Preharvest Detection and Postharvest Incidence of *Phlyctema vagabunda* on ‘Cripps Pink’ Apples in South Africa

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Abstract

Phlyctema vagabunda is responsible for significant postharvest losses in ‘Cripps Pink’ apples in South Africa. The first objective of this study was to determine the presence and incidence of *P. vagabunda* on stored commercial ‘Cripps Pink’ apple in five major pome fruit growing regions in the Western Cape. As the fungus remains latent until postharvest, the second objective was to develop a rapid molecular detection tool to determine the presence of *P. vagabunda* on asymptomatic ‘Cripps Pink’ apples from two commercial orchards. Postharvest disease incidence in ‘Cripps Pink’ apples in the Western Cape ranged from 0 to 73% in the 2010/2011, 0 to

6% in 2011/2012, and 0 to 30% in 2012/2013 seasons. *P. vagabunda* spores were also detected on ‘Cripps Pink’ fruit from December to February and from March to April. In December and January, *P. vagabunda* spores were detected on the ‘Hillieri’ crab apple pollinator. The knowledge that *P. vagabunda* conidia are present on the surface of ‘Cripps Pink’ apples and ‘Hillieri’ crab apples during the growing season could be applied to improving current management practices in the orchards.

Keywords: lenticel decay, pome fruit, RFLP, pathogen detection

Phlyctema vagabunda (Guthrie) Verkley (syn. *Neofabraea vagabunda* (E.J. Guthrie) Verkley) is a latent fungal pathogen causing postharvest lenticel decay of pome fruit worldwide (Bompeix and Cholodowski-Faivre 1997; Henriquez et al. 2004; Verkley 1999). It forms part of the bull’s-eye rot disease complex with *N. malicorticis* (Jackson) Nannfeldt, *N. perennans* (Kienholz) Dugan, Roberts & Grove, and *N. kienholzii* (Seifert, Spotts & Lévesque) Spotts, Lévesque & Seifert (Verkley 1999). First reported in South Africa in the 1960s by its synonym *Gloeosporium album* Osterw., the pathogen caused storage rot of apple in Elgin and Piketberg (Vorster 1965), and was reported in most of the apple growing regions in the Western Cape in the 1980s (Matthee 1982).

Although *P. vagabunda* was reported to cause significant postharvest decay on the late maturing ‘Cripps Pink’ apple variety in the Ceres and Grabouw regions of the Western Cape since 2009 (Den Breeyen and Lennox 2011; Lennox et al. 2009), no information exists regarding its distribution or incidence in the pome fruit growing regions in the Western Cape. Determining the distribution and incidence of *P. vagabunda* will be valuable in determining high-risk areas where management strategies, storage periods, and timely marketing of apple fruit can be reviewed and revised to reduce losses.

P. vagabunda, *N. malicorticis*, and *N. perennans* have been reported on a wide variety of plants other than pome fruit, including different crab apple varieties (*Malus fusca*, *Malus rivularis* (L.) Douglas, *Malus baccata* (L.) Borkhausen), quince (*Cydonia oblonga* L.), and Hawthorne (*Crataegus* spp.) (Creemers 2014; Kienholz 1939; Verkley 1999). These plant species may act as alternate sources of inoculum or as hosts for survival of the *Phlyctema* and *Neofabraea*

species during their lifecycle. Kienholz (1939) noted that native crab apples are believed to be the original host of the lenticel decay fungi and that the alternative hosts might serve as important sources of inoculum if they neighbor apple or pear orchards (Childs 1929).

Although methodologies such as spectral imaging (Adamiak et al. 2012), immunological and molecular methods (Gariépy et al. 2003; Křížková et al. 2008) have been developed to detect the presence and/or identify *Phlyctema* species from diseased tissue of mature fruit, there are no techniques available to detect *P. vagabunda* on asymptomatic fruit in the orchard.

The primary objective of the study was to determine the prevalence and incidence of *P. vagabunda* on stored commercial ‘Cripps Pink’ apples in the five major pome fruit growing regions in the Western Cape. The second objective was to develop a rapid molecular detection tool to ascertain if and when *P. vagabunda* was present on preharvest ‘Cripps Pink’ apples from two commercial pome fruit orchards in the Western Cape.

