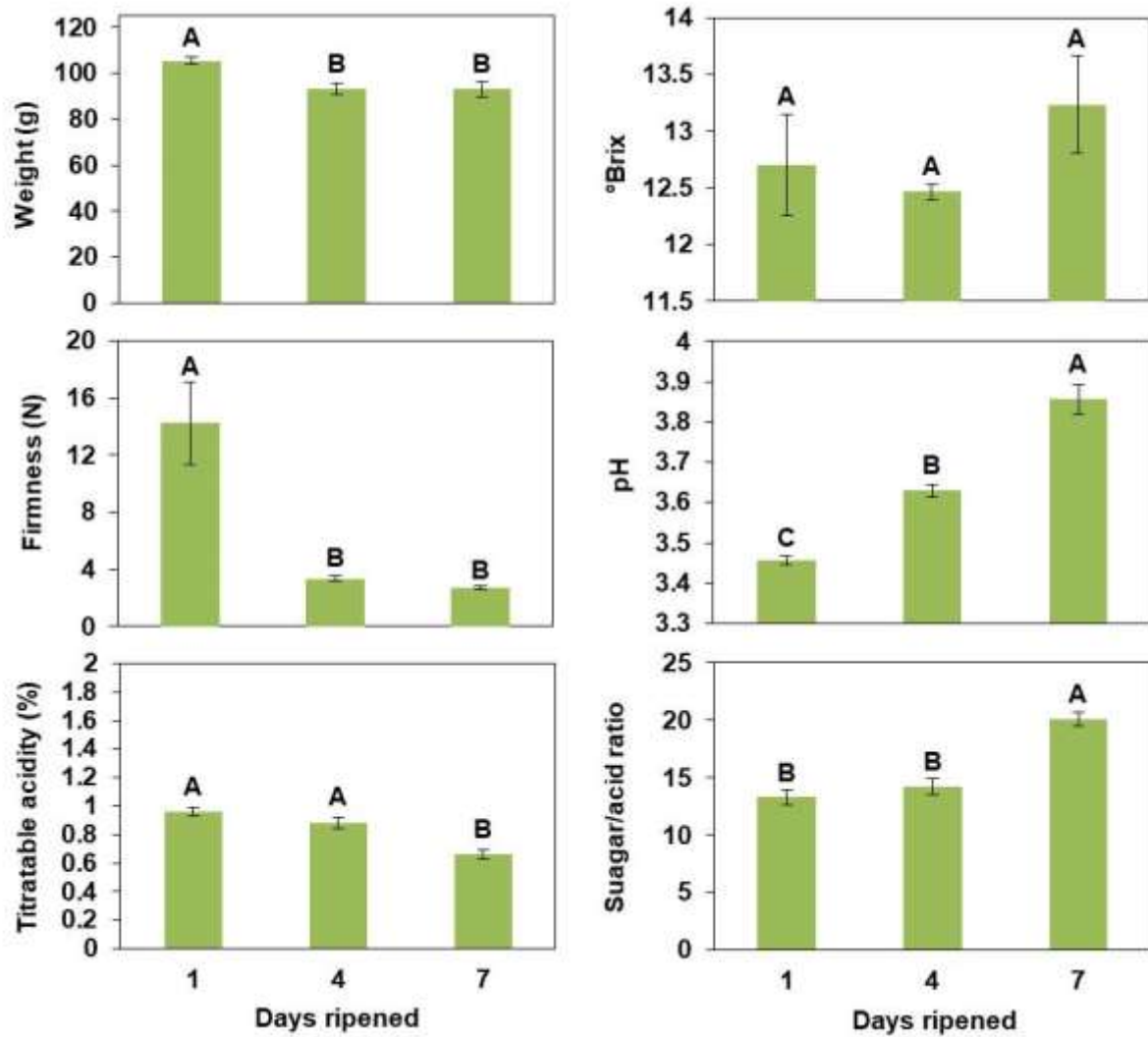


Fig. 1 Fruit indices of nectarine (cv. Sunlite) stored at ambient conditions (1, 1d postharvest; 4, 4d postharvest; 7, 7d postharvest). Vertical bars indicate standard error. Different letters (only letters of similar case are comparable) indicate treatments that are significantly different ($P < 0.05$)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

