Impact of Ripeness on the Infection and Colonization of *Penicillium digitatum* and *P. expansum* on Plum

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Highlights

• Ripeness had a significant effect (disease incidence and severity) on *P. digitatum*.

• *P. expansum* was unaffected by fruit ripeness.

• Ripeness, inoculum load and storage conditions affected *Penicillium*-plum interactions.

• *P. digitatum*, an opportunistic pathogen of plum, is a realistic threat to stone fruit.

• First report of reverse transcription ddPCR (RT-ddPCR) in postharvest fruit pathology.

Abstract

Penicillium digitatum was recently identified a postharvest pathogen of plum. Although little is known of this host-pathogen association, an increased disease occurrence and severity was noted on older fruit. This study 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. Disease incidence and severity of *P. digitatum* was significantly affected by ripeness, cold storage and inoculum load. Both species acidified tissue and advanced host ripening. Host ripening had a small effect on gene expression (*P. digitatum*: ACC deaminase decreased; *P. expansum*: pacC and creA increased). 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 at lesions was similar to the natural pH of the host. Alkalinisation via accumulation of ammonium/ammonia can 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. *P. 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 gene expression and the increase in disease incidence and severity on riper fruit. Future work should consider the decline of host resistance during ripening.

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

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 (Louw and Korsten, 2016).

Penicillium expansum Link is a well-known pathogen that contribute 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 fruit 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; 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. The accumulation of sugar, change in pH and decline of antifungal compounds 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 polygalacturinases (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 fruit) is resent and limited. This study 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. Material and methods

2.1. Fruit source and handling

'Fortune', a Japanese plum (*Prunus salicina* L.) 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 (Argenta et al., 2003); 1 d, 4 d, 8 d and 12 d postharvest. Fruit was ripened by incubation under ambient conditions (25.50 ± 0.67 °C; 59.89 ± 2.81% RH) over the required days prior to inoculation.

2.2. Fungal cultures

P. digitatum and *P. expansum* isolates originated from 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 5–7 d 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.

2.3. 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 (N), soluble solids/sugar content (SSC) (%), pH, titratable acidity (TA) (% malic acid) and sugar/acid ratio (SSC/TA) were recorded/determined. Fruit firmness was measured using a Turoni TR 53205 penetrometer (T.R. Turoni S.R.L., Forli, Italy) with a 5 mm 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.

2.4. Effect of fruit ripeness on infection and colonisation of Penicillium **spp**

Fruit (2015) were surface sterilised by dipping into 0.5% sodium hypochlorite (NaOCl) solution for 5 min, double rinsed by dipping into sterile tap water (≥5 min 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 (1 d, 4 d and 8 d postharvest) were wounded (1.5 × 3 mm) on opposite sides (two wounds; each on a side) using a sterile micropipette tip (20–200 µl). Ten fruit per ripeness level were inoculated (20 µl 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 under ambient conditions (25.34 ± 0.64 °C; 61.31 ± 3.63% RH) for 5 d. Horizontal and vertical (calyx axis vertical) lesion diameters were recorded 2 d, 4 d and 5 d post-inoculation. Some fruit (≥3) were incubated longer to study advanced symptom development. The trial was completed in triplicate.

2.5. 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 fruits (2016) from each ripeness level (1 d, 4 d, 8 d and 12 d after harvest) were inoculated with each concentration of *Penicillium* spp. or control solution for each storage condition. Fruit for ambient storage was randomised and incubated for 5 d 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 26 d. 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.

2.6. Firmness and pH of infected sites over time

Fifteen fruits (2015) from each ripeness level (1 d, 4 d and 8 d 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 5 d incubation period for each ripeness level and *Penicillium* spp. 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 5 mm diameter stainless steel cylindrical probe into inoculated sites (skin not removed). The trial was done in triplicate.

