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

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Abstract

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

caused by *P. digitatum*, *P. crustosum* and *P. solitum* on nectarine and plum. Rapid decay caused by *P. digitatum* highlighted the potential of the species to contribute to losses in the stone fruit industry. Future research should investigate the presence and impact of *P. digitatum* in the stone fruit supply chain. The role of fruit maturity in fruit-*Penicillium* interactions requires further investigation.

Key words: *Penicillium*, stone fruit, green mould, blue mould, virulence, SEM

Introduction

Nectarine and plum are climacteric fruit that are highly perishable and easily wounded, leading to a short shelf-life and representing a high risk crop in terms of postharvest decay (Crisosto and Mitchell 2011; Kader 2011). South Africa (SA) is a small, but important stone fruit producer that traditionally exports a significant volume of its crop. Fresh nectarine (*Prunus persica* (L.) Batsch var. *nucipersica* (Suckow) C. Schneider), peach (*Prunus persica* (L.) Batsch var. *persica*) and plum (*Prunus* L. spp.) exports reached 66 493 ton (~29% of total production) during the 2013/14 season. Plum occupied the largest export volume (~75%). Export of these fruit types contributed over €74.1 million in net export realisation for SA over the 2013/14 season (HORTGRO 2014).

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Numerous postharvest pathogens can cause decay of stone fruit. *Monilinia*, *Rhizopus*, *Penicillium*, *Alternaria*, *Botrytis*, *Cladosporium*, *Colletotrichum* and *Stigmina* include some of the most common fungal disease causing genera (Snowdon 2010).

According to Wells et al. (1994) and Snowdon (2010), brown rot is the most important disease of stone fruits, although *Penicillium expansum* Link can cause significant losses (>50%) when fruit (prunes) are wounded (Ceponis and Friedman 1957; Wells et al. 1994). *P. expansum* is the only *Penicillium* spp. reported to cause rot on commercial nectarine (*Prunus persica* var. *nucipersica*) and plum (*Prunus* spp.) (Pitt and Hocking 2009). Other reported *Penicillium* pathogens of stone fruit include *Penicillium crustosum* Thom on peach (*Prunus persica* var. *persica*) (Restuccia et al. 2006) and *Penicillium chrysogenum* Thom on blackplum (plum-like fruit of *Vitex doniana* Nielson) (Eseigbe and Bankole 1996). Lesions caused by *P. chrysogenum* on blackplum were small (≥8mm) after 8 days incubation (28±2ºC) and the fungus was infrequently isolated (19%) from the rotten fruit. No further disease symptoms were provided (Eseigbe and Bankole 1996).

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

Different fruit types often have overlapping export seasons and are usually handled and stored together from the point of distribution up to the market-end (Vermeulen et al. 2006; PPECB 2013). Complex fresh produce chains are also characterised by products from

different countries. These factors create an environment at risk to inoculum build-up, cross-contamination, cross-infection and ultimately losses. Risk can be higher at the end of a season (higher inoculum loads) and end of the fresh produce chain (increased susceptibility of older/riper fruit) so that even non-host pathogens can become a threat (Vilanova et al. 2012a; 2012b; 2014; Louw and Korsten 2014; 2015). For instance, when citrus increase inoculum levels of *P. digitatum* in a facility where pears are handled, *P. digitatum* can reach the pears via aerial dispersal or other means of cross-contamination, cause decay and contribute to losses (Louw and Korsten 2014; 2015). The study aims to determine the pathogenicity and aggressiveness of *Penicillium* spp. isolated from citrus and pome fruit supply chain environments on nectarine and plum cultivars, and partly examine the disease cycles (infection to reproduction) of *Penicillium* spp. on nectarine and alternative hosts (lemon and pear) via scanning electron microscopy (SEM). Observing this segment of the disease cycles on different host might prove helpful to illustrate the potential of inoculum build-up, cross-contamination and cross-infection.

Materials and methods

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

Table 1 Postharvest handling and storage practices of stone fruit cultivars

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

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

Fruit origin and handling. Postharvest practices for nectarine (*P. persica* var. *nucipersica*) and Japanese plum (*P. salicina*) cultivars collected for trials differed (Table 1).