Materials and Methods

Postharvest disease incidence and distribution. Asymptomatic ‘Cripps Pink’ apples were collected from commercial pack houses and Pg producers in 2010, 2011, and 2012. The fruit was not exposed to any postharvest treatments. One hundred fruit were obtained from a specific orchard block for each producer. The orchards were located in Elgin, Grabouw, Witzenberg Valley, and the Hemel-and-Aarde Valley in the Western Cape province, and the Koue Bokkeveld in the Eastern Cape province. Availability of fruit varied between orchards, producers, seasons, and regions (Supplementary data). In total, fruit was collected from 24 producers in the first season, and from 41 producers for the subsequent two seasons.

The fruit was packed on polystyrene fruit trays disinfected with 0.002% Sporekill (active ingredient: Didecylidimethylammonium Chloride 120 g/liter; ICA International Chemicals, South Africa). Moist paper towels were placed at the base of commercial pome fruit boxes lined with polyethylene bags, and the fruit trays were carefully packed. The moist paper towels ensured favorable humidity for the development of decay. The packed fruit was stored at –0.1°C in regular atmosphere (RA). After 4 months in RA, the fruit were stored at 25°C for 7 days to allow for optimal disease symptom expression.

The fruit was visually evaluated for bull’s-eye rot symptoms, and the frequency and characteristics of lenticel decay were identified per producer orchard. Subsequently, symptomatic fruit were transferred

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Funding: This study was funded by Hortgro Pome THRIP and National Research Foundation.

*The e-Xtra logo stands for “electronic extra” and indicates that supplementary data is published online.

The author(s) declare no conflict of interest.

Accepted for publication 26 September 2019.

to 4°C (RA) for an additional 30 days to allow for the development of fruiting bodies (acervuli).

Pathogenicity tests. A total of 5 *P. vagabunda* isolates, obtained from symptomatic fruit sampled in orchards located in the Witzenberg Valley, Elgin, and Vyeboom, in the Western Cape province, were grown on malt extract agar (MEA⁺) at 21°C in darkness for 30 days and tested for their pathogenicity on the apple cultivars ‘Fuji’, ‘Golden Delicious’, and ‘Cripps Pink’. Prior to inoculation, the fruit were surface sterilized with 70% ethanol (EtOH) for 30 s, and air-dried inside a laminar flow cabinet. A 5- to 10-mm-deep plug of external apple tissue was removed from each fruit using a 6-mm-diameter sterile cork borer (Romero et al. 2018). Subsequently, a 6-mm plug was taken from the margin of an actively growing colony and transferred into the wound. The inoculation wounds were sealed with petroleum jelly, and the inoculated fruit were placed in a clear Perspex moisture chamber at ambient room temperature (24°C ± 2°C; 80% relative humidity). Six fruits per cultivar were inoculated, and each fruit was inoculated twice, on alternate sides of the same fruit. Fruit from each cultivar were inoculated with clean MEA⁺ plug as controls. Lesion diameters were measured after 14 days. Reisolations were made from the resulting lesions to confirm the causal organism as *Phlyctema*. This trial was repeated once.

Orchard sample collection. Two commercial ‘Cripps Pink’ apple orchards, one located in the Witzenberg Valley and the other in Grabouw, Western Cape, each with a history of *P. vagabunda* infection were selected. Asymptomatic ‘Cripps Pink’ fruit from within the tree canopy were collected on a monthly basis during the growing season, from one-month post full bloom in November until harvest in April, in 2011, 2012, and 2013. ‘Hillieri’ crab apples (*Malus hupehensis* (Pamp.) Rehder), used as cross-pollinators in the Witzenberg Valley orchard, were collected at the same time.