2.7. Absolute quantification of regulatory genes

Up to twenty fruit (2016) from each ripeness level (1 d, 4 d, 8 d and 12 d 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 24 h and 48 h post-inoculation using a sterilised 8 mm 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 1 d and 12 d 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 100–150 mg homogenised tissue according to RNeasy® Plant Mini Kit (Qiagen®, Hilden, Germany) specifications. Initial RNA quantity was evaluated using the Qubit® 2.0 Fluorometer with Qubit RNA HS Assay Kit (Invitrogen™, Life Technologies™, Carlsbad,

CA). RNA analysis was conducted using the Agilent 2100 bioanalyzer (Agilent Technologies, Inc., Palo Alto, CA). 50 ng of total RNA was used for cDNA synthesis according to iScript™ Advanced cDNA Synthesis Kit for RT-qPCR (Bio-Rad Laboratories, Inc., Hercules, CA) specifications. Thermocycler conditions for cDNA synthesis were 30 min at 42 °C for reverse transcription (RT) and 5 min 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 (50 ng) 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 1) for *PG*, 1-amino-cyclopropane-1-carboxylic acid deaminase (*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, 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 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

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) in ddPCR 96-well PCR plates and sealed in a PX1 PCR Plate Sealer (Bio-Rad Laboratories, Inc.) according to manufacturer's instructions. Droplets were generated in the QX200 AutoDG (Bio-Rad Laboratories, Inc.). Generated samples were sealed and placed into a C1000 Touch thermal cycler (Bio-Rad Laboratories, Singapore). PCR cycle conditions were one cycle of 5 min at 95 °C (enzyme activation), 40 cycles of 30 s at 95 °C (denaturation) and 1 min at 54.5 °C (annealing/extension), and one cycle of 5 min at 4 °C and 5 min at 90 °C (signal stabilisation). Samples were held at 4 °C. Plates were transferred to a QX200 Droplet Reader (Bio-Rad Laboratories, Inc.) for analysis. Data were examined using

QuantaSoft™ Software (Bio-Rad Laboratories, Inc.).

2.8. Reisolation, preservation and identification

Two symptomatic fruits from each experiment of the ripeness trials were isolated for each *Penicillium* spp. Isolations were plated on MEA and incubated as described earlier. Cultures were assessed for morphological similarity once sufficient growth 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). Identity of the cDNA sequences were confirmed prior to downstream application. Sequences analysis was conducted using the ABI3500/3500XL Genetic Analyzer (Applied Biosystems, Foster City, CA). 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.

2.9. Statistical design and data analysis

The trial evaluating the multifactorial effect of fruit ripeness, inoculum load and cold storage was completed twice. The remainder of the trials were completed three times. Fruit was randomised according to the complete randomised design with factorial arrangements. 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 StatisticalAnalysis System (SAS) (version 9.4; SAS Institute Inc., Cary, NC). Bartlett's test for homogeneity was used to disclose similarity among repeats of trials (independent experiments). Trial repeats were pooled if proven nonsignificantly different ($P > 0.05$). Means were separated using Fisher protected Least Significant Difference.

3. Results

3.1 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. 1). Firmness decreased, °Brix and pH increased while the remainder of the indices stayed relatively consistent over the incubation period.