All fruit were harvested at a mature stage according to industry guidelines (DAFF 2013a; 2013b). Fruit from 2011/12 were collected from farms and packhouses in the Waterberg region of Limpopo Province (one of the major production regions in SA). Fruit from 2012/13 were collected at the local market (Tshwane Fresh Produce Market) and originated from the Western Cape Province (the major production region in SA). Stone fruit are not commercially treated with any postharvest fungicides. Immediately after collection and transport, fruit were placed into cold storage (4-7°C; ±60% RH) at the University of Pretoria plant pathology laboratories.

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

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

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

Aggressiveness of *Penicillium* spp. on nectarine and plum cultivars. Nectarine and Japanese plum cultivars (Table 1) were inoculated with conidial suspensions of *P. expansum*, *P. crustosum*, *P. solitum* and *P. digitatum*. Isolates from the citrus export chain environment were used. Ten surface sterilised fruit of each cultivar were inoculated with each *Penicillium* spp. (10 fruit for every unique cultivar-*Penicillium* combination). Infected wounds (%), lesion diameter (*ld*) and symptom expression were recorded on the third, fifth and seventh day of incubation. Nectarine and plum cultivars from season 2012/13 (Table 1) were evaluated for first signs of mycelial growth and sporulation. Forty lesions were evaluated per cultivar-*Penicillium* interaction.

Scanning Electron Microscopy. Colonisation and sporulation differences between the most aggressive of the three pathogens assessed in this study (*P. expansum*, *P. crustosum* and *P digitatum*) were evaluated using SEM on nectarine, lemon and pear. Lemon and pear fruit were added to compare infection of nectarine with that of alternative hosts. Some of these host-pathogen associations were recently reported (Louw and Korsten 2014; 2015) and potentially serve as cross-contamination sources for stone fruit in the fresh produce chain. Fruit available during the same period ['Crimson Glo' nectarines (retail bought), 'Forelle'

pears (retail bought) and 'Eureka seeded' lemons (end-market and non-treated)] were wound-inoculated on the same day at two-to-three sites with 20µl conidial suspension of *P. expansum*, *P. crustosum* and *P. digitatum*. Two sets of three fruit were inoculated with each *Penicillium* spp. Control fruit (only wounded) were also included. One set was incubated for 24h and the other for 48h. Each set of fruit was randomised. Inoculated sites were cut out (5mm x 5mm) after incubation, placed into fixing solution [2.5% Glutaraldehyde ($OCHCH_2CH_2CH_2CHO$) in 0.075M phosphate buffer, pH 7.4] and held overnight in a refrigerator. The next day, samples were rinsed (x3) with 0.075M phosphate buffer for 10min, treated with 0.5% aqueous osmium tetroxide (OsO_4) (SPI Supplies Division Structure Probe, Inc., West Chester, USA) for 2h in a fume hood and rinsed another three times with phosphate buffer (10min each). Samples were dehydrated by submergence in an increasing range of ethanol concentrations [30%, 50%, 70%, 90%, 100% (x3)]. Samples were submerged for 10min in each concentration, except for the final 100% step in which case samples were held prior to critical drying in a Bio-Rad E3000 critical point dryer (Bio-Rad, Watford, UK). Samples were mounted on an aluminium stub, coated with carbon in an EMITECH K950X carbon coater (EM Technologies Ltd, Ashford, UK) with a BOC Edwards EXT 70H 24V pump (BOC Ltd, Crawley, UK) and viewed using a Zeiss Ultra Plus SEM (Ultra High Resolution FEG SEM) equipped with a Gemini column (Carl Zeiss NTS GmbH, Oberkochen, GER).

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

cover >25% and ≤50% of scan area, >5 and ≤10 conidiophores, chains of three conidia; **4** – mycelia cover >50% and ≤75% of scan area, >10 and ≤20 conidiophores, chains of four conidia; **5** – mycelia cover >75% of scan area, >20 conidiophores, chains of five or more conidia.

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

Statistical design and data analysis. All trials, excluding SEM, were repeated using a complete randomised design (CRD). Trials comparing different *Penicillium* spp. isolates and stone fruit cultivars had factorial arrangements. Four measurements (two inoculation sites, each with horizontal and vertical diameter measurements) were taken from each fruit (including wound sites on control fruit). The four measurements were averaged to account as one rep. The mean of wounds made on control fruit were subtracted from lesions on inoculated fruit, allowing lesions to be expressed without wounding effect. Data (lesion diameters with wounds subtracted) were subjected to analysis of variance (ANOVA) in Statistical Analysis System (SAS) (version 9.2; SAS Institute Inc., Carry, NC, USA).

