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Infection of apple fruit by *Sphaeropsis pyriputrescens* in the orchard in relation to Sphaeropsis rot in storage

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Abstract Sphaeropsis rot, caused by *Sphaeropsis pyriputrescens*, is an important postharvest disease of apple in the United States. The objectives of this study were to determine the timing of apple fruit infection in the orchard in relation to development of Sphaeropsis rot in storage and to identify infection courts and mode of penetration by *S. pyriputrescens* on apple fruit. Fruit of apple cvs Red Delicious, Golden Delicious, and Fuji were inoculated in the orchard from 3 weeks after petal fall to 2 weeks before harvest at 5 to 6-week intervals in three consecutive seasons. All fruit were harvested and stored at 0ºC to monitor decay development. Light and scanning electron microscopy were used to examine the infection courts and mode of penetration of the fungus on/in the host tissues. At harvest, the fungus was re-isolated from the stem (pedicel), sepal, anther, or filament of the inoculated fruit, but decay did not develop on fruit. Sphaeropsis rot developed on inoculated fruit during cold storage beginning 1–3 months after harvest. Stem-end rot was prevalent on cv. Golden Delicious, whereas calyx-end rot was prevalent on cv. Fuji. Both stem- and calyx-end rots were common on cv. Red Delicious. Infection also occurred at lenticels on fruit skin, particularly on cv. Golden Delicious, but at low incidence. Relationships between the incidence of Sphaeropsis rot in stored apple fruit and the timing of inoculation in the orchard varied with cultivar and year. On cv. Red Delicious apples, the incidence of Sphaeropsis rot generally increased as the timing of infection approached harvest. Histological studies indicated that infection took place through natural openings of plant organs such as stomata on stems and sepals and lenticels on fruit skin. Fungal penetration also was observed at micro-cracks on the stem and sepal and at trichome sockets where mechanical damage occurred in sepals. Direct penetration was observed on the stem and sepal of fruit, but most invasions were restricted between the cuticle and the epidermis. Our results indicate that wounding is not required for infection of apple fruit by *S. pyriputrescens*, though it may facilitate infections.

Keywords Apple · Latent infection · Postharvest disease · *Sphaeropsis pyriputrescens* · Sphaeropsis rot

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Introduction

Sphaeropsis rot, caused by *Sphaeropsis pyriputrescens* C.L. Xiao & J.D. Rogers, is a recently recognized postharvest fruit rot disease of apple and pear in the United States (Xiao and Rogers 2004; Xiao et al. 2004). A

survey conducted in Washington State from 2004 to 2006 showed that Sphaeropsis rot was observed on stored apple fruit originating from 73 % of the orchards sampled, accounting for 17 % of the total decayed fruit (Kim and Xiao 2008). Sphaeropsis rot has the potential to cause significant economic losses. In one instance, 24 % of cv. Red Delicious apple fruit were decayed by *S. pyriputrescens* after 7 months of storage (Xiao and Rogers 2004). Sphaeropsis rot has since also been reported in the state of New York (Kim et al. 2013) and British Columbia, Canada (Sholberg et al. 2009).

S. pyriputrescens also causes a twig dieback and canker disease of apple trees and crabapple trees that are commonly used as pollinizers in commercial apple orchards in the region (Xiao and Boal 2005). Pycnidia of *S. pyriputrescens* commonly form on these cankers and killed twigs in affected orchards, but the teleomorph state of the fungus has not been observed (Xiao and Boal 2005). Viable pycnidia of the fungus are available throughout the fruit-growing season in apple orchards (C. L. Xiao, *unpublished data*). Because *S. pyriputrescens* inoculum is present in orchards but no inoculum sources have been identified in postharvest environments, it is believed that infection of apple fruit by the fungus occurs in the orchards (Kim and Xiao 2008; Xiao and Boal 2005).

Since inoculum availability of *S. pyriputrescens* appears not to be a limiting factor for infection of apple fruit in the orchard, environmental conditions and/or fruit susceptibility are presumably important factors determining the timing of fruit infections. Although the effects of environmental factors on in vitro mycelial growth and conidial germination of the fungus have been studied (Kim and Xiao 2010; Kim et al. 2005), when and how *S. pyriputrescens* infects apple fruit in the orchard has not been determined. In addition to environmental conditions, fruit maturity, cultivar susceptibility, and susceptibility windows for specific host tissues during the growing season may also affect the incidence of fruit rot diseases of apple caused by fungi (Hwang 1983; Kim et al. 2001; Sitterly and Shay 1960). It is generally accepted that immature fruit are less susceptible to fruit infection by fungal pathogens. A previous study showed that in addition to stem-end rot and calyx-end rot, Sphaeropsis rot can also originate from infection of fruit skin, especially in apple cv. Golden Delicious (Kim and Xiao 2008). The presence of necrotic or dead tissues on floral parts of fruit is involved in the initiation of other fruit rot diseases. *Botrytis cinerea* that causes calyx-end fruit rot of strawberries mainly infects necrotic tissues of sepals (Powelson 1960), and calyx-end Phacidiopycnis rot of pears has also been associated with infections via necrotic sepals (Liu and Xiao 2009). The presence of necrotic tissue at the tip of sepals is common on apple fruit; however, its association with infection of apple fruit by *S. pyriputrescens* rot is unknown.

The objectives of this study were to determine the timing of apple fruit infection in the orchard in relation to development of Sphaeropsis rot during storage and infection courts and mode of penetration by *S. pyriputrescens* on apple fruit.