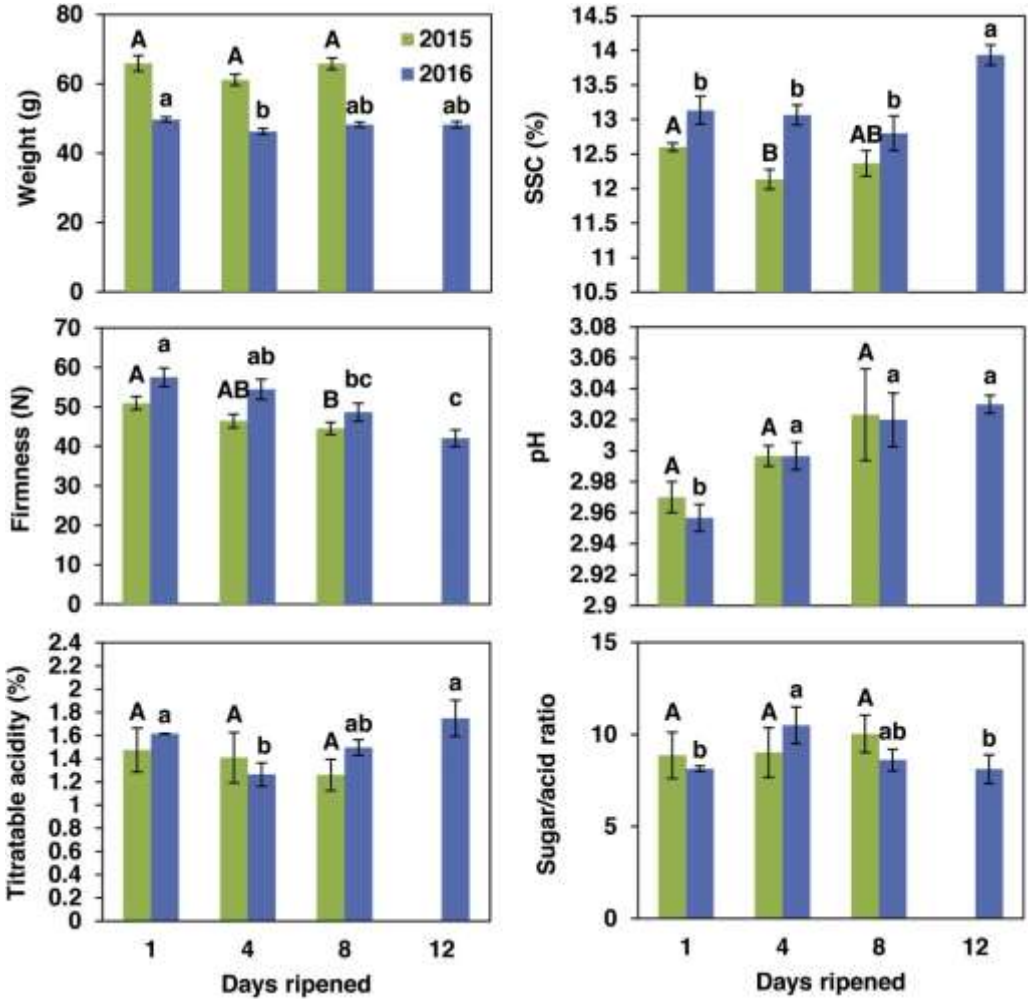

Fig. 1. Fruit indices of plum (cv. Fortune) ripened at ambient conditions (**1,** 1 d postharvest; **4,** 4 d postharvest; **8,** 8 d postharvest; **12,** 12 d postharvest). Vertical bars indicate standard error. Different letters (only letters of similar case are comparable) indicate treatments that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

3.2. Effect of fruit ripeness on infection and colonisation of *Penicillium* spp.

Repeat of trials 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. 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. 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.

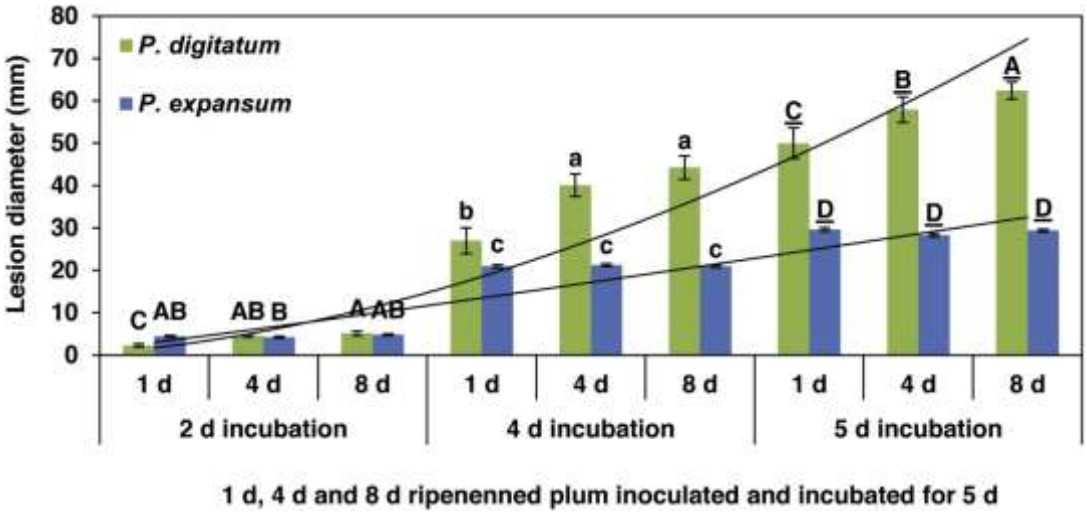

Fig. 2. Lesion growth of *Penicillium* digitatum and **P. expansum** on plum (cv. Fortune) of different fruit ripeness levels over 5 d ambient incubation. Wounds have been subtracted from lesion diameters. Vertical bars indicate standard error. Different letters (only letters of similar case and underlining are comparable) indicate treatments that are significantly different (**P** < 0.05) based on Fisher protected Least Significant Difference.