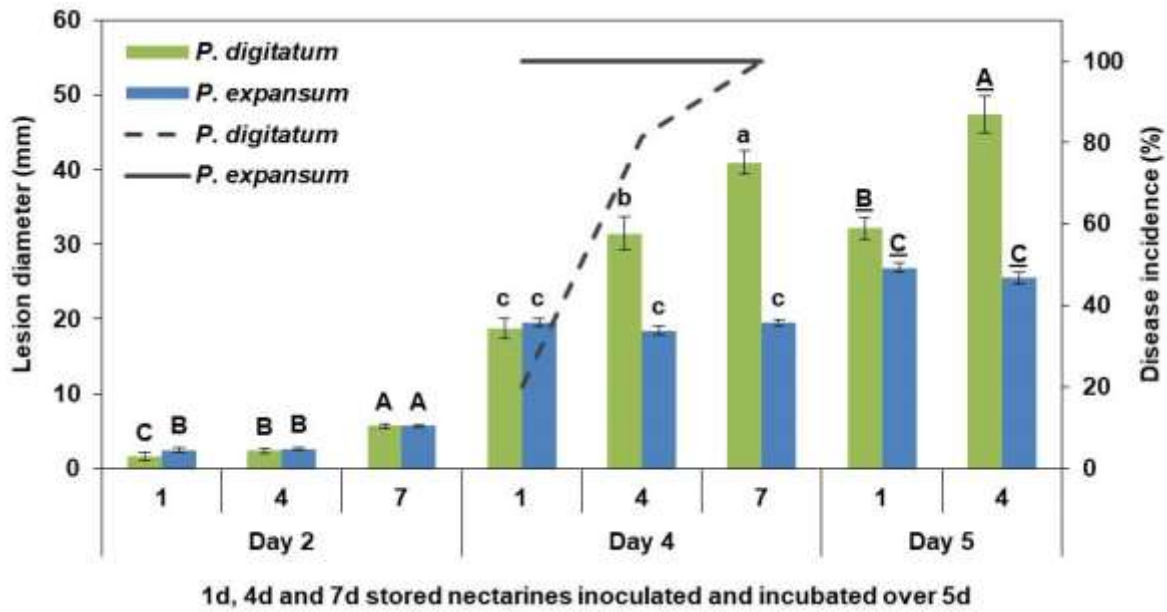


Fig. 2 Lesion development of *Penicillium* spp. on nectarine (cv. Sunlite of different postharvest storage periods incubated for 5d under ambient conditions). Control (wound size) was subtracted from lesion diameters. Vertical bars indicate standard error. Different letters (only letters of similar case and underlining are comparable) indicate treatments that are significantly different ($P < 0.05$) based on Fisher protected Least Significant Difference.

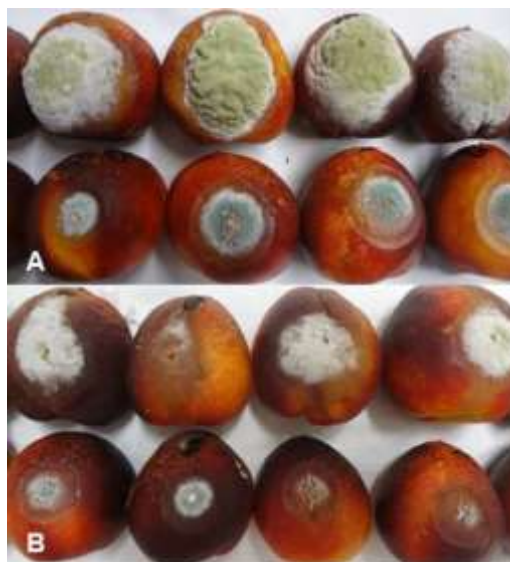


Fig. 3 Nectarine (cv. Sunlite) inoculated with *Penicillium digitatum* (top) and *P. expansum* (bottom) and incubated at ambient conditions. **A**, 4d postharvest stored fruit incubated for 5d; **B**, 7d postharvest stored fruit incubated for 4d.

Effect of storage 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., storage period 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. 4). Lesions were larger for both species when fruit were longer stored 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.