For the collection of the fruit, a randomized sampling within each orchard was followed. For each orchard, tree rows were numbered as well as the trees within each row. A set of 25 nonunique numbers were generated for the tree rows using Research Randomizer (<https://www.randomizer.org>; Urbaniak and Plous 2013). An additional set of 25 nonunique numbers was generated for trees within the rows selected in the first number set. Both sets excluded the first and last rows and trees to avoid external influences from surrounding orchard blocks. For each month, new row-tree number sets were generated for each orchard. Twenty-five sample sets comprising 20 fruit covering the top, middle, and bottom of each tree were kept in marked brown paper bags in cooler boxes in the field. At the laboratory, the fruit was stored at -0.1°C in RA until they were processed.

Climatic factors. Rainfall volume and duration, minimum and maximum temperature, and relative humidity were collected from 2009 to 2013 from the Witzenberg Valley and Grabouw orchards, where the monthly sampling was undertaken. Statistical analysis of the hourly data collected from weather stations situated close to each orchard and the relationship of climatic factors between the two orchards and the sampling seasons was undertaken with Statistica (v. 10; StatSoft Inc., U.S.A.).

Removal of *P. vagabunda* conidia from orchard fruit. The sampled orchard fruit was subdivided into five manageable sets according to the tree numbers sampled. One hundred fruit from the first five trees grouped as the first set, the fruit of the next five trees in the second, and so forth.

Each fruit set was sonicated, using an adapted methodology from Spotts and Holz (1996) and Narayanasamy (2011), to dislodge spores. The fruit was washed in 1 liter of sterile distilled water (sdH₂O) amended with 1% Tween 80 and sonicated for 10 min at a frequency of 50 Hertz (Hz) in a water bath (UMC2, Ultrasonic Manufacturing Company, South Africa). Fruit sampled in November and December (100 fruit/subset) were sonicated together. For the remaining months, when the fruit in a set was too large and could not be processed once-off, the fruit was sonicated in batches, recycling the same sonication-water for that particular set. The fruit was discarded, and the spore-fruit-wash solution was poured through a clean sieve and stored at -0.1°C in sterile 1-liter bottles. The step was repeated for the remaining fruit sets.

The spore-solution of each set was distributed into three 250-ml Beckman jars (Beckman Coulter Inc., Fullerton, CA, U.S.A.), and were centrifuged for 20 min at 10,000 rpm in a J2-21 Beckman centrifuge (Beckman Coulter). The supernatant was discarded, and the remaining spore-pellets transferred to 50-ml conical Falcon tubes (Thermo Fisher Scientific, Waltham, MA, U.S.A.). The tubes were filled to the maximum volume of 50 ml with sdH₂O, briefly vortexed and spun for 10 min at 4,000 rpm. The supernatant was discarded, and the wash step repeated. The spore pellets were transferred to 15-ml Falcon tubes, filled with sdH₂O, and briefly vortexed. The tubes were centrifuged for 10 min at 10,000 rpm. The supernatant was discarded, and the wash step repeated. Resulting spore-pellets were transferred to 2-ml tubes, filled with sdH₂O, vortexed and spun for 5 min at 13,000 rpm. The supernatant was discarded, and the wash step repeated twice. The resulting spore-pellets were stored at -20°C for DNA extraction.

DNA was extracted from the spore-pellets, amplified with the Neo8tub primer set and pathogen identity determined according to the PCR and RFLP sections in the following section.

The PCR-RFLP detection tool.

Isolates. DNA extracted from monosporic *P. vagabunda* isolates: STE-U 7659, 7663, 7664, and 7666, were obtained for Department of Plant Pathology, Stellenbosch University culture collection. The following *Phlyctema* and *Neofabraea* species were obtained from the Westerdijk Fungal Biodiversity Institute, Utrecht, the Netherlands, namely: *P. vagabunda* (CBS 304.62), *N. perennans* (CBS 453.64), *N. malicorticis* (CBS 141.22), and *N. kienholzii* (CBS 355.72, syn. *Neofabraea* sp. nov.). The isolates were grown on potato dextrose agar plates (PDA, Biolab, Gauteng, South Africa) at 23°C in the dark. After 21 days, the resulting mycelium was scraped from the plates and transferred to 2-ml microcentrifuge tubes for DNA extraction.