Fig. 3. Plum (cv. Fortune) inoculated with *Penicillium* spp. and incubated at ambient conditions. **A,** 4 d ripened fruit incubated for 5 d (left) and 8 d (right); **B,** 1 d ripened fruit incubated for 10 d.

3.3. 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. Lesions were greatly affected by all variables (Fig. 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. *P. expansum* was less affected by ripeness, cold storage (largest lesions) and inoculum load. However, the combined effect of inoculum load and cold storage yielded large differences. Disease

incidence of *P. expansum* was 100%. Disease incidence of *P. digitatum* varied, increasing as ripeness and inoculum load increased (Table 2).

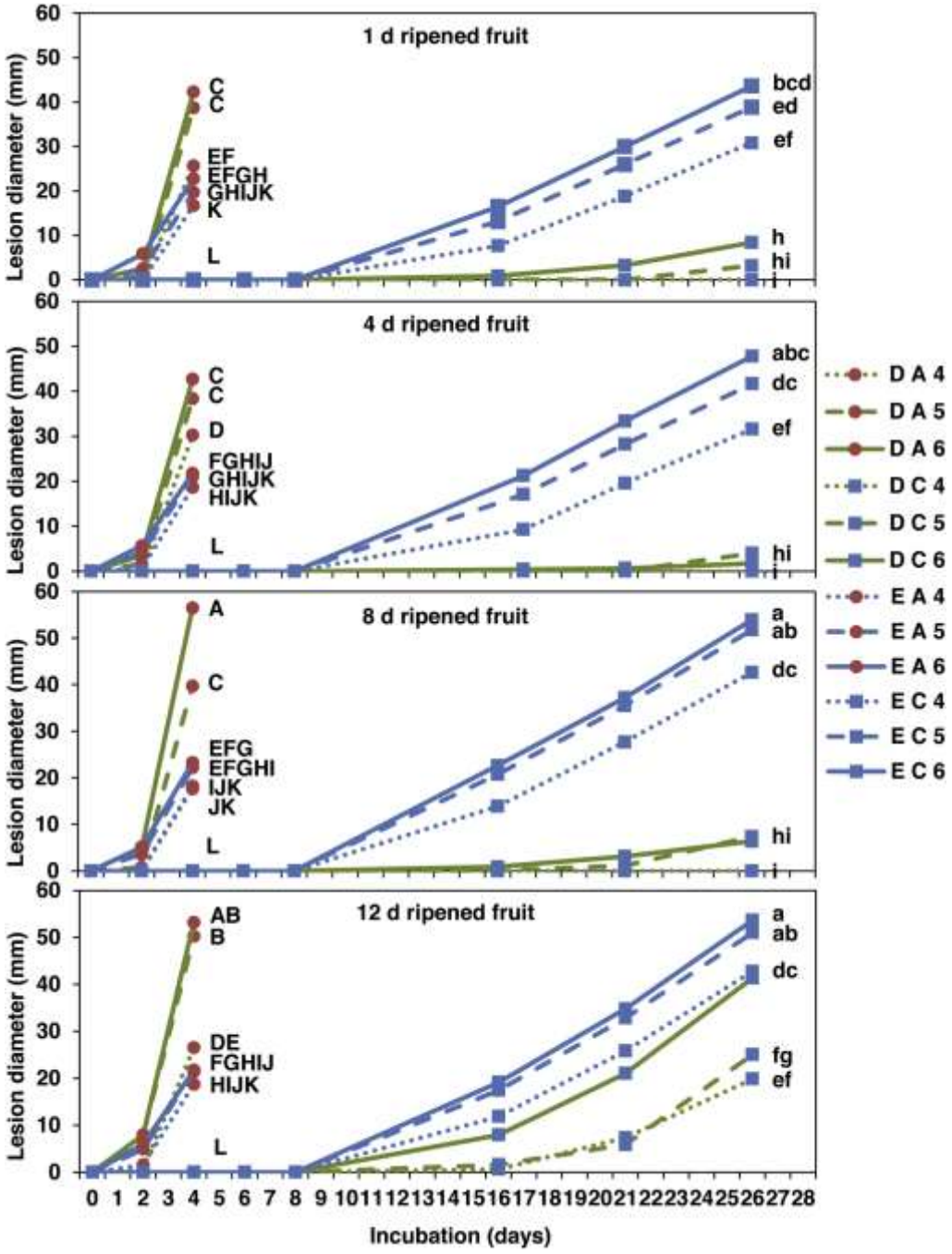

Fig. 4. Lesion diameter caused by different concentrations (**4**–**6**) of *Penicillium* digitatum (**D**) and *P. expansum* (**E**) on plum (cv. Fortune) at ambient (**A**) and cold (**C**) conditions. **4,** 10^4 conidia/ml (200 conidia); **5,** 10^5 conidia/ml (2 000 conidia); **6,** 10^6 conidia/ml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

Table 2 Disease incidence (%) of *Penicillium* spp. at 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

3.4. 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 decrease faster on riper fruit (Fig. 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.