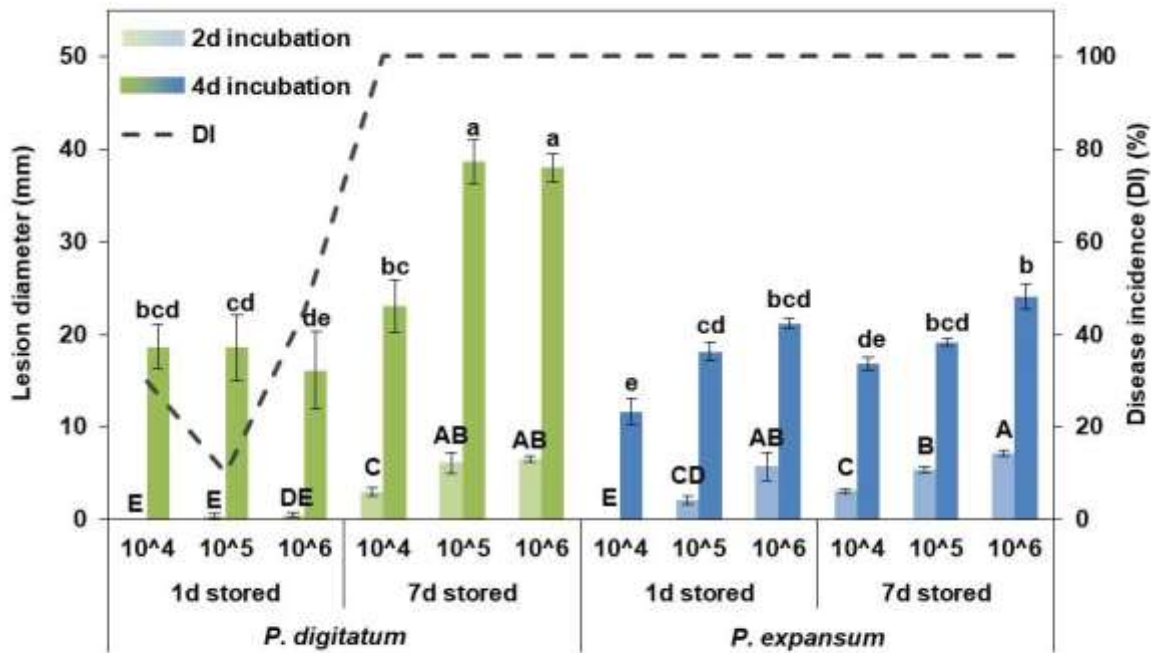


Fig. 4 Lesion diameters caused by different concentrations (10^4 – 10^6 conidia/ml; 200–20 000 conidia) of *Penicillium* spp. on nectarine (cv. Sunlite) stored for different periods (**1d**, 1d postharvest storage; **7d**, 7d postharvest storage). Wounds have been subtracted from lesion diameters. Vertical bars indicate standard error. Different letters (for each pathogen evaluated 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). *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.

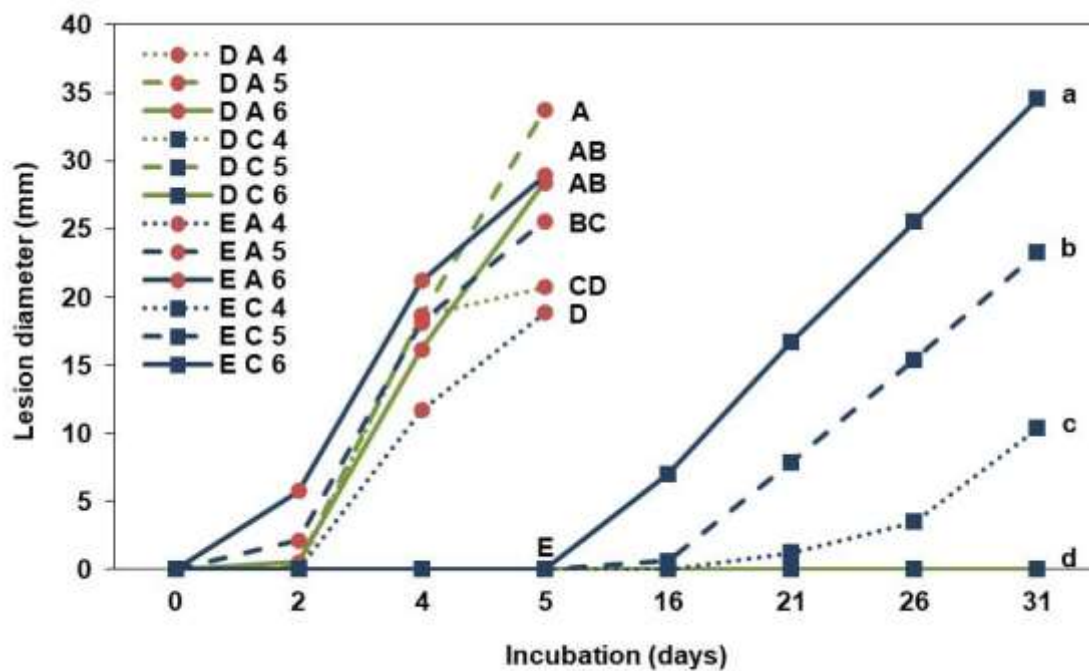


Fig. 5 Lesion diameter caused by different concentrations (4–6) of *Penicillium digitatum* (D) and *P. expansum* (E) on nectarine (cv. Sunlite) (1d postharvest storage) at ambient (A) and cold storage (C) conditions. 4, 10^4 conidia/ml (200 conidia); 5, 10^5 conidia/ml (2 000 conidia); 6, 10^6 conidia/ml (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.

