Isolation, spore production and Koch’s postulates of Elsinoe pyri

R.W.A. Scheper, P.N. Wood and B.M. Fisher

The New Zealand Institute for Plant & Food Research Limited, Private Bag 1401, Havelock North, New Zealand
Corresponding author: reiny.scheper@plantandfood.co.nz

Abstract Elsinoe leaf and fruit spot is a minor disease of apple and pear. Very little is known of the biology and life cycle of the causal agent Elsinoe pyri. The fungus was isolated from spots on apple fruit, and grew very slowly on potato dextrose agar (PDA). The conditions needed for spore production were examined using different culturing media, plating techniques and culture ages. When small pieces of a 2- to 6-week-old culture from PDA were sub-cultured onto corn meal agar for 2 days, viable conidia were produced. Conidial germination occurred between 10°C and 26°C, with the highest germination percentage at 20°C and 26°C, and greatest germination tube elongation at 20°C. At least 200 conidia per leaf were required to infect ‘Royal Gala’ leaves. Typical elsinoe spots were visible 6 weeks after inoculation. Four months after inoculation, conidia from the spots were re-isolated onto PDA and grew into typical colonies of E. pyri.

Keywords Elsinoe spot, fungus, conidia, spore production, apple, disease, pathogen.

INTRODUCTION
Elsinoe spot of apple and pear (Elsinoe pyri (Wor.) Jenkins (anamorph Sphaceloma pirinum (Pegl.) Jenkins)) is economically important in some organic orchards, but it is rarely observed in orchards with a conventional fungicide regime. It was first recorded in New Zealand in 1951 (Atkinson 1971).

Spots on leaves are whitish-grey with a brown margin, round, 1-2 mm in diameter, or oval, up to 4 × 2 mm, and may occasionally coalesce. In the centre of the spots, the dark brown fruiting structures may be visible (Woronichin 1914). On yellow and green fruit, spots can vary in colour from white to pale yellow brown, to brown in the centre and surrounded by a dark red margin (Jenkins 1932, 1946). In the centre of the spots, dark fruiting structures may protrude through the ruptured epidermis (Jenkins 1946). As many as 100 spots, up to 2 mm in diameter, have been counted on a single small fruit (Jenkins et al. 1945). Spots on leaves and fruit are sometimes confused with spots caused by Phoma sp. However, unlike phoma spot, the smaller elsinoe spots have a dark border and when examined with a hand lens, acervuli instead of pycnidia may be visible (Jenkins & Watson 1972). Small, aseptate, hyaline conidia (4-6 × 2.5-4 µm) may be present on acervuli on leaves and fruit, but they are not always abundant (Jenkins 1932).

Very little is known about the biology and life cycle of E. pyri. A study was conducted to isolate and examine the fungus, and determine the conditions needed for conidium production in culture and infection of apple leaves in a glasshouse.
MATERIALS AND METHODS

Isolation of *Elsinoe pyri*

Leaves and apples with roughly circular spots, 2–3 mm in diameter, were collected from Hawke’s Bay orchards. The lesions were silver-grey in the centre, surrounded by a charcoal grey border and a purple-brown margin, and were typical of spots caused by *E. pyri*. Tissue was collected at different times of the year, over a number of years, to determine when conidia were present in orchards, and identify a method to isolate this extremely slow growing fungus. Leaves were collected at three different times, and fruit at three different times.

In December 2007, approximately 70 leaves with spots were collected from an unsprayed orchard. No acervuli were observed in the lesions on these leaves. The leaves were surface sterilised (60 s in 95% ethanol followed by two 30-s rinses in sterile distilled water (SDW) and blot drying on sterile paper towels) and placed in sterile Petri dishes containing two sterile filter papers moistened with 3 ml SDW, one large leaf or two small leaves per Petri dish. The leaves were placed on two sterile pipette tips to avoid direct contact with the wet filter papers in the bottom of the Petri dish. The plates were sealed with plastic film (Glad®wrap) and placed on the laboratory bench at approximately 20°C. The lesions were checked weekly using a dissecting microscope, for the development of acervuli.

In January 2008, approximately 200 leaves with elsinoe spots that had acervuli, were collected from an unsprayed orchard. The leaves were collected over a period of 3 weeks, and placed in sealed plastic bags, containing moist tissues to maintain high humidity, on the laboratory bench at 20°C for 1-4 weeks. Acervuli of 100 lesions were placed in 1 ml Tween solution. The suspensions were examined for the presence of conidia using a compound microscope, and, when present, the conidia were streaked onto antibiotic PDA and the plates were incubated at 20°C in the dark.