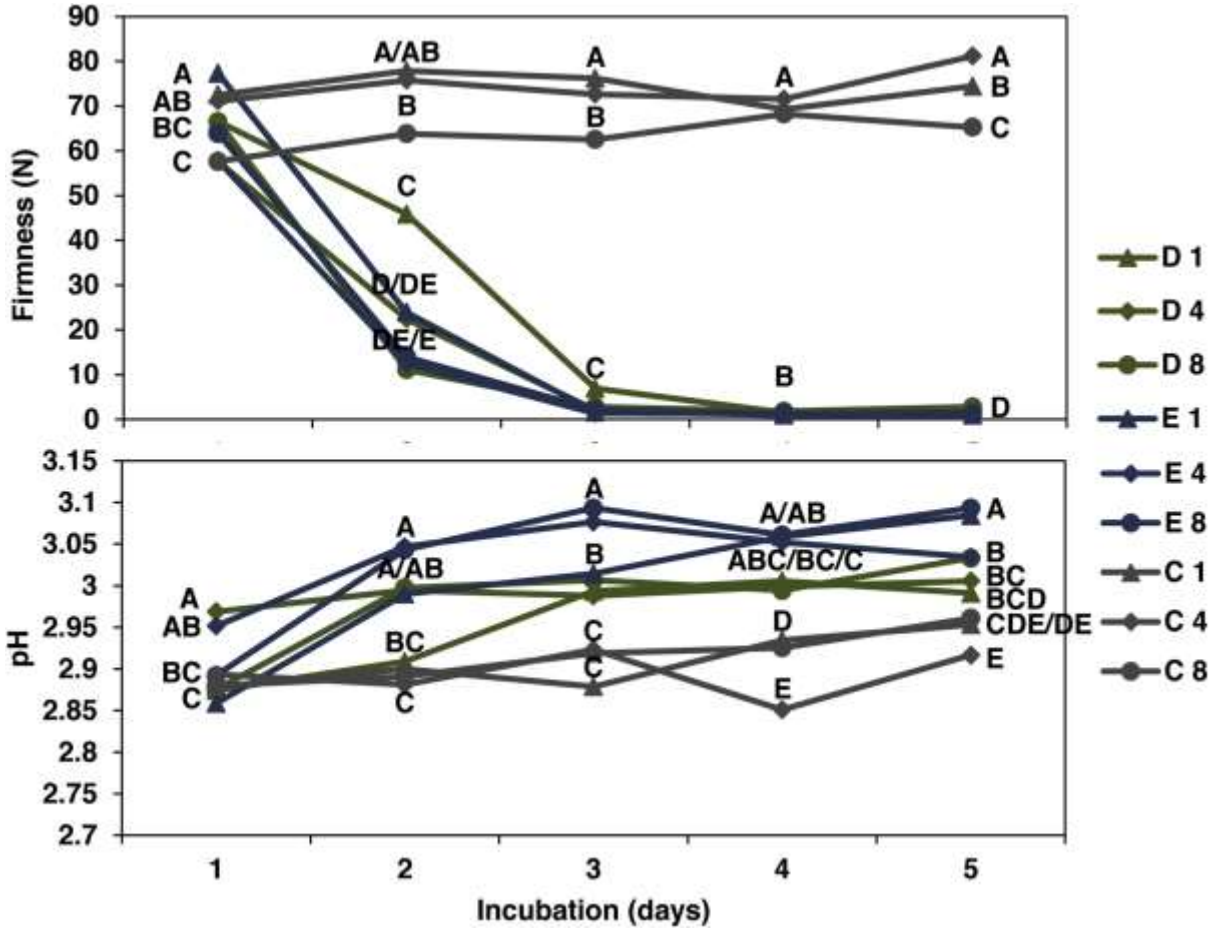

Fig. 5. Firmness and pH of lesions caused by *Penicillium* digitatum (**D**) and *P. expansum* (**E**) on plums (cv. Fortune) of different ripeness levels (**1,** 1 d ripened; **4,** 4 d ripened; **8,** 8 d ripened) over 5 d incubation. **C,** control. Different letters (only letters within a specific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

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 were not only darker in colour (Fig. 3A), but the pH of the 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 colonized and uncolonized tissue. In the case of the larger lesions of *P. digitatum* the difference between colonized and uncolonized tissue became less than what was observed with *P. expansum*. Most *P. digitatum* inoculated fruit were completely colonized 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).

3.5. 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 genes were higher, resulting in negative log values of normalised data (Fig. 6). From normalised data, *PG* of *P. digitatum* was significantly higher, *ACCD* and *creA* significantly lower and *pacC* similar to that of *P. expansum*. The ripeness of fruit did not have a significant effect on gene expression (P = 0.26–0.92). For *P. digitatum*, only *ACC*D decreased. For *P. expansum*, only *pacC* and *creA* increased. Incubation time had a significant effect on the expression of most genes. For *P. digitatum*, *PG* and *ACCD* significantly increased and *pacC* and *creA* significantly decreased. In the case of *P. expansum*, *PG*, *pacC* and *creA* decreased and *ACCD* increased.