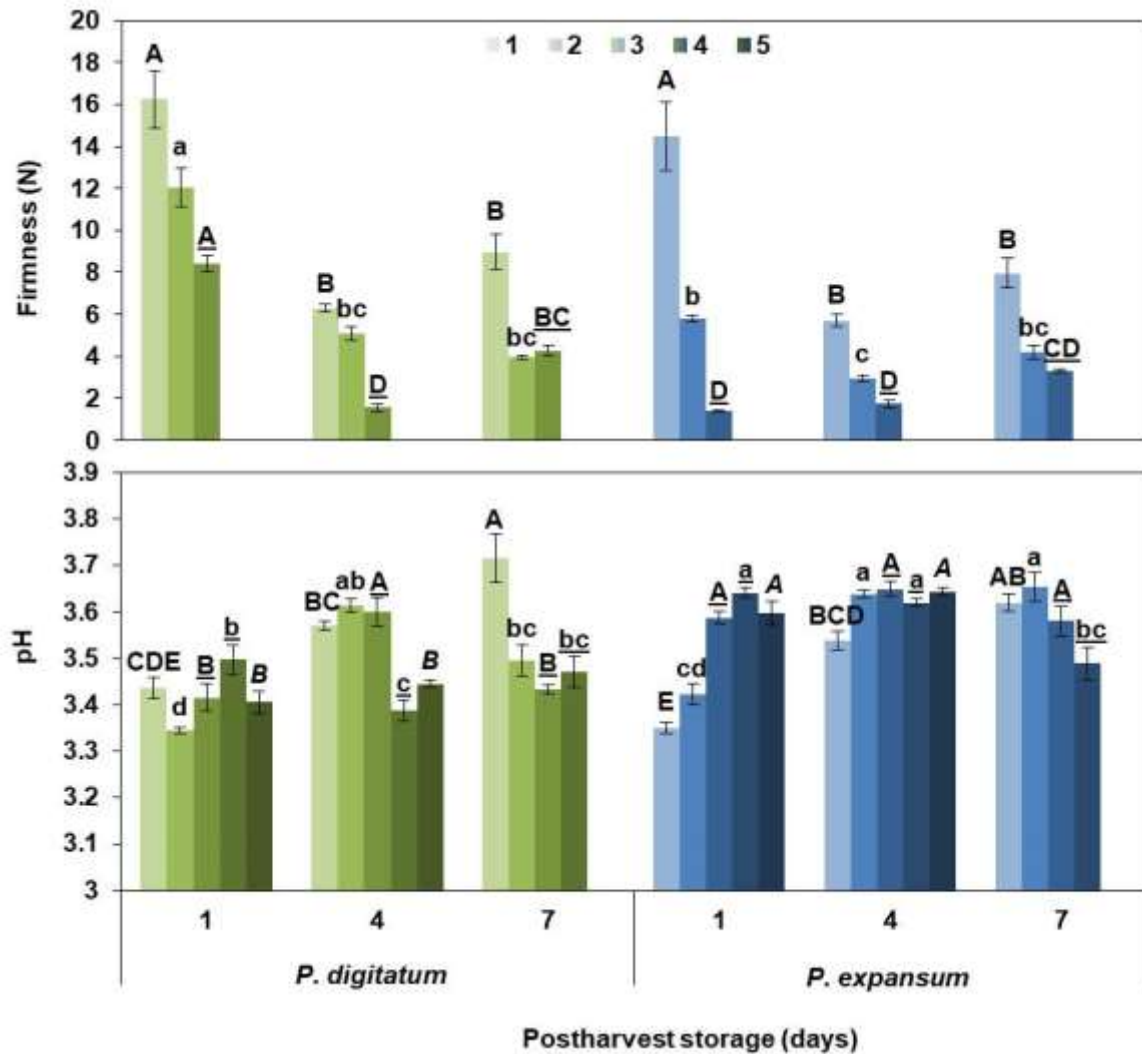


Fig. 6 Firmness and pH of lesions caused by *Penicillium* spp. on nectarine (cv. Sunlite) stored for different periods (1, 1d postharvest storage; 4, 4d postharvest storage; 7, 7d postharvest storage) and incubated for 5d (1–5). Vertical bars indicate standard error. Different letters (for each pathogen evaluated 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.