Bartlett's test for homogeneity was used to reveal similarity among trial repeats (independent experiments). Nonsignificant differences resulted in trial repeats being pooled. Means were separated using Fisher protected Least Significant Difference.

Results

Confirming pathogenicity and comparing citrus isolates to pear isolates. The diameter of lesions from independent pathogenicity experiments were not significantly different (P = 0.91). All *Penicillium* spp. were detected pathogenic on both nectarine and plum cultivars (Table 2). Based on lesion sizes, the interactions between fruit type and *Penicillium* spp. were significantly different ($P < 0.0001$). *P. expansum* caused the largest lesions on Sunburst, *P. digitatum* the second largest and *P. crustosum* and *P. solitum* thereafter. Disease incidence was low for *P. digitatum* and *P. solitum* on Sunburst. Only 25% of lesions caused by *P. digitatum* on nectarine were significantly larger than control fruit. The largest lesion was 83.90±8.49mm in diameter (diameter of wound subtracted) after seven days incubation. *P. digitatum* caused the largest lesions on African Rose, thereafter *P. expansum*, *P. crustosum* and *P. solitum*. Disease incidence was 100% for all *Penicillium* spp. on the plum cultivar.

Independent experiments of the isolate comparison trial were not significantly different (P = 0.72). However, a distinct difference was noted for *P. digitatum* in the independent experiments (Fig. 1). Both isolates of *P. digitatum* failed to cause lesions on Bright Pearl in the initial experiment, yet large lesions were produced in the second experiment [citrus isolate ld = 24.28±2.65 (20%); pear isolate ld = 22.95±3.10mm (60%)]. Because only mean disease severity data are reported in Figure 1, results from *P. digitatum* in the first experiment (no lesions) are not observable. Disease incidence for *P. expansum* and *P. crustosum* were 100% for both isolates, whereas that for *P. solitum* was 65% (citrus isolate) and 70% (pear

isolate). Control fruit yielded no lesions. Citrus and pear chain isolates were not significantly different based on *ld* ($P = 0.60$).

Table 2 Lesions caused by *Penicillium* spp. on nectarines and plums after seven days incubation