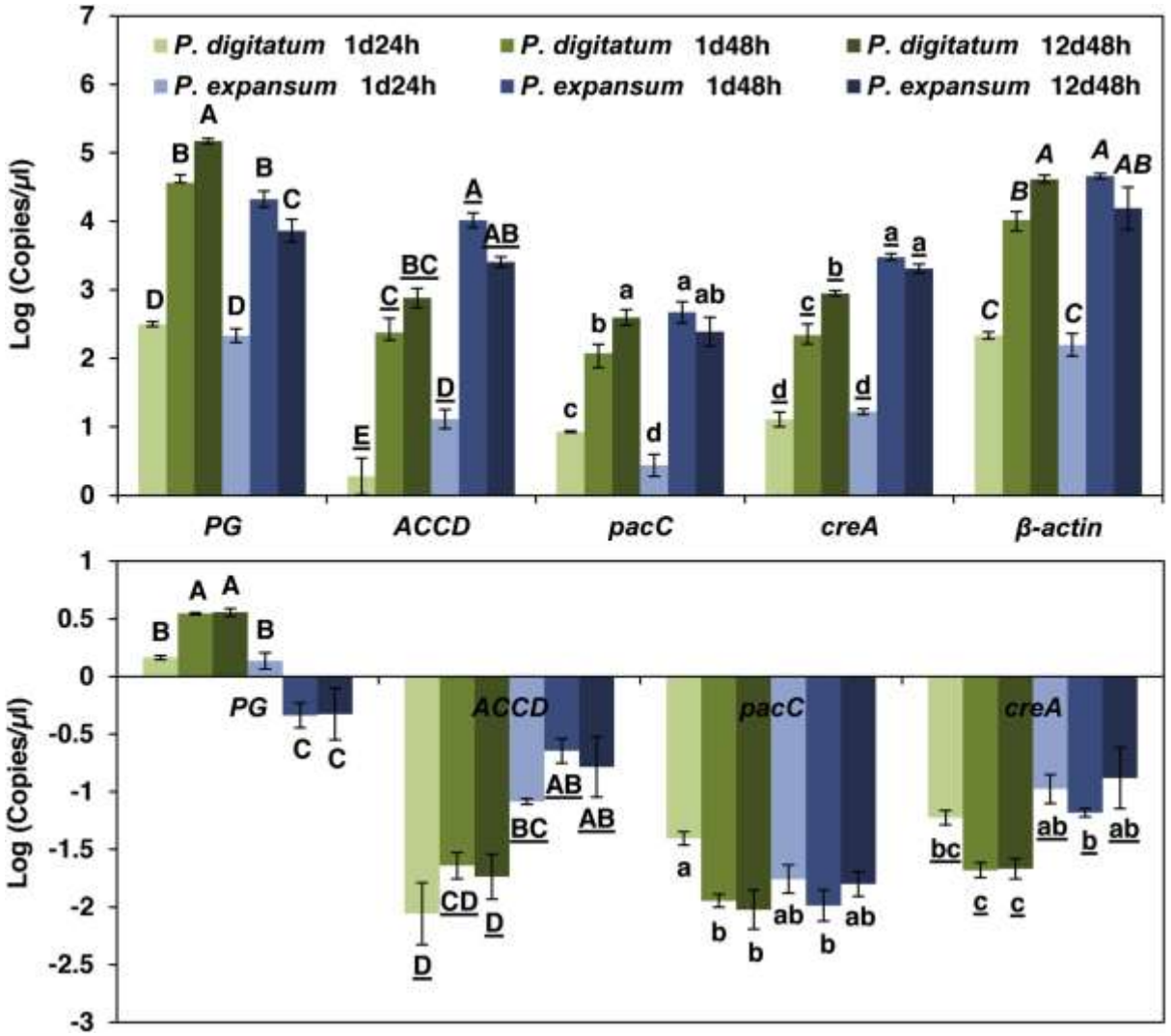

Fig. 6. Gene expression of *Penicillium* digitatum and *P. expansum* when inoculated in plum (cv. Fortune) of different ripeness levels (1 d and 12 d postharvest) after 24 h and 48 h incubation. Raw data (top) was normalised (bottom) using the reference gene (*β-actin*). Vertical bars indicate standard 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.

3.6. 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 the β-tubulin gene. 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 fruit ripeness stages. The connection between decay caused by *P. digitatum* on older fruit was originally not as prevalent on plum (Louw and Korsten, 2016). In this study, lesion diameter and disease incidence of *P. digitatum* showed a significant increase as fruit ripened. This was not the case with *P. expansum*. Louw and Korsten (2016) were the first 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 aggressiveness of different *Penicillium* spp. on different plum and nectarine cultivars (Louw and Korsten, 2016). In this study, we could specifically make the link between disease incidence and severity, and fruit ripeness.

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. 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)] compare to other apple cultivars (Louw and Korsten, 2014).

Prusky et al. (2004) reported that *P. expansum* caused 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. 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 of 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 we would thus expect larger lesions on less acidic cultivars or at least on cultivars with pH ≥ 3.5. This does not correspond with **in vivo** work of Prusky et al. (2004) or this study (pH of plums far below 3.5).

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 apples 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 depend 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 our 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 corresponds with our findings. 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, we can confirm acidification of colonised plum tissue.