Firmness and pH of infected sites. Trial repeats were not significantly different based on the firmness of infected tissue (lesions) ($P = 0.32$ – 0.43). Measurements for fruit that incubated for 4d and 5d were disregarded (Fig. 6). The firmness at the lesion sites of these fruit increased due to large mycelial masses that developed (growth) under the inoculated sites. In the case of control fruit, longer incubated fruit was dryer and skin more elastic (skin

not removed for these experiments). Storage 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 for 1d postharvest fruit but not necessarily for 4d or 7d postharvest fruit when comparing it to that of *P. expansum*. For instance, with 1d postharvest fruit *P. digitatum* lowered firmness by 27.14% over 2d incubation whereas *P. expansum* lowered it by 64.89%. Results were, however, comparable for the *Penicillium* spp. on 7d postharvest fruit (58.70% vs 55.97% respectively).

Trial repeats were not significantly different based on pH measurements from 2–5d incubation ($P = 0.30–0.97$). Similar to firmness, storage 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 4d and 7d postharvest fruit (Fig. 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 equalization 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 colonized tissue (lesions) was generally lower than that of uncolonized (infected fruit) and healthy tissue (control fruit). There were some exceptions with 1d postharvest fruit (Table 2). Differences in pH between colonized and uncolonized tissue ($\Delta_{\text{pH colonized-uncolonized}}$) increased (4d incubation) and differences between $\Delta_{\text{pH colonized-uncolonized}}$ and colonized vs control ($\Delta_{\text{pH colonized-control}}$) were small ($\Delta\Delta_{\text{pH}}$).

Table 2 pH of uncolonized and colonized tissue of *Penicillium*-inoculated nectarine (cv. Sunlite) of different storage periods

Incubation (days)	<i>Penicillium</i> spp.	Tissue	Postharvest storage (days)			
			1d	4d	7d	
4	<i>P. digitatum</i>	Uncolonized	3.56±0.1 DEFG	3.60±0.09 DEFG	4.00±0.20 AB	
		Colonized	3.5±0.1 EFG	3.39±0.07 G	3.47±0.1 FG	
		Δ_{pH1}	0.06	0.21	0.53	
		Δ_{pH2}	0.01	0.31	0.51	
		$\Delta\Delta_{pH}$	0.01	0.1	0.02	
		<i>P. expansum</i>	Uncolonized	3.75±0.13 BCDE	3.78±0.21 BCD	3.81±0.17 BCD
	Colonized		3.64±0.03 DEFG	3.62±0.03 DEFG	3.49±0.11 EFG	
	Δ_{pH1}		0.11	0.16	0.32	
	Δ_{pH2}		0.15	0.08	0.49	
	$\Delta\Delta_{pH}$		0.04	0.08	0.17	
	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	<i>P. digitatum</i>	Uncolonized	3.45±0.14 cde	3.77±0.33 a	.
			Colonized	3.41±0.07 e	3.45±0.03 cde	.
			Δ_{pH1}	0.04	0.32	
Δ_{pH2}			0.11	0.24		
$\Delta\Delta_{pH}$			0.07	0.08		
<i>P. expansum</i>			Uncolonized	3.43±0.05 de	3.63±0.14 abc	.
		Colonized	3.6±0.08 abcd	3.64±0.02 ab	.	
		Δ_{pH1}	0.17	0.01		
		Δ_{pH2}	0.08	0.05		
		$\Delta\Delta_{pH}$	0.09	0.04		
		Control*	Unwounded	3.62±0.06 abcd	3.69±0.25 ab	.
Wounded			3.52±0.17 bcd	3.69±0.18 ab	.	

Δ_{pH1} , pH difference between uncolonized and colonized; Δ_{pH2} , pH difference between colonized and control (wounded); $\Delta\Delta_{pH}$, difference between Δ_{pH1} and Δ_{pH2} .

*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.