DNA extraction. For DNA extraction, a Wizard Genomic DNA Purification Kit (Promega Corporation, Madison, WI, U.S.A.) was used. The ‘tissue culture cell lysates’ purification method of the supplier was followed but was preceded by a homogenization step. The homogenization step consisted of the following: (i) approximately 0.5 g glass beads and 400 µl lysis buffer were added to each sample and shaken for 5 min at 30 Hz in a Retsch mill (Verder Scientific, Haan, Germany), (ii) lysated spores were incubated in a 65°C water bath for 10 min, and centrifuged for 4 min at 14,000 rpm. Following extraction, the DNA concentration of each sample was determined on a NanoDrop spectrophotometer (Thermo Fisher Scientific). DNA samples were stored at -20°C.

Primer design. A selected subset of partial sequences of the β-tubulin gene region, generated for *Neofabraea* species by de Jong et al. (2001), were obtained from the nucleotide database GenBank (NCBI, <https://www.ncbi.nlm.nih.gov/nucleotide>) (Table 1). Sequences were aligned using MAFFT sequence alignment program version 6 (Kato and Toh 2008) followed by manual adjustments of the alignments in Sequence Alignment Editor v. 2.0a11 (Rambaut 2002), examined, and a 411 base pair (bp) conserved region unique to *P. vagabunda* was selected. The primer pair Neo8tubF (5'-ACC ACTT GGT CTC CGC AGT-3') and Neo8tubR (5'-GGG ATC CAC TCG ACG AAG TA-3') was designed based on the 411-bp region using Primer3 (version 0.4.0; Untergasser et al. 2012).

Polymerase chain reaction (PCR) amplification. DNA, obtained from the STE-U and CBS *Phlyctema* and *Neofabraea* cultures and from a subsection of the fruit wash sets, was amplified with the Neo8tub primers in a 40-µl reaction. Each reaction volume contained 1× reaction buffer, 1.0 µg/ml bovine serum albumin (BSA), 2.25 mM MgCl₂, 0.2 mM dNTPs, 0.15 µM of each primer, 2 U Biotaq DNA polymerase (5 U/µl; Bioline, London, UK), and 10–100 ng of DNA. All PCR reactions were conducted in an Applied Biosystems 2700 PCR machine (Applied Biosystems, Carlsbad, CA, U.S.A.). The amplification conditions were an initial denaturing step of 3 min at 96°C, followed by 40 cycles consisting of 1 min at 96°C, 1 min at 62°C, and 90 s at 72°C, and a final extension step of 5 min at 72°C.

PCR products were visualized alongside a 100-bp ladder (GeneRuler, Thermo Fisher Scientific) with ultraviolet fluorescence on a 1.5% agarose gel stained with ethidium bromide.

The amplified 411-bp PCR products were purified with an MSB Spin PCRapace Kit (Invitex, Hayward, CA, U.S.A.). The purified PCR-products were prepared for forward and reverse sequencing in a Big Dye Terminator 3.1 sequencing kit (Applied Biosystems). Samples were analyzed on an ABI Prism 3730xl genetic analyzer (Applied Biosystems). To confirm the identity of the resulting nucleotide sequences, the sequences were run through the Basic Local Alignment Search Tool (BLAST) of the National Centre for Biotechnology Information (NCBI; <https://blast.ncbi.nlm.nih.gov/Blast.cgi>). In addition to the designed Neo8tub primer set, the amplification of DNA was performed with species-specific primers in a multiplex-PCR as described by Gariépy et al. (2003) with the improved forward primer by Soto-Alvear et al. (2013).

Restriction fragment length polymorphism analysis (RFLP). In order to differentiate between *P. vagabunda*, *N. malicorticis*, *N. perennans*, and *N. kienholzii* RFLP-analysis, single nucleotide polymorphisms unique to each of the species were identified within the 411-bp region amplified by the Neo8tub primer set in Geneious (v5.3.3; Kearse et al. 2012). Enzymes able to cut at these polymorphisms were chosen for the restriction analysis.