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

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

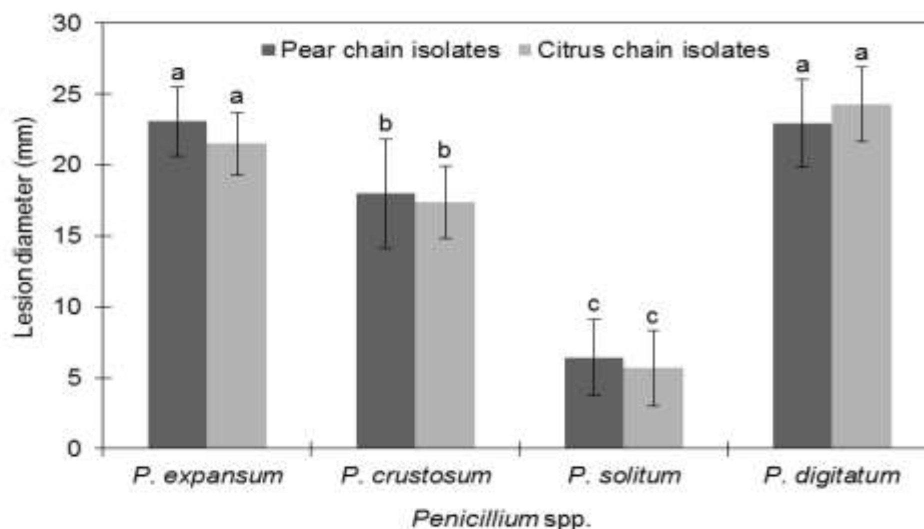
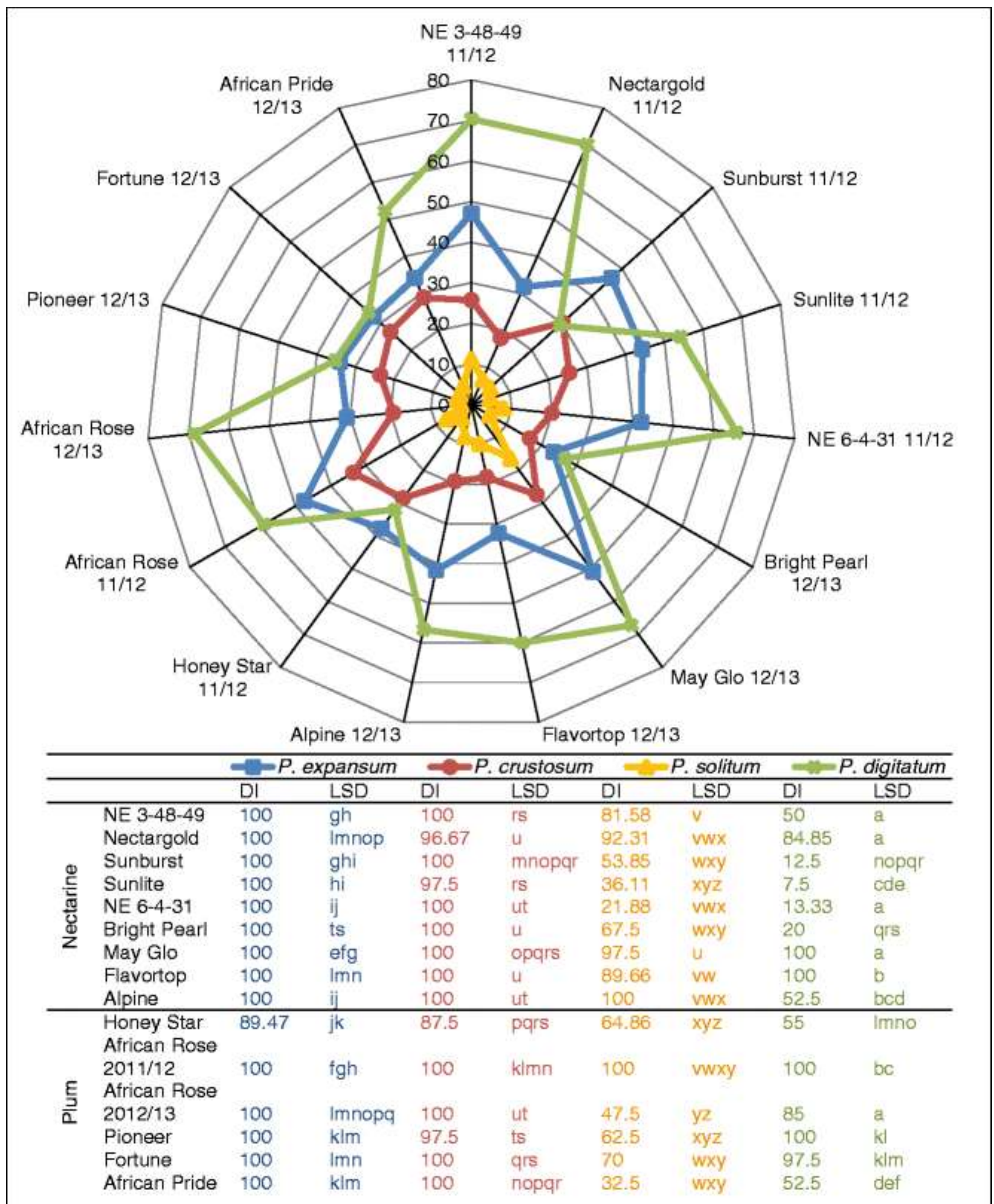


Fig. 1 Comparing *Penicillium* citrus -and pear chain isolates in terms of mean lesion sizes (10 fruit) produced on Bright Pearl nectarines. Mean diameter of control was subtracted from lesion diameters. Vertical bars represent standard deviation. Different letters are significanly different ($P < 0.05$) according to Fisher protected Least Significant Difference.

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

The different interactions between cultivar and *Penicillium* spp. were significantly different based on lesion size (P < 0.0001). *P. digitatum* caused the largest lesions on most cultivars, however disease incidence varied (Fig. 2). Low incidence was recorded on Sunburst, Sunlite, NE 6-4-31 and Bright Pearl. *P. expansum* and *P. crustosum* caused lesions throughout both cultivar ranges at high incidence. *P. solitum* caused the smallest lesions and disease incidence was low on some cultivars (Sunlite, NE 6-4-31, African Rose 2012/13 and African Pride).