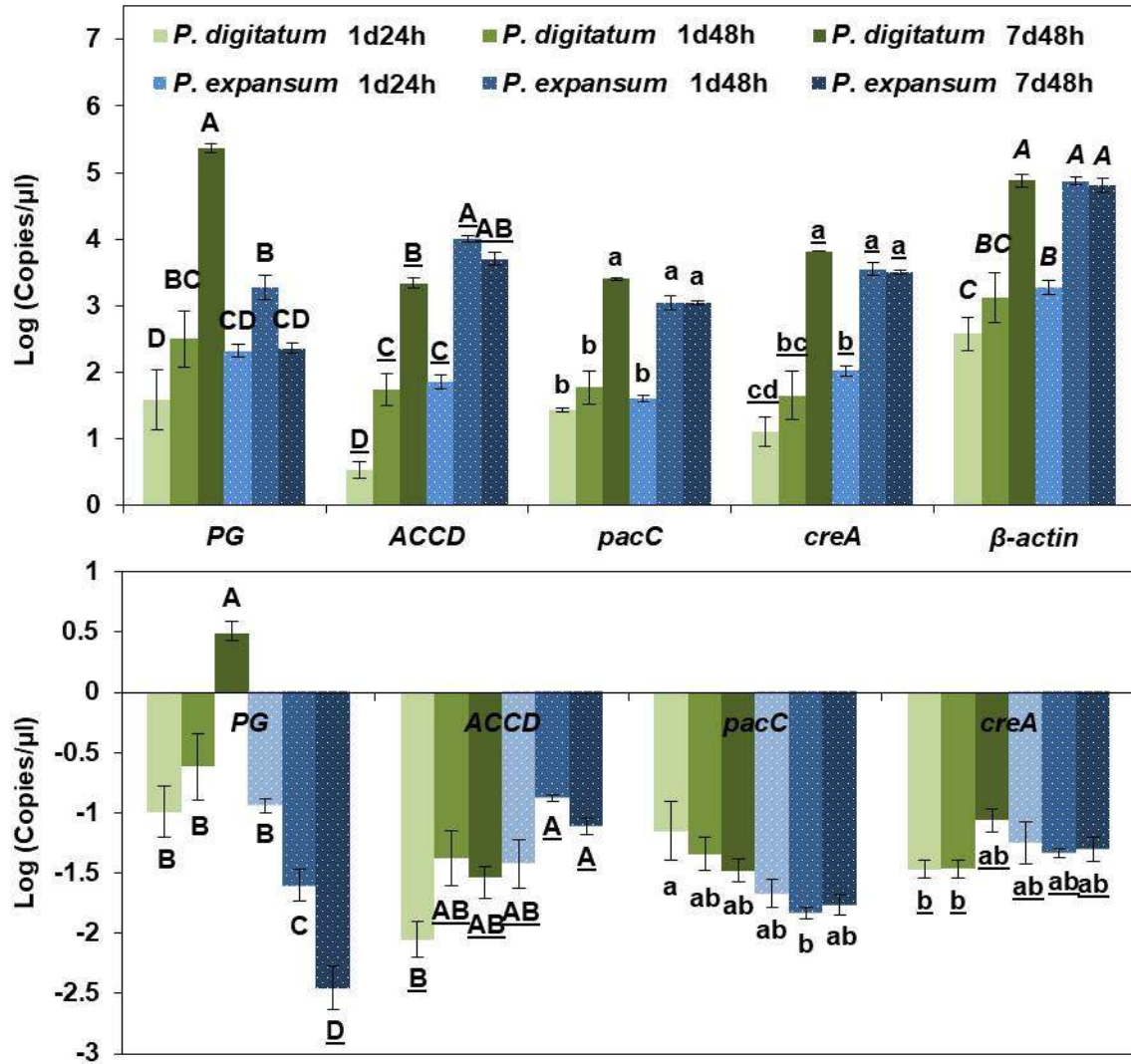


Fig. 7 Gene expression of *Penicillium digitatum* and *P. expansum* when inoculated in nectarine (cv. Sunlite) stored for different periods (1d and 7d postharvest storage) after 24h and 48h incubation. Raw data (top) was normalized (bottom) using the reference gene (*β-actin*). Vertical bars indicate 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.

Absolute quantification of genes. Trial repeats were not significantly different based on gene expression profiles ($P = 0.51–0.94$). Absolute and normalized data (reference gene) are displayed (Fig. 7). The high expression of the reference gene led to negative log values when normalization was completed. Only normalized 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 storage period 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 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 *Penicillium* culture collection at UP.

Discussion

Nectarines became riper as fruit were stored for longer (days postharvest). The increase in sugar/acid ration should be noted. The pH and sugar are important regulatory factors impacting on infection and colonization 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 colonization of the *Penicillium* spp. This was clearly the case with *P. digitatum* but not necessarily with *P. expansum*. The effect of storage 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 storage.