Materials and methods

Inoculum preparation

A single-spore isolate of *S. pyriputrescens*, CLX2380, recovered from a decayed cv. Red Delicious apple fruit collected from a commercial packinghouse in Manson, WA was used in this study. For inoculum preparation, the isolate was reactivated from a culture stored in 15 % glycerol at −80 °C and grown on potato dextrose agar (PDA; Difco Laboratories, Detroit, MI) for 3 weeks under a 12-h light/12-h dark cycle (10 Wm^{-2}) at 20 °C. A previous study indicated that oatmeal agar was more suitable than PDA for production of *S. pyriputrescens* pycnidia (Kim et al. 2005). To produce a large quantity of inoculum, oozing conidia from pycnidia on PDA were streaked on oatmeal agar. Inoculated plates were sealed with Parafilm and incubated at 20 °C under a 12-h light/12-h dark cycle for 3 weeks. Each plate was flooded with 20 ml sterile water and oozing conidia from pycnidia were scraped off the surface of the medium. The resulting conidial suspensions were filtered through two layers of cheesecloth. Haemacytometer counts were used to adjust the final concentration to 1×10^5 conidia/ml in 2004 and 5×10^5 conidia/ml in 2005 and 2006. Tween 20 was added into the conidial suspension at a final concentration of 0.001 %.

Time of infection on fruit in the orchard in relation to Sphaeropsis rot in storage

Experiments were conducted in research orchards of cv. Red Delicious trees planted in 1996 and cv. Golden

Delicious trees planted in 1985 located at the Washington State University Tree Fruit Research and Extension Center, Washington State University (WSU-TFREC), Wenatchee, WA and in a research orchard of cv. Fuji planted in 1991 near Orondo, WA. At least 60 fruit were randomly selected as a replicate from two trees for cvs. Red Delicious and Golden Delicious and one tree for cv. Fuji, and there were three replicates for each cultivar on each inoculation date. All trees were under-tree irrigated, and insects and weeds were controlled following the recommendations for apple production in the region (Smith et al. 2004). In 2004 and 2005, one application of thiophanate-methyl (Topsin M 70WP at 1.1 kg/ha with a spray volume of 1,871 l/ha) and two applications of trifloxystrobin (Flint 50 W at 175 g/ha) were made on cv. Red Delicious trees at least 3 weeks before the first fruit inoculation, and no fungicide was used on cv. Golden Delicious and cv. Fuji trees. In 2006, trifloxystrobin (Flint 50 W) was applied twice (one in late April and another in early June) to all three cultivars for control of powdery mildew. Trifloxystrobin was used because it is less inhibitory to *S. pyriputrescens* than the other fungicides commonly used in the region for control of powdery mildew in apple (Y. K. Kim and C. L. Xiao, *unpublished data*).

Starting 3 weeks after petal fall and continuing until 2 weeks before harvest, fruit of cvs. Red Delicious, Golden Delicious, and Fuji, were periodically inoculated with conidial suspensions of *S. pyriputrescens* at 5 to 6-week intervals during 2004 to 2006. In total, four inoculations per year were carried out for Red Delicious and Golden Delicious and five inoculations were done for Fuji. Fruit were inoculated with conidial suspensions by spraying them to run-off using a hand sprayer. Control fruit were sprayed with sterile water. The fruit were then covered with moistened plastic bags to maintain high humidity. All inoculations were made near sunset to avoid high daytime temperatures that could have affected viability of the conidia. Furthermore, the plastic bags where covered with white paper bags to prevent heating from early morning sunlight. After approximately 15 h of incubation, the bags were removed the following morning. During the incubation period, temperature and relative humidity were measured with a small data logger [Watchdog® 450, Spectrum Technologies, Inc., Plainfield, IL, USA]) placed inside a plastic bag in each cultivar block. The experimental design was a randomized complete block with three replications per treatment on each inoculation date.

To determine whether the fungus had infected the fruit, 10 inoculated fruit per replicate were randomly collected at 2 weeks after inoculation and at harvest. The fruit were surface-disinfested in 0.6 % NaOCl for 5 min, rinsed twice with sterile deionized water, and then dried in a laminar-flow hood. Pedicels (hereafter referred to as stems) were excised from each fruit and placed on acidified PDA (APDA; 4 ml of a 25 % solution of lactic acid per litre of PDA). Sepals, anthers, and filaments from the flower parts in the calyx ends of fruit were excised separately for re-isolation of the fungus. Necrotic and non-necrotic tissues of each sepal were separated with a sterile scalpel and placed on APDA. All plates were incubated at 20 °C in the dark, and the presence or absence of *S. pyriputrescens* was initially recorded after 3–4 days and confirmed after 10–14 days of incubation based on the morphology of the fungus (Xiao and Rogers 2004).

All fruit not used for re-isolation were harvested at commercial maturity and were transferred onto sterilized fiberboard apple-trays wrapped in perforated polyethylene bags, and stored in cardboard apple boxes at 0 °C in regular atmosphere. Decay development on the fruit, infection sites (stem, calyx, or skin) on the fruit and percentage of fruit with the symptoms of Sphaeropsis rot were recorded monthly after harvest for up to 9 months. Isolations were made from all decayed fruit to confirm the causal agent. Stem and calyx tissues from fruit that remained symptomless after 9 months of storage were also plated to determine whether these tissues were infected. In 2005 and 2006, isolations were also made from the symptomless stems or calyx tissues on the decayed fruit that were removed during the monthly evaluations.