Symptom expression of *Penicillium* spp. on nectarine and plum cultivars. Symptom expression was relatively consistent across the cultivars evaluated (Fig. 3). Lesions commenced with browning and softening of underlying tissue. Symptoms were more visible on light coloured cultivars. Softening of tissue resulted in a slightly sunken appearance of lesions. White mycelial growth developed from brown infected tissue, subsequently yielding conidiophores producing conidia. In the case of *P. digitatum* infected fruit, cellular collapse caused a wrinkled appearance of rotten areas. The wrinkling appearance became apparent



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

Fig. 2. Figure of mean lesion diameter (mm), and table of least significant difference (LSD) and disease incidence (DI) (%) of pathogenic *Penicillium* spp. on nectarine and plum cultivars (20 fruit per cultivar) after 7 days incubation (5 days incubation for Nectargold) at room conditions. Means of wounds from control fruit were subtracted from means of lesions. Letters that are dissimilar are significantly different ($P < 0.05$) based on mean of lesion diameter according to Fisher protected Least Significant Difference

Fig. 3. Symptoms caused by *Penicillium* spp. on nectarine and plum cultivars after 7 days incubation at room conditions

when fruit surfaces were overlaid with conidia. *P. digitatum* was able to produce copious amounts of lime green conidia within the incubation period. The other *Penicillium* spp. yielded blue to blue-grey conidia. *P. expansum* produced the highest amount of conidia of the blue mould causing *Penicillium* spp. Overall, more conidia were produced on nectarines than on plums.

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

Table 3 Days of incubation for first visible signs of mycelia and conidia

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

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

Observations from SEM images. All interactions were compatible for germination of conidia and the development of mycelia within 24h (Table 4). The degree of germination and mycelial growth depended on the host-*Penicillium* spp. interaction. Only microphotographs revealing new findings are presented in Figure 4. No conidiophores were produced at 24h or

on pears at 48h. The 48h incubation period showed a significant progression in the life stages of all *Penicillium* spp. on nectarine. *P. expansum* was the only species that did not produce conidia on nectarine within the 48h incubation period, although mycelial growth was abundant and conidiophore development was observed (Table 4 + Fig. 4d). *P. crustosum*

Table 4 Scanning electron microscopy observations of lesions caused by *Penicillium* spp. on fruit after 24h and 48h incubation at room conditions