In May 2008, an additional 50 leaves with spots and acervuli were collected and stored in a plastic bag in the dark at 1°C until October 2008 when they were examined. Individual acervuli from approximately 100 lesions were each transferred to 50 µl 0.005% Tween®20 (Tween solution). All suspensions were examined for the presence of conidia using a compound microscope and, when present, the conidia were streaked onto antibiotic PDA, and plates were incubated at 20°C in the dark.

In September 2008, four over-ripe apples were collected and placed on a laboratory bench for 1 to 2 weeks at approximately 20°C. Individual acervuli from approximately 10 lesions were each transferred to 50 µl Tween solution. All suspensions were examined for the presence of conidia using a compound microscope and, when present, the conidia were streaked onto antibiotic PDA and the plates were incubated at 20°C in the dark.

A yellow apple with one elsinoe spot was collected from an orchard in June 2009. Individual acervuli were transferred to 50 µl Tween solution, the suspensions examined for the presence of conidia and streaked onto antibiotic PDA as described above.

A ‘Granny Smith’ apple with elsinoe spots was collected from an orchard in July 2011. The fruit surface was swabbed with 70% ethanol and two lesions were excised and each transferred to 50 µl SDW. The tissue was macerated and left to stand for 1 h on the laboratory bench. The suspension was streaked onto antibiotic PDA and the plates were incubated at 20°C in the dark.

Cultures of *E. pyri* were sub-cultured onto fresh antibiotic PDA as soon as they were visible.

Conidium production and viability

A series of experiments was conducted to determine the optimal conditions for conidium production in culture. The method described by Jenkins (1932) was applied by sub-culturing onto corn meal agar (CMA, Sigma), small (1 mm²) pieces of an *E. pyri* colony (1 cm²) that had been growing on PDA for 2 weeks. SDW (1 ml) was pipetted onto the CMA prior to sub-culturing (5 pieces of *E. pyri* colony per plate). Plates were sealed with plastic film and placed at 20°C in the dark for 20 h. Subsequently, small *E. pyri* colonies
were removed from the CMA and placed in Tween solution (5 colonies/100 µl). Conidia were counted and their viability was ascertained by determining the percentage conidial germination of a $10^6$ conidia/ml suspension in three ways: (1) six 100-µl drops of suspension were placed on glass microscope slides without cover slips, (2) six 25-µl drops on glass microscope slides with cover slips and (3) 300 µl was spread evenly over 1% water agar, CMA and PDA plates. The slides were placed on grids in eight Sistéma® KLIP IT™ meat keeper boxes, eight slides per box, with water below the grid to ensure high humidity (humidity box). Slides and plates were incubated at 20°C for 48 h, after which lacto aniline blue stain was pipetted onto the slides and plates. On slides with cover slips, the stain was pipetted next to the cover slips, and allowed to mix with the conidial suspension through diffusion. On slides without cover slips, the stain was added before the cover slips were placed on the drops. Three drops of stain were randomly placed on each plate. The numbers of germinated and un-germinated spores on the glass slides and the plates were counted using a microscope.

To optimise conidium production and simplify the method described by Jenkins (1932), the method was modified by sub-culturing small pieces of a 4-weeks-old *E. pyri* colony on PDA, onto CMA without SDW. In one test, 12 small (1 mm$^2$) pieces of *E. pyri* PDA culture per plate were incubated at 20°C in the dark for 5 days. In another test, 30 small (1 mm$^2$) pieces of *E. pyri* PDA culture per plate, spaced approximately 1 cm apart, and 30 slightly larger pieces (2 mm$^2$) per plate, were incubated in the same conditions for 2 days. The method that yielded the largest numbers of conidia was repeated several times, using *E. pyri* cultures on PDA that varied in age from 2 to 12 weeks old at the time they were cut into small pieces and placed onto CMA. For each test, the viability of the conidia was determined by placing six 25-µl drops (1-2 x $10^6$ conidia/ml) on glass microscope slides with cover slips, placed in a humidity box, and incubated at 20°C for 48 h.

In addition, conidium production on a dense ‘mat’ of *E. pyri* cultures was examined on different agar media, to determine whether a conidium production method could be developed that does not involve laborious sub-culturing and removal of large numbers of small colonies. To create a dense *E. pyri* mat on CMA, PDA and malt extract agar (MEA, Merck), a suspension of $3 \times 10^4$ conidia per plate was spread evenly over the agar, and sealed plates were placed at 20°C in the dark for 7 days. In addition, on CMA, $10^5$ conidia per plate were spread evenly over the agar to grow an even denser *E. pyri* mat. Conidial suspensions were produced by flooding each plate with 2 ml Tween solution and pipetting the liquid four or five times over the culture to harvest conidia. The number of conidia produced on each medium was compared with conidium production using the methods based on Jenkins (1932).