In addition to the fruit inoculation, flowers at full bloom were also inoculated in 2007 as described above. The fruit were then harvested and stored at 0 °C. The development of calyx-end Sphaeropsis rot on the fruit was recorded after 9 months of storage.

Mode of entry and penetration

Fruit of the three cultivars were inoculated in June, August and September with conidial suspensions (5×10^5 conidia/ml) of *S. pyriputrescens* in the laboratory and orchards. An additional inoculation was conducted for cv. Fuji in October. For the laboratory inoculation, fruit were picked on each inoculation date from the research orchards where the field inoculations were

conducted. Stem- and calyx-ends of fruit were surface-disinfested with 70 % ethanol for 1 min, rinsed twice with sterile deionized water and air-dried. Stems and sepals of the fruit were inoculated with the conidial suspension using a hand sprayer until run-off. Fruit were placed on aluminum muffin trays in a plastic crisper containing approximately 200 ml of deionized water in the bottom to maintain high humidity. Fruit were incubated at 20 °C for 12 h, 1, 2, 3, 7, and 14 days.

Five fruit inoculated in the orchard as described above were removed from trees and examined with a light microscope 1, 3, 5, and 7 days after inoculation, and thereafter every 2 weeks until harvest. For specimen preparation, stems and sepals were excised from the inoculated fruit with a sterile scalpel. The samples were fixed in formalin-acetic acid-alcohol (FAA) for at least 12 h before being dehydrated in an ethanol series. The samples were then embedded in paraffin wax and sectioned at 10 μm in thickness with a rotary microtome (Spencer lens Co., Buffalo, NY, USA). The sections were attached to a Poly-L-Lysine coated slide (LabScientific, Inc., Livingston, NJ, USA) and dried on a slide warmer. The sections were then stained with lactophenol-cotton blue solution (Sigma Chemical Co., St. Louis, MO, USA) for 3 min. Excess stain was gently washed from the slides with deionized water and air-dried. The slides were observed under a light compound microscope, and digital images were taken with a Zeiss Axiocam.

For scanning electron microscopy (SEM) observations of conidial germination and penetration, stems and sepals were excised from the inoculated fruit and frozen in liquid nitrogen for 10 min. The frozen samples were then freeze-dried in a lyophilizer for 18 h after which they were stored in a desiccator. Dried samples were removed from desiccator storage and, under a stereomicroscope, affixed to a 10 mm aluminum stub using double-sided carbon tape by pressing only on opposite edges of the tissue with a stainless steel microprobe. Mounted tissue was coated with gold/palladium using a Desk II cold sputter coater (Denton Vacuum Inc., Morristown, NJ, USA) fitted with a tilting omni-rotating head. Coated samples were returned to the desiccator and held under low vacuum at 1.3×10^{-4} Pa and 10 °C until examined microscopically using a Tescan Vega II LS SEM (Tescan, s.r.o., Brno, Czech Republic). Images were obtained at 10 kV and 7.5×10^{-3} Pa.

Data analysis

Analysis of variance was performed with SAS PROC GLM (version 9.2, SAS Institute, Cary, NC, USA) to compare the incidence of infection on different floral parts (sepal, anther, and filament), decay development on different infection sites, and necrotic versus non-necrotic tissues of sepals. The test indicated that there were no interactions between timing of inoculation and tissue types in all three comparisons ($P>0.05$) and thus, all further analyses were conducted on pooled data. However, there were significant interactions between cultivars and tissue types ($P<0.05$). Mean separations were conducted within a cultivar by the Waller-Duncan K-ratio t test ($P=0.05$), while the means of necrotic and non-necrotic tissue were separated by Fisher's protected least significant difference ($P=0.05$). Linear regression analysis was conducted using SAS PROC REG to determine relationships between the incidence of Sphaeropsis rot in storage and time of fruit inoculation in the orchard (number of days before harvest). All percentage data were arcsine square root-transformed prior to statistical analysis.

Results

Timing of apple fruit infection in relation to Sphaeropsis rot in storage

Relative humidity inside the plastic bags remained near saturation within 2–3 h of inoculation and ranged from 90 to 100 % during the entire incubation periods after inoculation on all inoculation dates (data not shown). In all 3 years, average temperatures during the incubation periods ranged from 15 to 25 °C from May to July (data not shown). The average temperatures of September and October inoculations were below 15 °C. Maximum temperatures exceeding 30 °C during the incubation periods in June, July or August inoculations were encountered infrequently. The highest temperature was 37.5 °C in the cv. Fuji orchard in June 2006, but it lasted for less than 1 h when fruit were exposed to direct sunlight in the morning. Temperatures below 10 °C were often recorded during June, September and October inoculations.