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 study, the pH measurements of uncolonised tissue of inoculated apples were much higher than that of tissue at the inoculated sites ($\Delta_{pH} = 0.31–0.88$) and freshly harvested fruit ($\Delta_{pH} = 0.34–0.9$) [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 at the point of colonisation (leading edge of lesion). At the same time, they maintain an acidic environment within colonised tissue while pH of uncolonised tissue increase [$\Delta_{pH} = 0.31–0.88$ on apples (Prusky et al., 2004) and $\Delta_{pH} = 1.64–1.86$ 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 was 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).

Polygalacturinases 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 and thus also ACCD (Bi *et al.*, 2016). 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 nitrogen source, modulate environmental pH, and regulate certain genes, pectolytic enzymes and toxins (Barad *et al.*, 2016a; 2016b). The upregulation of ACCD in this study 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 at 48h and 72h whereas pepg2 decreased over time. Differential expression of pepg1 and pepg2 could be due to 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 besides 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) 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 upregulation 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 ACC will be induced on fresh fruit and not necessarily on riper fruit. The riper fruit was possibly already at a state where there wasn't a need for 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 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 vastly 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 up-regulated in colonised apple tissue but down-regulated *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 study, cold storage and inoculum load (facility hygiene) had a greater impact on *P. digitatum* than *P. expansum* on plum. If managed correctly, it could prove sufficient to control *Penicillium* green mould but not blue mould (*P. expansum*) 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* (6.3×10^4 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 dominate 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 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***.** *P. 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 reverse transcription ddPCR (RT-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 increase in ammonia) but as colonisation takes place (acidification) accelerated ripening will follow thus increasing 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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