Conidium production in culture, as described above, was compared with the production and viability of conidia on leaves from the field (field inoculum), to determine whether the production of field inoculum would be more effective and efficient than inoculum produced from *E. pyri* cultures. To produce field inoculum, apple leaves with elsinoe spot and acervuli were collected from an unsprayed orchard in January 2008, over a period of 3 weeks. Approximately 200 leaves were placed in sealed plastic bags, containing moist tissues to maintain high humidity, on the laboratory bench at 20°C for 1-4 weeks. Acervuli of 100 lesions were placed in 1 ml Tween solution. Conidia were enumerated and the percentage conidial germination of a $10^5$ conidia/ml suspension was determined, by placing six 25-µl drops on glass microscope slides with cover slips in a humidity box, and incubated at 20°C for 48 h.

**Temperature range for conidial germination**

Conidia were produced using a modified version of the method described by Jenkins (1932). Small pieces of a 6-week-old *E. pyri* colony growing on PDA were sub-cultured onto CMA; 30 pieces of colony were used per plate. Small 5-day-old *E. pyri* colonies were removed from the CMA and placed in Tween solution (30 colonies/ml) to produce a conidial suspension, which was diluted to $3 \times 10^5$ conidia/ml. Droplets (70 µl)
of the conidial suspension were pipetted onto 64 slides, two droplets per slide, and cover slips were placed on the droplets. The slides were placed in humidity boxes. Two boxes were placed at each of the following temperatures: 10°C, 15°C, 20°C and 26°C. After 6, 12, 18, 24, 30, 36, 42 and 48 h, lacto aniline blue stain was pipetted onto one slide in each box, next to the two cover slips, and allowed to mix with the conidial suspension through diffusion. Conidial germination rates and the length of the longest germination tube were determined using a compound microscope. Analysis of variance (ANOVA) and Fisher’s protected least significant differences of means (LSD, =0.05) were used to determine statistical differences in conidial germination rates at different temperatures after 24 and 48 h.

Inoculation of the host and re-isolation of *Elsinoe pyri*

To fulfil Koch’s postulates, five unsprayed potted ‘Royal Gala’ trees, with two shoots each, were inoculated with conidia of *E. pyri* grown on a culture that had been isolated from a typical ellsinoe spot on an apple fruit. In addition, the effect of inoculum concentration on leaf infection was investigated.

In December 2008, twenty leaves per tree (10 leaves per shoot) were spray inoculated with conidial suspensions of $2 \times 10^5$ conidia/ml, $2 \times 10^4$ conidia/ml or $2 \times 10^3$ conidia/ml, using a hand-pumped atomiser. Each leaf was inoculated with one puff of approximately 100 µl inoculum. The twenty leaves on the negative control tree were sprayed with Tween solution.

Conidia were produced using a modified version of the method described by Jenkins (1932). Small pieces of a 6-week-old *E. pyri* colony growing on PDA were sub-cultured onto CMA; 30 pieces of colony were used per plate. Small 2-day-old *E. pyri* colonies were removed from the CMA and placed in Tween solution (30 colonies/ml) to produce a conidial suspension, which was diluted to the required concentration.

The leaves were inoculated 2 h before dark and were misted heavily for 15 min immediately after inoculation. The humidity in the glasshouse was maintained at approximately 90% for 1 week after inoculation and was set at 80% for the remainder of the trial.

Conidial germination rates of the two highest concentrations of inoculum were determined by placing six 70-µl drops of each conidial suspension on glass microscopy slides and incubating them at 22°C for 24 h in a humidity box. In addition, each conidial suspension was plated onto two PDA plates, 200 µl per plate, to check the viability of the inoculum. The plates were sealed with plastic film and incubated at 20°C in the dark for 5 days.

The leaves were observed for symptom development with the aid of a 10× hand lens every 4 days until the first symptoms were visible, and then weekly until 8 weeks after inoculation, followed by a final assessment 16 weeks after inoculation.

At the final assessment, all inoculated leaves were detached from the trees, stored for 2 weeks at 2°C in a plastic bag, and examined in the laboratory using a dissecting microscope (40×), to detect the presence of acervuli in the lesions.