None of the fruit inoculated in the orchard showed symptoms of Sphaeropsis rot at harvest. The symptoms began to appear after 2–3 months of storage at 0 °C on

cvs Red Delicious and Golden Delicious fruit and 1–2 months after harvest on cv. Fuji. Sphaeropsis rot did not develop on control fruit sprayed with sterile water. In all 3 years, Sphaeropsis rot developed on some inoculated fruit from all of the inoculation dates during the fruit-growing season from 3 weeks after petal fall to 2 weeks before harvest (Fig. 1). Incidence of Sphaeropsis rot generally increased as the timing of inoculation approached harvest on cv. Red Delicious in 2005 and 2006 but not in 2004 (y=66.3+0.32x, R^2=0.62, P=0.21 in 2004; y=72.5+0.26x, R^2=0.94, P=0.03 in 2005; and y=94.1+0.43x, R^2=0.88, P=0.06 in 2006) and on cv. Golden Delicious in 2005 but not in 2004 and 2006 (y=45.5+0.25x, R^2=0.72, P=0.15 in 2004; y=90.2+0.21x, R^2=0.99, P=0.01 in 2005; and y=27.1+0.11x, R^2=0.62, P=0.22 in 2006). The highest incidence of Sphaeropsis rot occurred on the fruit inoculated at 2 weeks before harvest. The regression analysis indicated that the incidences of Sphaeropsis rot on cv. Fuji fruit were similar regardless of the inoculation time during the fruit-growing season (y=14.3–0.02x, R^2=0.02, P=0.81 in 2004; y=24.8+0.05x, R^2=0.11, P=0.58 in 2005; and y=24.8+0.004x, R^2=0.004, P=0.92 in 2006). The levels of Sphaeropsis rot incidence on cvs. Red Delicious and Fuji were similar in all 3 years.

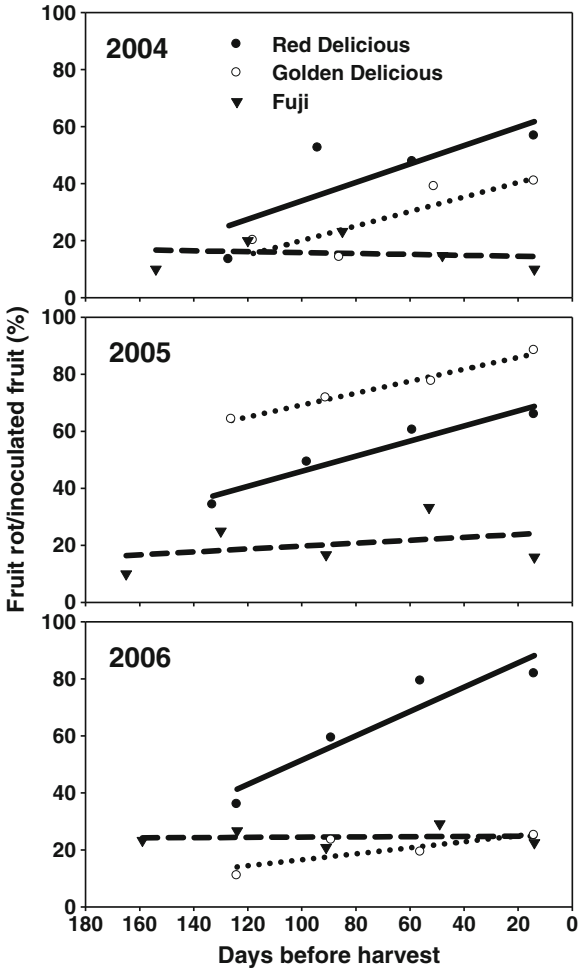

Fig. 1 Percentage of fruit with symptoms of Sphaeropsis rot during storage on three apple cultivars that were inoculated with conidial suspension of *Sphaeropsis pyriputrescens* in the orchard from 3 weeks after petal fall to 2 weeks before harvest at 5 to 6-week intervals during 2004–2006. After harvest, fruit were stored for 9 months at 0 °C in regular atmosphere

In 2005 and 2006, *S. pyriputrescens* was recovered from the symptomless stem and calyx tissues of decayed fruit after monthly evaluations and after 9 months of storage. On average in the 2 years, infection rates on symptomless tissue after monthly evaluations were 40.3 %, 43.7 %, and 25.7 % on cvs. Red Delicious, Golden Delicious and Fuji, respectively. After 9 months of storage, the fungus was re-isolated from 64.9 %, 62.6 %, and 21.6 % of the symptomless fruit on cvs. Red Delicious, Golden Delicious, and Fuji, respectively. The infection rates on specific tissues (stem or calyx) varied with cultivar and evaluation time.

In a separate experiment conducted in 2007, flowers were inoculated with a conidial suspension of *S. pyriputrescens* at full bloom, and fruit were harvested on commercial harvest dates. After 9 months of storage at 0°C, incidences of Sphaeropsis rot were 4.2 %, 17.5 % and 7.2 % on cvs. Red Delicious, Golden Delicious and Fuji, respectively.

Infection courts

Visual observations during the early stage of symptom development on stored apples indicated that stem-end rot originated primarily from the proximal-end of the stem, and necrosis of the stem (browning or blackening) progressed toward the stem-bowl and eventually reached the fruit flesh (Fig. 2a). The early stage of symptom development showed that calyx-end Sphaeropsis rot started mainly from infected sepals (Fig. 2b). Sphaeropsis rot also developed on fruit skin, particularly on cv. Golden Delicious (Fig. 2c).