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

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

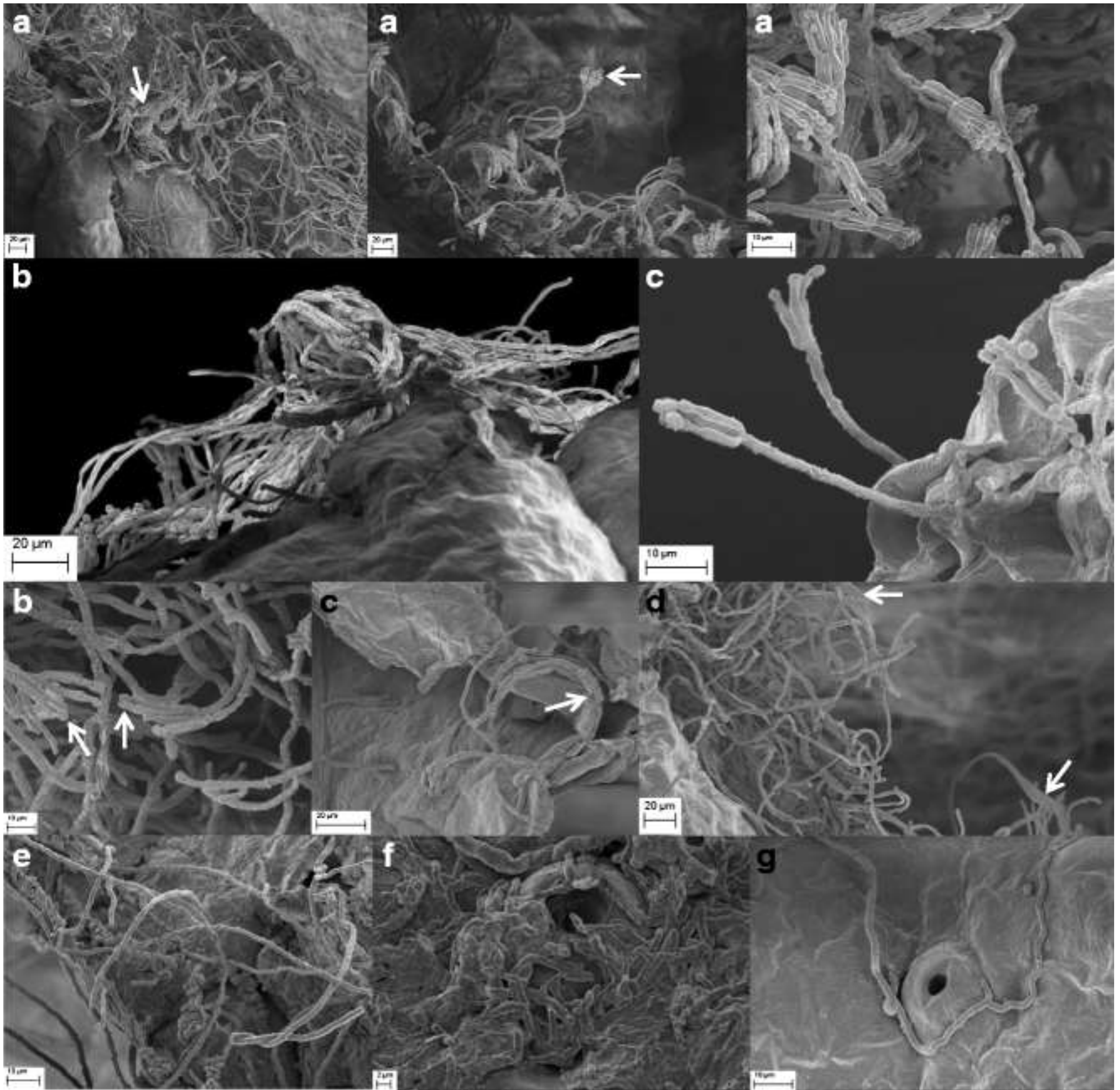


Fig. 4. Scanning electron micrographs of *Penicillium* spp. on nectarine, pear and lemon. **a** *P. crustosum* sporulating on nectarine (48 h). **b** *P. digitatum* sporulating on nectarine (48 h). **c** *P. expansum* sporulating on lemon (48 h). **d** *P. expansum* producing metula on nectarine but no conidia (48 h). **e** Coiling and twisting of *P. expansum* mycelia on pear (48 h). **f** *P. digitatum* not penetrating open stomata of lemon (24 h). **g** *P. digitatum* mycelium growing around open stomata of lemon (48 h)

produced the most conidia within 48h on nectarine (Table 4 + Fig. 4a), followed by *P.digitatum* (Table 4 + Fig. 4b). Severe twisting and coiling of *P. expansum* mycelia was observed on pears after 48h (Fig. 4e). *P. expansum* and *P. crustosum* showed little

germination on lemons within 24h (also observed from *P. expansum* on pear), yet *P. expansum* was able to sporulate after 48h (Fig. 4c). *P. expansum* was able to produce more conidia on lemons within 48h than *P. digitatum*, although *P. digitatum* produced the most abundant mycelia within the same period. *P. crustosum* was unable to produce conidiophores on lemons within 48h (Table 4). Observations revealed that *P. digitatum* did not grow towards or penetrate open stomata of lemon fruit (Fig. 4f-g). This was also observed from *P. crustosum* on lemons after 48h (mycelium growing over open stomata). Stomatal interactions were not observed on other hosts. No conidia or fungal activity was observed on control fruit.

Table 5. Identity of β-tubulin gene sequences and GenBank accession numbers

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

N, Nectarine; P, Plum.

Reisolation from fruit and species identification. Isolates made from symptomatic fruit were successfully grouped according to PCR-RFLP and positively identified using sequencing (Table 5). This confirmed Koch's postulates by identifying the isolated *Penicillium* spp. as the species previously inoculated into the fruit.