For *P. vagabunda*, the enzymes *Bbs*I, *Ssp*I, *Ksp*AI, *Bsu*RI (Fermentas, Waltham, MA, U.S.A.) were individually tested in a 20- μ l reaction volume containing 10 units (U) of restriction enzyme, 10 $\times$ corresponding restriction buffer, and 10 μ l Neo8tub PCR-product. The reactions were incubated in a 37°C water bath for 3 to 16 h, as specified for the particular enzyme by the manufacturer.

The same procedure was followed for the *Hinf*I enzyme (Fermentas) for *N. perennans*, *N. malicorticis*, and *N. kienholzii*. Restricted fragments were separated and visualized alongside a 100-bp DNA ladder (GeneRuler, Thermo Fisher Scientific) on a 3% agarose gel stained with ethidium bromide.

Additionally, two enzymes, *Bsu*RI and *Hinf*I, were evaluated as a double digest reaction. Each 25- μ l reaction contained 10 U of each enzyme, 10 $\times$ corresponding reaction buffer, and 10 μ l of PCR-product. The reaction was incubated in a 37°C water bath for 16 h. Restricted fragments were separated alongside a 20-bp ladder (Lonza, Rockland, ME, U.S.A.) on a 3% agarose gel stained with ethidium bromide and visualized under ultraviolet light.

Results

Postharvest disease incidence and distribution. Characteristic *P. vagabunda* fruit decay symptoms were observed in all regions sampled for each season (Fig. 1). As there was varying availability of fruit each season, comparisons between disease incidence and distribution could not be made between orchard, region, or season. The occurrence of *P. vagabunda* per producer orchard ranged from 0 to 73% in 2010, 0 to 6% in 2011, and 0 to 30% in 2012, with the Witzenberg Valley having the highest incidence of *P. vagabunda* decay per 100 fruit in 2010 and 2012 (Supplementary data). The average disease incidence was 0–36.8% in 2010, 0–1.2% in 2011, and 0–6.8% in the 2012 season (Table 2). Mean disease incidences for

Table 1. Partial sequences of the β -tubulin gene region including *Phlyctema vagabunda* and *Neofabraea* species, GenBank accession number, collection reference number, and location used in this research (adapted from de Jong et al. 2001)

Species	Accession number	Reference number ^a	Location
<i>Phlyctema vagabunda</i>	AY 064702	CBS 109875	USA
<i>P. vagabunda</i>	AF 281457	CBS 452.45	UK
<i>P. vagabunda</i>	AF 281458	CBS 628.72	Yugoslavia
<i>P. vagabunda</i>	KR 859310	CBS 304.62	South Africa
<i>P. vagabunda</i>	MH 877785	CBS 452.64	UK
<i>Neofabraea malicorticis</i>	AF 281468.1	CAL 198	Canada
<i>N. kienholzii</i>	KR 866107	CBS 355.72	Portugal
<i>N. perennans</i>	AF 2814747	CBS 453.64	UK

^a CAL, Collection of C. André Lévesque, Agriculture and Agri-Food Canada, Ottawa; CBS, Centraalbureau voor Schimmelcultures.

Fig. 1. *Phlyctema vagabunda* on 'Cripps Pink' apples showing **A**, characteristic bull's eye lesion with alternating light and dark brown circles and light center; and **B**, sporulating lesions with acervuli.

the Witzenberg Valley were 74, 4, and 40%, while Grabouw had 1, 1, and 2% for 2011, 2012, and 2013, respectively.

A total of 996 monosporic cultures, collected from fruit obtained from pack houses, were morphologically identified as *P. vagabunda* according to descriptions by Verkley (1999) and Soto-Alvear et al. (2013). Representative isolates were identified as *P. vagabunda* with the PCR-RFLP identification method developed.

Pathogenicity. The five isolates produced characteristic lesions that resembled the characteristic *Phlyctema* and *Neofabraea* species on the three apple cultivars inoculated. The ‘Cripps Pink’ and ‘Fuji’ cultivars were more susceptible to *P. vagabunda* compared with the ‘Golden Delicious’ cultivar for the Witzenberg Valley, Elgin, and Vyeboom regions (Fig. 2). Noninoculated control fruit were asymptomatic. Fungi isolated from the leading edge of the lesion were morphologically similar to the original *P. vagabunda* isolated from infected ‘Cripps pink’ apples. The identity was confirmed as *Phlyctema vagabunda* by PCR-RFLP identification, thereby completing Koch’s postulates.