Although symptoms of Sphaeropsis rot developed primarily at the stem-end and calyx-end of fruit, incidence of stem-end rot and calyx-end rot varied with cultivars. On cv. Red Delicious, both stem-end rot and calyx-end rot were common in 2004 and 2005, while

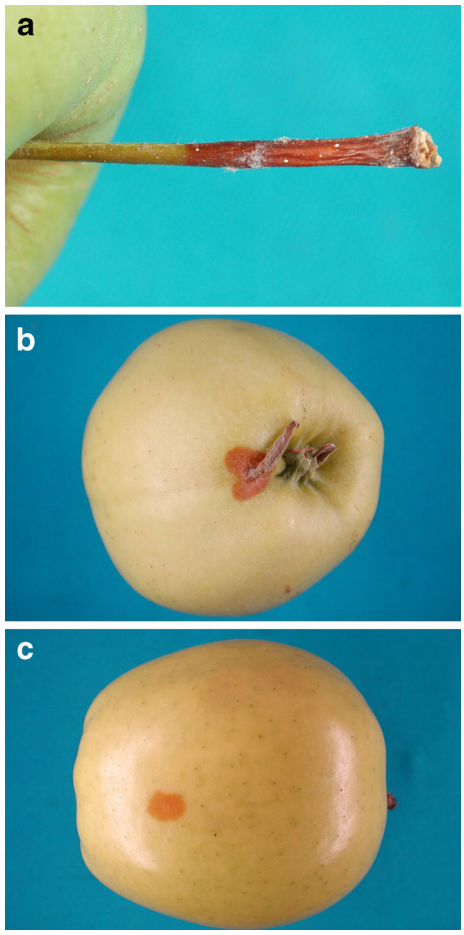

Fig. 2 Early stage of Sphaeropsis rot on different infection sites: **a**, Fruit stem infected by *Sphaeropsis pyriputrescens* showing necrotic tissue progressing toward the stem bowl area of the fruit; **b**, Calyx-end Sphaeropsis rot initiated from an infected sepal. Infections of sepals occur regardless of whether the sepals are disconnected or attached to the calyx. **c**, Sphaeropsis rot on fruit skin initiated from infection through a lenticel

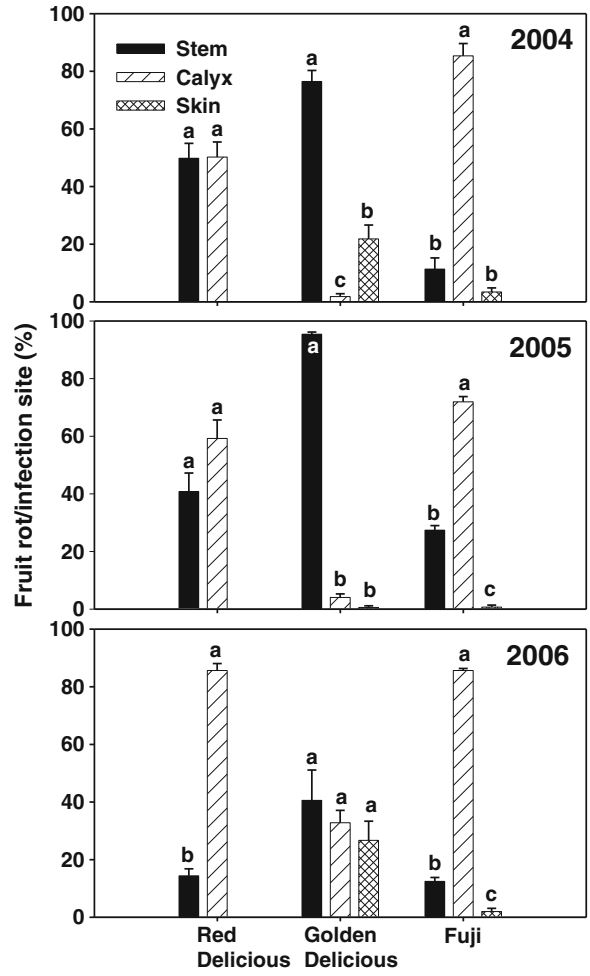

Fig. 3 Percentage of Sphaeropsis rot originating from infections at the stem, calyx, and fruit skin of three apple cultivars (no rot observed from infections of fruit skin on cv. Red Delicious) by *Sphaeropsis pyriputrescens*. Fruit were inoculated periodically with conidial suspensions of *S. pyriputrescens* in the orchard from 3 weeks after petal fall to 2 weeks before harvest during 2004–2006. After harvest, all fruit were stored for 9 months at 0 °C. Values are means of pooled data from the decayed fruit with Sphaeropsis rot symptoms for each cultivar from all inoculations. Bars (standard errors) with the same letters within the same cultivar are not significantly different according to the Waller-Duncan K-ratio *t* test (P=0.05)

calyx-end rot was significantly higher than stem-end rot in 2006 (Fig. 3). Stem-end rot was significantly higher than either calyx-end rot or skin rot on cv. Golden Delicious in 2004 and 2005, but the differences among these infection sites were not significant in 2006. In all 3 years, calyx-end rot was prevalent on cv. Fuji (Fig. 3).

To determine whether *S. pyriputrescens* had established in inoculated fruit, re-isolation of the fungus was attempted from the stem and floral parts including sepals, anthers, and filaments at 2 weeks after inoculation and at harvest in 2005 and 2006. The fungus was recovered from more than 90 % of the inoculated fruit 2 weeks after inoculation (data not shown). At harvest, regardless of the cultivars, the infection rate in sepals was significantly higher than that on anthers or filaments in both years, except for anther on cv. Golden Delicious in 2006 (Table 1).