Penicillium digitatum caused larger lesions at higher disease incidences on the longer stored fruit. Lesion diameter was even similar in size at 1d postharvest to *P. expansum* but quickly surpassed it on 4d and 7d postharvest fruit. Previous work 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 was particularly because fruit was 1d/2d postharvest. In this study, disease incidence on 1d postharvest fruit was higher (20%) and lesion diameter showing similarities (32.13mm after 5d incubation). This is one of the first studies demonstrating a significant shift in lesion diameter and disease incidence for *P. digitatum* due to postharvest storage. Vilanova et al. (2014) reported *P. digitatum* able of causing lesions of ± 45 mm in diameter on over-mature but not immature or commercially mature ‘Golden Smoothee’ apples. *Penicillium expansum*, similar to what was observed in previous (Louw and Korsten 2016) and current work on plum (Louw and Korsten 2019), was relatively unaffected by fruit age/ripeness. Vilanova et al. (2017) recently reported that *P. expansum* alter the ethylene biosynthesis pathway of apple differently than *P. digitatum* and abiotic stresses (i.e. wounding). These findings indicate that these species utilize very different strategies during infection and colonization.

Scanning electron microscopy (Louw and Korsten 2016) 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 (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 current work on plum (Louw and Korsten 2019) 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 storage had no effect on lesion diameter. Other factors are interacting on the response.

Modulation of pH was affected by fruit storage. This can primarily be ascribed to the varying pH values (initial) of the fruit from the different storage periods. 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. Colonized tissue of an acidic host can be maintained or further acidified by acidification (organic acid production and ammonium/ammonia uptake). Alternatively, the mechanism can be inverted to increase pH (Barad et al. 2016a; Bi et al. 2016; Prusky et al. 2004). A dual pattern of pH modulation has been discussed for *P. expansum* on apple (Barad et al. 2016a; Bi et al. 2016; Sánchez-Torres and González-Candelas 2003).

It was noted in our study that the *Penicillium* spp. did not modulate pH to a state that would be optimal for PG expression [expression of *pepg1* (endopolygalacturonase gene) of *P. expansum* was optimal at pH 4 and much lower at pH 3–3.5 (*in vitro*) (Prusky et al. 2004)] or PG activity [PG isolated from *P. expansum* was active over pH 3~6.5 but optimal at pH 4–5.5 (*in vitro*) (Jurick et al. 2010; Yao et al. 1996)]. The pH at infected sites of nectarine equalized 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 [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)] and *P. digitatum* infected citrus fruits [‘Navel’ = 3.12 ± 0.07 , ‘Oro Blanco’ = 3.10 ± 0.14 (7d incubation) (Prusky et al. 2004) and *Citrus unshiu* = 3.22 ± 0.15 (4d incubation) (Zhang et al. 2013)].

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. (Jurick et al. 2009; Jurick et al. 2010). The pH of *P. digitatum* colonized tissue of nectarine, plum (Louw and Korsten 2019) and citrus (Prusky et al. 2004; Zhang et al. 2013) was lower than that of *P. expansum* on nectarine, plum (Louw and Korsten 2019) and apple (Prusky et al. 2004). It is thus possible that the *PG* activity of *P. digitatum* is optimal at lower pH levels than *P. expansum* and *P. solitum*. Future studies are needed.

The pH values of colonized sites were similar to the initial pH of fresh nectarine (1d postharvest). The pH value prior to inoculation was 3.46 compared to 3.5 (*P. digitatum*) and 3.64 (*P. expansum*) after 4d incubation. The differences remained relatively small for 4d postharvest fruit (*P. digitatum* $\Delta_{\text{pH}} = 0.24$ and *P. expansum* $\Delta_{\text{pH}} = 0.01$) but increased considerably for 7d postharvest fruit ($\Delta_{\text{pH}} = 0.39$ and $\Delta_{\text{pH}} = 0.38$ 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 maintain an environmental pH similar to that of the host. In cases where the pH is too high (i.e. type, cultivar, storage or ripeness), a lowering in pH was evident. The pH of decayed tissue seems to be host- (Bi et al. 2016; Prusky et al. 2004) and species-specific.

$\Delta_{\text{pH}}^{\text{colonized-uncolonized}}$ for both *Penicillium* spp. increased from 1d postharvest to 7d postharvest storage (0.06-0.11 to 0.32-0.53) but the $\Delta\Delta_{\text{pH}}$ s were similar (0.01-0.17). This does not correspond with results of *P. expansum* on apple (Prusky et al. 2004) or *Penicillium* spp. on plum (Louw and Korsten 2019). $\Delta_{\text{pH}}^{\text{colonized-uncolonized}} = 0.31-0.88$ for apples (Prusky et al. 2004) whereas $\Delta_{\text{pH}}^{\text{colonized-control}} = 0.02-0.24$ [combining data from Keller et al. (2004) and Prusky et al. (2004)], thus $\Delta\Delta_{\text{pH}} = 0.29-0.64$. With plum, *P. digitatum* $\Delta\Delta_{\text{pH}} = 0.16-1.8$ and *P. expansum* $\Delta\Delta_{\text{pH}} = 0.6-1.55$. The primary reason for the large difference observed with plum was the rapid ripening of infected plum as compared to slower (natural) ripening of uninfected fruit. The interference of *Penicillium* spp. on fruit ripening also seems to be host- and species-specific (Vilanova et al. 2017).