PCR-RFLP method development. The Neo8tub primer set yielded the expected 411-bp fragment for *P. vagabunda*. Coincidentally, the primer set amplified *N. malicorticis*, *N. perennans*, and *N. kienholzii* and was genus-specific rather than species-specific.

Table 2. Average postharvest incidence of *Phlyctema vagabunda* decay from ‘Cripps Pink’ apples collected from five growing regions in the Western Cape over three consecutive seasons

Region	Disease incidence (%)		
	2010	2011	2012
Elgin	0.8	1.2	2.0
Grabouw	3.2	0.3	3.9
Hemel-en-Aarde Valley	1.0	1.2	2.8
Ceres	1.5	0.6	4.7
Witzenberg Valley	36.5	0.5	6.8

However, *P. vagabunda* could be distinguished from the other *Neofabraea* species with the *Bbs*I, *Ssp*I, *Ksp*AI, and *Bsu*RI enzymes. The *Bsu*RI enzyme along with *Hinf*I, in a double-digest reaction, yielded distinct fragment profiles for the individual bull’s-eye rot species.

PCR-RFLP detection. *P. vagabunda* was detected on asymptomatic ‘Cripps Pink’ apples in all three seasons in the Witzenberg Valley and Grabouw orchards, and the pathogen was detected on the ‘Hillieri’ crab apple variety used for ‘Cripps Pink’ pollination in the Witzenberg Valley orchard (Table 3). However, *P. vagabunda* was not detected repeatedly in a season and varied from month to month with regards to the season as well as the region. For both regions across all seasons, *P. vagabunda* was not detected at the start of the season in November. The detection rate increased in December, January, and February and was the highest in March and April (Table 3). *P. vagabunda* was detected most often in the Witzenberg Valley and toward the end of the season. In Grabouw, *P. vagabunda* was only detected in February and April in 2012 and April in 2013. *P. vagabunda* spores were detected on the ‘Hillieri’ crab apples in December 2012, and in January 2011 and 2012 (Table 3).

Climatic factors. No clear relationship between the detection of *P. vagabunda* in the orchard and climatic factors could be determined (data not shown). There was no difference in the average rainfall volume (100 mm) and precipitation duration (between 20 mins and 4 h) throughout the season for each orchard. No significant differences were observed in the daily temperatures. It was observed that the Witzenberg Valley orchard experiences colder winters (2 to 4°C) compared with the Grabouw orchard (6 to 8°C). The average relative humidity was similar during the winter and spring for both orchards, but the humidity in the Grabouw orchard is 2 to 4% higher during summer and autumn compared with the humidity of the Witzenberg Valley orchard.

Discussion

Postharvest evaluation of ‘Cripps Pink’ apple fruit indicated that *P. vagabunda* was present in the five different pome fruit growing

Fig. 2. Pathogenicity of *Phlyctema vagabunda* on ‘Fuji’, ‘Golden Delicious’, and ‘Cripps Pink’ cultivars from the Witzenberg Valley, Elgin, and Vyeboom apple production region in the Western Cape. Means represented by bars designated with the same letter are not significantly different ($P < 0.05$).

regions in Western Cape over the three consecutive seasons. However, the average incidence of postharvest *P. vagabunda* between the seasons and the regions sampled was variable. Similar variable incidence of the *Neofabraea* pathogens was reported on apples in the United Kingdom, with the average decay caused by *Neofabraea* on susceptible apple cultivars ranging between 80 and 100% in 2010 and 2011, while dropping to 56% in 2013 and 16% in 2014 (Saville 2014).