On sepals, necrotic and non-necrotic tissues of each sepal were cultured separately (Fig. 4). In 2005 and 2006, the incidence of recovery of *S. pyriputrescens* from necrotic tissues was significantly higher than that from non-necrotic tissues on cvs Red Delicious and Fuji

Table 1 Incidence of successful harvest time re-isolation of *Sphaeropsis pyriputrescens* from sepal, anther, and filament tissues of apple fruit that had been inoculated at different stages of development in the orchard in 2005 and 2006

Infection court	Incidence (%) in 2005			Incidence (%) in 2006		
	Red Delicious	Golden Delicious	Fuji	Red Delicious	Golden Delicious	Fuji
Sepal	67.8 a[z]	91.7 a	88.0 a	70.8 a	76.7 a	83.3 a
Anther	23.3 b	46.7 b	40.7 c	48.3 b	61.7 a	64.7 b
Filament	8.9 c	60.0 b	66.7 b	20.0 c	41.7 b	57.3 b

[z] Ten inoculated fruit per replicate for each inoculation time were randomly collected at harvest for re-isolation of the fungus. There were four inoculation times per season for Red Delicious and Golden Delicious and five per season for Fuji. There were a total of 360–450 attempted re-isolations per cultivar per season. Data were pooled for analysis as there were no interactions between timing of inoculation and tissue types. Values within a column followed by the same letter are not significantly different according to the Waller-Duncan K-ratio *t* test (P=0.05)

(P<0.044). On cv. Golden Delicious, the recovery from necrotic tissues was higher than that from non-necrotic tissues in 2005 (P=0.029), but the difference was not significant in 2006 (P=0.228).

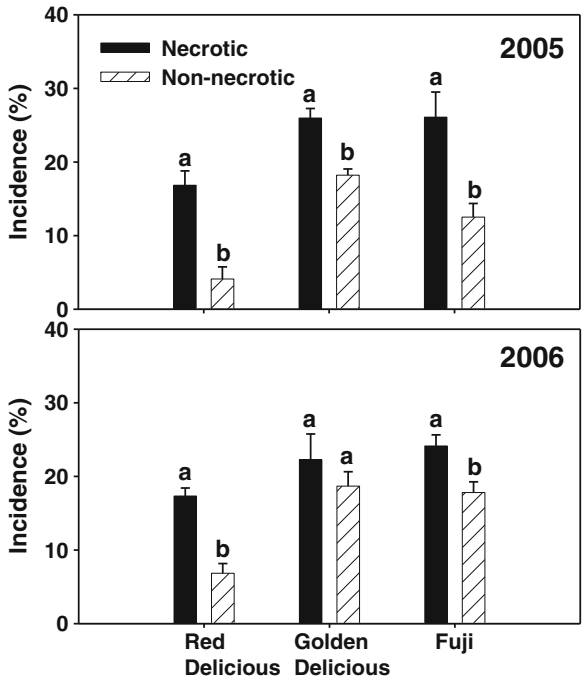

Fig. 4 Incidence of *Sphaeropsis pyriputrescens* re-isolated from necrotic and non-necrotic sepal tissues of apple fruit after inoculation at different stage of fruit development. Re-isolation was conducted 2 weeks after inoculation and at harvest, each time from 10 fruit per replicate (5 sepals per fruit). Fruit were inoculated with conidial suspensions of *Sphaeropsis pyriputrescens* in the orchard from 3 weeks after petal fall to 2 weeks before harvest in 2005 and 2006, a total of 150–180 attempted re-isolations per cultivar per season. Data were pooled for analysis as there were no interactions between timing of inoculation and tissue types. Bars (standard errors) with different letters within the same cultivar are significantly different according to Fisher's protected significant difference (P=0.05)

Mode of entry and penetration

Conidia of *S. pyriputrescens* germinated and germ tubes entered or penetrated stem, sepal, and skin tissues on the inoculated fruit within 24 h of inoculation in both laboratory and orchard conditions (Figs. 5 and 6). Germ tubes from germinated conidia entered through natural openings of plant organs, such as stomata on fruit stems and sepals (Fig. 5a) and lenticels on apple fruit (Fig. 5e). Penetrations were also observed at naturally occurring microcracks on the plant tissues (Fig. 5b, d, and f) and at a trichome socket where mechanical damage occurred (Fig. 5c). Direct penetration and infection were observed by light microscopy (Fig. 6). Infrequently, penetrations were observed in epidermal layers (Fig. 6a), but most penetrations were restricted between the cuticle and the epidermis (Fig. 6b). No deeper penetration by the fungus was observed even after 14 days of incubation at 20 °C in the laboratory and at harvest in the orchard.

Discussion

Infection of apple fruit by *Sphaeropsis pyriputrescens* could occur as early as during bloom or shortly after bloom and continue until harvest, but symptoms of Sphaeropsis rot developed only after at least 1–3 months of cold storage, depending upon cultivar. Sphaeropsis rot initiated mainly from infections at the stem and calyx of fruit. Histological studies showed that *S. pyriputrescens* was able to enter stem, calyx and skin of fruit through natural openings, such as stomata and lenticels, and micr-cracks and was capable of penetrating stem and sepal tissues directly. *S. pyriputrescens*