This study is one of the first to use ddPCR to quantify the expression of genes in postharvest pathology of 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* genes (*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 likewise observed on plum (Louw and Korsten 2019) 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). 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. 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, however, not similarly affected by these factors. *ACCD* expression is affected more 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, b). 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 ($\Delta_{\text{pH}} = 0.07\text{--}0.1$).

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, b; 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 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 (Louw and Korsten 2019), the definite connection between infection and increased ripening could not be made since control fruit had similar pH values to uncolonized tissue of infected fruit. The nectarine fruit were also harvested at an

advanced mature stage (unlike with plum). The upregulation of *ACCD* (24h vs 48h) was an indicator of increased ripening. Ripening could have been due to ACC synthesized 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 7d postharvest fruit at 48h indicates synthesis of more ACC early after harvest rather than later. It is possible that the host environment at 7d postharvest was already at a favorable state (very ripe). Testing the nutritional composition of infected fruit (colonized and uncolonized tissue) should be considered in future work.

Fruit storage 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 storage. 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 corresponds with the higher °Brix value of the 7d postharvest fruit. CreA regulates the carbon catabolite repression [ensures preferentially utilization of certain carbon sources (i.e. glucose) (Fernandez et al. 2012, 2014)] but is also involved in acidification and alkalization processes (Bi et al. 2016).

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). Acidification can be induced under excess sugar, even for pathogens classified with alkalizing lifestyles. Conversely, alkalization can be induced under carbon deprived conditions, even by acidifying pathogens

(Alkan et al. 2013; Bi et al. 2016; Ment et al. 2015). Bi et al. (2016) showed higher sucrose levels will cause *P. expansum* to produce less ammonia and more gluconic acid (*in vitro*). This environment will cause a decrease in expression of *ACCD* and *pacC* (Barad et al. 2016a, b). 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. This corresponds with the increase in expression of *pacC* (*P. expansum*). The strong *in vitro* reaction observed by (Bi et al. 2016) can be ascribed to the sucrose levels being very high (excess). Other factors (i.e. pH, ammonium) possibly played a larger part in the interaction of *P. expansum* on nectarine. The effects environmental pH, and nitrogen and carbon sources have on the host-pathogen interactions of different *Penicillium* spp. is more complex 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 colonized 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 stored or 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 colonized tissue at lesions on nectarine.

Storage or ripeness not only affected lesion diameter but also disease incidence of *P. digitatum*. Many host factors (physical, biochemical and molecular) change during postharvest storage. Although host physiology (i.e. firmness, sugar content, pH, nitrogen levels) provided some explanation for the increased disease incidence and lesion diameters, it is still not clear what specific factors trigger/s the increase on the longer stored fruit. The low and sharp decline in fruit firmness of 7d postharvest fruit could suggest advancement in fruit senescence, thus deterioration in host resistance and an increase in infection and colonization of *P. digitatum*. Studying host resistance [decline as fruit ripen (Prusky et al. 2016)] might reveal the true cause for the opportunistic lifestyle expressed by *P. digitatum* on nectarine.

The lifestyle of *P. digitatum* on nectarine makes it less of a concern early in a fresh produce chain but more so at the end (long storage and fruit tend to be riper). *Penicillium expansum* does not follow this lifestyle on nectarine, constantly causing lesions of similar size with complete 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. None the less, the sensitivity of *P. digitatum* to cold storage and its opportunistic lifestyle on nectarine was confirmed. This can however not be said when *P. digitatum* infect and colonize citrus (Eureka seeded lemons). Louw and Korsten (2015) revealed that even with low concentrations (6.3×10^4 conidia/ml) *P. digitatum* could cause lesion of 43.8 ± 5.6 mm in diameter after 26d cold storage ($5.0 \pm 0.7^\circ\text{C}$ and $86.4 \pm 4.5\%$ RH). The host and environment *P. digitatum* are exposed to thus determine its opportunistic nature.

This is one of the first reports 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 colonize 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 colonization of *P. digitatum* on nectarine (more acidic new host) to what is already known on citrus [i.e. citrus peel (Zhang et al. 2013)].

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