Overall, the Witzenberg Valley orchards had higher levels of *P. vagabunda* fruit decay per fruit lot than fruit evaluated from other regions with the exception of the 2011 season, and this could possibly be ascribed to the period in which the fruit were harvested. For all three seasons, the 'Cripps Pink' apples from the Witzenberg Valley orchards were harvested 1 to 2 weeks later than the prescribed harvest period. Fruit harvested 1 to 2 weeks later than the optimum maturity date had higher incidences of bull's-eye rot compared with fruit harvested at the optimal time or earlier (Børve et al. 2013; Neri et al. 2005). Prolonged exposure to rainfall and inoculum favors the sporulation, dispersal, and infection of *P. vagabunda* (Edney et al. 1977; Soto-Alvear et al. 2013). In addition, as apples mature they are more susceptible to pathogen infections of the lenticels (Curry 2003). Aguilar et al. (2017) determined that although *N. perennans* and *N. kienholzii* were able to infect 'Red Delicious' and 'Fuji' apple cultivars throughout the growing season in the U.S. Pacific Northwest, both cultivars were most susceptible to infection during the final weeks prior to fruit harvest. Although there are several environmental and cultural factors that affect disease establishment and development, harvesting the fruit as early as possible may prevent further postharvest losses (Saville 2014).

Disease symptoms were observed in wounded fruit from the three apple cultivars tested 30 days after inoculation. No symptoms were observed on the wounded, control fruit. There was a difference in susceptibility between the apple cultivars, with the 'Cripps Pink' cultivar being the most susceptible, followed by 'Fuji', while the 'Golden Delicious' cultivar was the most resistant. The same susceptibility trend was observed in the three regions.

P. vagabunda was successfully detected on asymptomatic 'Cripps Pink' apples harvested throughout the growing seasons with the PCR-RFLP method in the Witzenberg Valley and Grabouw orchards. However, detection was variable within and between the two orchards and the growing season. Ceustermans et al. (2015) observed that *P. vagabunda* infection occurred on 'Pinova' apples as early as 12 weeks prior to the first harvest period and that infection levels increased as the harvest period approached; they observed that *P. vagabunda* infestations differed between fruit on individual trees.

P. vagabunda spores were detected on the 'Hillieri' crab apple fruit used as a pollinator in the Witzenberg Valley orchard. The presence of *P. vagabunda* detected in washes of the 'Hillieri' crab apple surfaces earlier or during the same month as conidia obtained from the 'Cripps Pink' apples in the Witzenberg Valley orchard could contribute to the increased disease incidence and severity. The crab apple could be considered as an alternative host and an important

contributing source of *P. vagabunda* inoculum. This is the first record of the detection of *P. vagabunda* on crab apples in South Africa. There are no reports of other crab apple varieties susceptible to *P. vagabunda* or acting as an alternative host and an inoculum source in South Africa. However, Kienholz (1939) observed in canker inoculation studies that the 'Oregon' and 'Siberian' crab apple varieties were susceptible to *N. perennans* and *N. malicorticis*. Whether those species occurred naturally on the crab apple varieties or their role in the disease cycle of bull's-eye rot was not investigated.

It is possible that the colder winter weather of the Witzenberg Valley is beneficial to *P. vagabunda* survival and the availability of inoculum during the subsequent growing season. Spotts (1985b) observed that *N. malicorticis* inoculated on pear fruit and stored at -1.1°C survived for 7.3 months. Henriquez et al. (2008) showed that the effect of temperature on the development of *N. perennans* in inoculated pear fruit prior to harvest was variable with the greatest disease incidence recorded at 10°C in one year but the greatest at 30°C in the second year. An in-depth investigation into the influence of climatic factors on the survival, proliferation, and infection of *P. vagabunda* in orchards is required before definite conclusions can be postulated.

Approaching harvest, increased susceptibility of apple fruit and greater *P. vagabunda* spore dispersal could play an important role in the incidence of bull's-eye rot in the orchard (Bompeix 1978; Grove et al. 1992; Spotts 1985a). Late-season apple varieties including 'Cripps Pink' and 'Golden Delicious' give rise to higher disease incidence (Soto-Alvear et al. 2013), with increased *P. vagabunda* infection observed approaching harvest (Ceustermans et al. 2015; Neri et al. 2009). Aguilar et al. (2017) confirmed that although 'Red Delicious' and 'Fuji' apple varieties were susceptible to infection by *P. perennans* and *N. kienholzii* throughout the growing season, these species were most susceptible during the final weeks prior to fruit harvest.