Discussion

All *Penicillium* spp. (*P. expansum*, *P. crustosum*, *P. solitum* and *P. digitatum*) inoculated into nectarine and plum proved to be pathogenic. *P. expansum* is a well-known postharvest pathogen of stone fruit (Ceponis and Friedman 1957). To our knowledge this is the first report demonstrating the pathogenicity of *P. crustosum*, *P. solitum* and *P. digitatum* on plum. One study demonstrated *P. crustosum* causing disease on peaches (cv. Late Peach of Leonforte) (Restuccia et al. 2006). They recorded 100% disease incidence after 15 days in a biological control experiment. The size of lesions and symptoms were not provided. The purpose of their study was to investigate the potential use of commercial biocontrol products to inhibit disease caused by *P. crustosum* and *Mucor circinelloides* Tiegh. on peaches. Nectarine is a mutant of peach and thus belongs to the same species but a different variety (Blake 1932). Pathogenicity of *P. crustosum* on nectarine was thus expected, but our study is the first to specifically provide evidence of *P. crustosum* pathogenicity on nectarine and deliver new information on decay caused by *P. crustosum* on *P. persica*. *P. solitum* has never been associated with or isolated from stone fruit before. Ma et al. (2003) previously made use of *P. digitatum* isolates from nectarine and plum, but no connections were made to decay and no pathogenicity trials were conducted. Navarro et al. (2011) report infection volumes of roughly 1300mm^3 and 1500mm^3 caused by *P. digitatum* on nectarine (Flavela and Flanoba). Large infection volumes were not recorded and no symptoms were illustrated or described.

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

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

In our study *P. solitum* was evaluated as the least aggressive species with low disease incidence (67.85%). The significance of the species in the fresh produce market is considered negligible. The species has a very small host range, it is not known to produce any significant mycotoxins (Frisvad and Samson 2004; Pitt and Hocking 2009) and only causes small lesions when pathogenic (pome fruit) (Louw and Korsten 2014). However, little is still known of the species (Pitt et al. 1991; Pitt and Hocking 2009) and it has the ability to sporulate rapidly from small lesions, which can contribute to higher inoculum loads in fruit storage environments. Higher inoculum loads of pathogens can lead to increased disease incidence and severity (Vilanova et al. 2012a; 2012b; 2014).

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

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

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

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

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

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

The pathogenicity and high aggression of *P. digitatum* on some pome fruit cultivars were linked to old and over-mature fruit (Louw and Korsten 2014; Vilanova et al. 2014). Fruit physiology was not evaluated in this study, but it was observed that fruit age played a similar role in the pathogenicity tests of *P. digitatum* on stone fruit. This was particularly noted when comparing interactions on freshly picked nectarines (cvs. Sunburst and Sunlite) to older fruit used in trial repeats (long stored) (cvs. NE 3-48-49, Bright Pearl and African Pride). Recently picked fruit inoculated with *P. digitatum* showed smaller lesions and very low

disease incidences. Disease incidence on NE 3-48-49 increased from 20 to 80% due to one day prolonged cold storage. Bright Pearl fruit used to compare different environmental isolates were stored four days longer in the trial repeat, resulting in the disease incidence increasing from 0 to 20% (citrus isolate) and 0 to 60% (pear isolate). Likewise, Bright Pearl used to compare aggressiveness on different cultivars caused a disease incidence shift from 0 to 40%. *P. solitum* was also affected by fruit age (cvs. Sunburst, Sunlite and African Pride), but to a lesser extent than *P. digitatum*. The remainder of the *Penicillium* spp. evaluated in this study was not affected by fruit age. Future work will focus on the influence of fruit maturity and ripeness on host defence mechanisms and decay caused by these pathogens. The significance of such research would depict pathogenic profile shifts as fruit mature and ripen.

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

Handling of citrus, pome and stone fruit in close proximity anywhere along the fresh produce chain can further contribute to decay caused by *P. digitatum* on these fruit types. The potential of cross-contamination and cross-infection taking place cannot be avoided. In SA, the start of the stone fruit export season overlaps with the end of the citrus export season and crosses with the pome fruit export season (PPECB 2013). Inoculum loads tend to increase as seasons progress. High inoculum levels of *P. digitatum* can thus be present in the

fresh produce chain at the end of the citrus season. Inoculum levels can be even higher if sanitary practices are neglected. These aspects can contribute to decay caused by *P. digitatum* on stone fruit, especially at the end of the export chain when fruit can be riper. Little to nothing is known of *P. digitatum* decay of stone fruit in the fresh produce chain. The causal agents of decay are rarely identified or identified based on symptom expression. Further research is needed to isolate and associate *P. digitatum* with postharvest losses in the fresh produce chain of stone fruit.

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Compliance with Ethical Standards

The authors of this manuscript declare that this work complies with the Ethical Standards of the journal, have no conflict of interest and agree on the publication of the work.

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