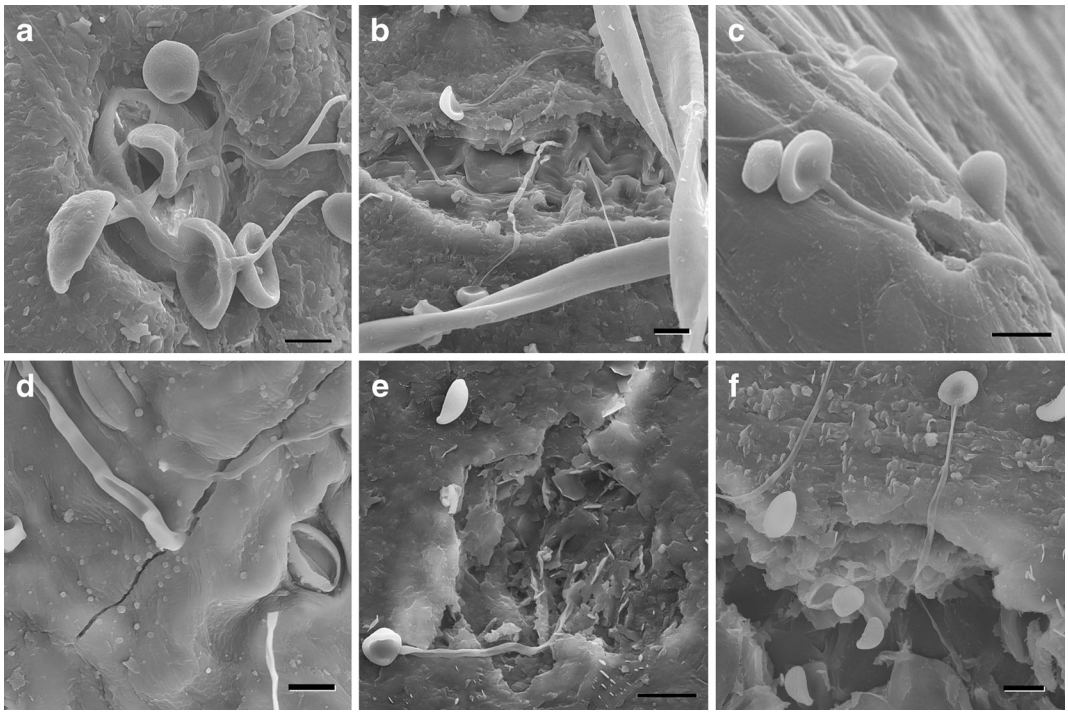

Fig. 5 Scanning electron micrographs of stem (**a**-**c**), sepal (**d**), and skin (**e** and **f**) of apple fruit inoculated with *Sphaeropsis pyriputrescens*: **a**, Germ tubes entering opened stomata; **b**, Penetration of germ tubes through a natural crack; **c**, Penetration of a germ tube through a hole where a trichome was attached; **d**, Hyphae entering a natural crack; **e**, Penetration of a germ tube through a lenticel; **f**, Conidia and germ tubes entering a natural crack. **d** and **f** were observed at 48 h after inoculation, **e** was observed at 72 h after inoculation, and all others were observed at 24 h after inoculation. Bars: A=5 μm, B, D, and F=10 μm, and C and E=6.67 μm

remained latent in apple fruit before harvest and survived over the fruit-growing season. These findings will have important implications in developing and implementing relevant measures for control of Sphaeropsis rot. Control of postharvest fruit rots often focuses on protection of wounds from infections by wound-invading pathogens such as *Penicillium expansum*, through either pre-harvest fungicide sprays or pre-storage fungicide treatments. Our results suggest that in order to control Sphaeropsis rot, fungicide treatments applied near harvest or as a pre-storage treatment need to be able to eradicate established latent infections by *S. pyriputrescens*.

S. pyriputrescens was able to establish infection on apple fruit that were inoculated at various times during the fruit-growing season, suggesting that environmental

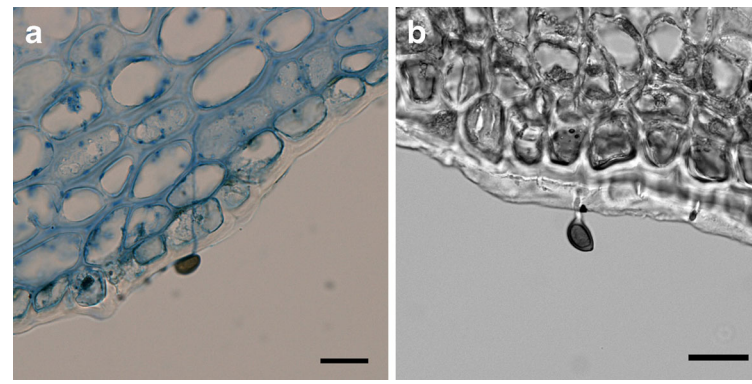

Fig. 6 Light micrographs of the stem and sepal of apple fruit inoculated with *Sphaeropsis pyriputrescens*: **a** and **b**, Longitudinal section of fruit stems showing direct penetration of a germ tube growing from a conidium. Bars=20 μm

conditions such as low temperatures in the early season and high temperatures in the summer, encountered during the course of fruit inoculation experiment in the orchards, did not limit the establishment of infections or the survival of latent infections in apple fruit. Temperatures during the 15-h incubation periods were generally within the range favourable for conidial germination and subsequent mycelial growth of the fungus (Kim and Xiao 2010; Kim et al. 2005). Although the temperatures in May, September and October inoculations were often below 15 °C, at which conidia of the fungus need more time to germinate, Sphaeropsis rot was observed on the fruit inoculated during these months in all 3 years of the study. High temperatures after inoculation in summer might be another concern related to the fruit infection. Our previous study found that *S. pyriputrescens* is not able to germinate or grow at temperatures higher than 30 °C but can survive at this temperature in vitro (Kim and Xiao 2010; Kim et al. 2005). The present study, however, indicated that high temperatures in June and July inoculations did not prevent infections of apple fruit by *S. pyriputrescens*. A possible explanation is that the duration of time the conidia were exposed to such high temperatures was short enough that it did not significantly affect the viability of conidia. However, because our experiments were not designed to determine the effect of temperature on the germination and survival of *S. pyriputrescens* in vivo, a further study could be to determine the survival of *S. pyriputrescens* on the fruit surface. Nonetheless, our results from the 3-year field inoculation studies clearly indicated that infections of apple fruit by *S. pyriputrescens* can occur during summer months under the field conditions in the region.