In South Africa, 'Cripps Pink' apples are predominantly grown in the Western Cape province which is a winter rainfall region. Trees flower and fruit-set occur in spring from late September to early November. Mature fruit is harvested in autumn from mid- to late April. In this study, the increase in detection frequency of *P. vagabunda* during autumn coincided with an increase in rainfall recorded at the two orchards. Harvest usually coincides with an increase in precipitation favoring *P. vagabunda* infection (Edney et al. 1977; Tan and Burchill 1972). Rainfall plays a key role in the sporulation, distribution, and infection of *P. vagabunda* with precipitation periods longer than 30 min ensuring infection (Edney 1974; Edney et al. 1977; Henriquez et al. 2008). Kienholz (1956) and Soto-Alvear et al. (2013) observed significantly higher incidences of *Phlyctema* species on apple in seasons or areas receiving higher rainfall. Moreover, Henriquez et al. (2008) observed higher incidences of *P. vagabunda* on fruit exposed to increased rainfall events in autumn.

The species-specific multiplex-PCR developed by Gariépy et al. (2003) could not be used to identify bull's-eye rot species from DNA obtained from the fruit washes in this study. Substitution of

Table 3. Detection of *Phlyctema vagabunda* present on 'Cripps Pink' apple fruit collected over three seasons from one-month post full bloom until harvest from two commercial orchards located in the Witzenberg Valley and Grabouw, respectively, and from 'Hillieri' crab apples from the Witzenberg Valley orchard

Region	Season	Growing season ^a					
		November	December	January	February	March	April
Witzenberg Valley	2011	... ^b	4 ^c	H ^d	...	1	5
	2012	...	H	4 + H	2	1	5
	2013	...	...	...	...	...	2
Grabouw	2011	...	...	...	1	...	...
	2012	...	...	...	...	...	5
	2013	...	...	...	...	...	4

^a The season starts one-month post full bloom in spring (November), continues in summer (December, January, February), and ends in autumn (March, April) when fruit are harvested mid to end of April.

^b ... = Pathogen not detected.

^c The detection frequency of *P. vagabunda* from five washed fruit sets comprising 100 'Cripps Pink' apples per set.

^d 'Hillieri' crab apples used as pollinators for 'Cripps Pink' apples in the Witzenberg Valley.

the forward primer (Neovagabunda-up) with the one developed by Soto-Alvear et al. (2013) allowed for amplification of samples, but nonspecific PCR-products were observed. Inspection of the primer showed the first nucleotide (5'-TGATGAGACCTTCTGTAT-3') not to be homologous to the majority of the *P. vagabunda* sequences (5'-CGATGAGACCTTCTGTAT-3') by de Jong et al. (2001) in GenBank. Single nucleotide polymorphisms (SNPs) can influence target-specificity of primers, even at the 5'-hydroxyl end (Ye et al. 2012). This could explain why nonspecific bands were obtained, and the inability to identify *Phlyctema* and *Neofabraea* isolates in this study. The PCR-RFLP was regarded as adequate for the screening of DNA from fruit surfaces.

In conclusion, *P. vagabunda* is the only confirmed pathogen causing fruit decay of 'Cripps Pink' apple in the Western Cape, and management strategies focus on controlling *P. vagabunda* only. The PCR-RFLP method developed in our study successfully differentiated between the causal agents of bull's-eye rot decay and should be implemented in the rapid detection of *Phlyctema* and *Neofabraea* species on pome fruit in South Africa. Insight into the period when *P. vagabunda* spores are present on the surface of 'Cripps Pink' apples and 'Hillieri' crab apples during the growing season was determined. Further evaluation is needed to discern the extent of the bull's-eye rot species on apple and pear varieties in South Africa toward improved pre- and postharvest disease management.

Acknowledgments

We thank A. Pretorius, E. Davids, and M. Leibrandt for technical and field assistance and the apple producers and pack houses from the Witzenberg Valley and Grabouw for fruit provision.

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