Relationships between the incidence of Sphaeropsis rot in stored apple fruit and the timing of infection (inoculation) in the orchard varied with apple cultivar and year. On fruit of cv. Red Delicious, the incidence of Sphaeropsis rot generally increased as the timing of infection approached harvest, and the highest incidence was observed on fruit inoculated at 2 weeks before harvest in all 3 years. It is possible that susceptibility of fruit to infection by the fungus increased as fruit development and maturity progressed toward harvest. Increased susceptibility as the growing season progresses has also been reported in pear fruit of cv. d'Anjou to infection by *Botrytis cinerea* (Lennox and Spotts 2004) and by *Potebniamyces pyri* (Liu and Xiao 2009). However, on cv. Golden Delicious, such a relationship was observed only in 2005. On cv. Fuji fruit there was no correlation between incidence of Sphaeropsis rot in stored fruit and timing of fruit inoculations in the orchard. The discrepancies in results among the 3-year experiments within the same cultivar may suggest that environmental factors may influence the development and survival of latent infections in the fruit. Further research could examine quantitative relationships between infection and survival in the orchard and environmental factors such as temperature and relative humidity. In addition, the vast majority of Sphaeropsis rot on cv. Fuji originated from infections of floral parts leading to calyx-end rot, whereas in general stem-end rot was common on fruit of both cvs Red Delicious and Golden Delicious. It remains to be determined whether the susceptibility of the stem of apple fruit to infection by *S. pyriputrescens* is responsible for the differences in decay incidence between Fuji and the other two cultivars.

S. pyriputrescens was re-isolated from floral parts of the fruit, but the infection was more prevalent on sepals than on either anthers or filaments. Similar results have been reported in different fruit-pathogen systems, such as infection of strawberry and kiwifruit by *B. cinerea* (Bristow et al. 1986; Michailides and Morgan 1996; Powelson 1960) and plum by *Monilinia laxa* (Schlagbauer and Holz 1990). Because our visual observations found that most calyx-end Sphaeropsis rots originated from infected sepals, further histological studies were then focused on sepal infections. Although *S. pyriputrescens* infected both necrotic and non-necrotic tissues of sepals, the presence of necrotic tissues on sepals favoured infection by the pathogen. Similar findings have been reported in Phacidiopycnis rot of pears caused by *Potebniamyces pyri* (Liu and Xiao 2009) and in strawberry fruit rot caused by *B. cinerea* (Powelson 1960). Direct penetration of the fungal hyphae through sepal tissues indicated that natural openings are not required for colonization of sound sepal tissues. This also may explain why calyx-end rot is one of the common symptoms, even though systemic colonization of filaments and styles was not commonly observed. Our results also showed that *S. pyriputrescens* colonized lenticels of fruit skin and remained latent in the orchard, leading to fruit rot in storage. Although Sphaeropsis rot originating from infections through lenticels of fruit skin was observed in cv. Golden Delicious, it was uncommon in cvs Red Delicious and Fuji. Re-isolation of *S. pyriputrescens* from symptomless sites

(stem or calyx tissue) of decayed fruit after the monthly evaluations and from symptomless fruit after 9 months of storage showed that co-infection of the stem and calyx was common, especially in cvs Red Delicious and Golden Delicious fruit. According to our visual observations, when infection occurred at both stem and calyx of the same fruit, calyx infections developed the decay symptom earlier than stem infections, causing the entire fruit to rot before the stem infection reached the fruit flesh. This may explain why calyx-end rot was higher than stem-end rot on Red Delicious in 2006, though many stems were also infected. The findings of the infection courts by *S. pyriputrescens* suggest that control of Sphaeropsis rot should be focused on protecting the stem and calyx of fruit.

Conidia of *S. pyriputrescens* germinated on the stem, sepal, and skin of apple fruit. On stems, conidia germinated and penetrated the stem tissue through stomata. We found that *S. pyriputrescens* was able to infect fruit through natural openings such as stomata and lenticels as well as micro-cracks and was also capable of penetrating fruit stem and sepal tissues directly through cuticles. Successful penetration occurred within 24 h after inoculation in both laboratory and orchard conditions. Cuticular micro-cracks on the surface of fruit stems and sepals provided alternative sites for penetration by *S. pyriputrescens*, which has been observed for *Monilinia laxa* and *Rhizopus stolonifer* on nectarines (Nguyen-The et al. 1989) and *B. cinerea* on grapes (Pucheu-Planté and Mercier 1983). Although the fungus was recovered from all floral parts, the relative contribution of infections in each floral part to calyx-end Sphaeropsis rot in storage remains unclear. Previous studies on *B. cinerea* reported that not all infected floral parts lead to fruit decay in other fruit crops. Infection of styles at bloom by *B. cinerea* in pear and strawberry does not result in fruit decay (Bristow et al. 1986; De Kock and Holz 1992; Powelson 1960). The SEM study showed that sepals were indeed the predominant site for calyx-end infection by *S. pyriputrescens*, whereas there is no clear evidence of penetration in anthers and filaments.

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