Acervuli from 10 lesions were transferred to 50 µl Tween solution and left to stand for approximately 2 h, and subsequently mixed with a pipette. Of each suspension, 20 µl was examined for the presence of conidia, using a compound microscope. If conidia were observed, the suspension was streaked out onto antibiotic PDA plates, 10 µl per plate, and incubated at 20°C. Agar plates were examined regularly for 3 weeks for colonies of *E. pyri*. Identification of *E. pyri* colonies was confirmed by sub-culturing onto CMA and examining the presence and morphology of conidia, using a compound microscope.

**RESULTS**

**Isolation of *Elsinoe pyri***

Two dark red, compact cultures of the fungus *E. pyri* were isolated from a single acervulus from a lesion on one of the over-ripe apples in September 2008. Many other cultures on the streaked plates were yeasts. One of the *E. pyri* isolates was deposited in the International Collection of Microorganisms from Plants (ICMP 18257). *Elsinoe pyri* was also
isolated from the lesion on the yellow apple in June 2009 and from both lesions on the 'Granny Smith' apple in July 2011. *Elsinoe pyri* was isolated only from spots that had mature, dark brown acervuli, which produced small, aseptate, hyaline conidia (4-6 × 2.5-4 µm, Figures 1a, 1b & 1c).

*Elsinoe pyri* was not isolated from leaves that were collected in December 2007, as the lesions on the incubated leaves did not develop acervuli. *Elsinoe pyri* was also not isolated from leaves that were collected in January 2008 or from leaves that were collected in May 2008 and stored at 1°C for 5 months, as the plates were overgrown with yeasts and other fast growing fungi.

The *E. pyri* cultures were slow-growing, convoluted, pulvinate, rust brown to dark red or purple in color, with a diameter of approximately 10 mm after 1 month's growth on PDA at 20°C (Figure 2a).

**Conidium production and viability**

Using the method described by Jenkins (1932), approximately $10^4$ conidia (5-6 × 2.5-3 µm) were harvested on average from each of the small colonies. The average conidial germination rate was 85% on glass slides with cover slip, 17% without cover slip, and 0%, 95% and 100% on WA, CMA and PDA, respectively.

When 12 small colonies on CMA were incubated for 5 days, approximately $2 \times 10^4$ conidia were harvested on average from each of the small colonies, but the cultures were difficult to remove from the CMA to harvest the conidia. However, 2 days of incubation of approximately 30 small (1 mm$^2$) colonies per CMA plate, resulted in approximately $3.5 \times 10^4$ conidia from each of the small colonies, whereas larger pieces (2 mm$^2$) of *E. pyri* produced, on average, $10^5$ conidia from each of the colonies. The 2-day-old cultures were easy to remove from the CMA.

Plates with a dense *E. pyri* mat due to inoculation with $3 \times 10^4$ conidia per plate, yielded $3 \times 10^5$ conidia per plate, after 7 days on MEA. However, on CMA and PDA fewer conidia than were applied to the plates were recovered. CMA plates inoculated with $10^5$ conidia, yielded $6 \times 10^5$ conidia after 7 days. The viability of the conidia that were produced using the method adapted from Jenkins (1932), where the small pieces of *E. pyri* on CMA were incubated for 2 days (approximately 30 pieces per plate), varied with the age of the culture on PDA. When the PDA culture was 2 weeks old, the conidial germination rate was 85%. In duplicate experiments using 4-week-old PDA cultures, the germination rates were 85% and 87%. In duplicate experiments using 6-week-old PDA cultures, the germination rates were 72% and 85%. Cultures on PDA that were 8-, 10- and 12-weeks old resulted in conidial germination rates of 55%, 12% and 3%, respectively.

The culture morphology of *E. pyri* on PDA also changed as the culture aged, with sectoring occurring when the cultures were 2 months old (Figure 2b). Elsinoe spots on leaves that were collected in January 2008 yielded, on average, $5 \times 10^3$ conidia per lesion. Lesions with large numbers of mature acervuli (Figures 3a, 3b & 3c) yielded approximately $8 \times 10^3$ conidia, whereas those with few acervuli yielded approximately $2 \times 10^3$ conidia. The conidial germination rate of the field inoculum measured after 48 h was 26%.

**Temperature range for conidial germination**

Conidia germinated at all temperatures tested between 10°C and 26°C, with the highest germination percentage occurring at 20°C and 26°C (Figure 4). However, the greatest germination tube growth occurred at 20°C.

After 24 h, significantly more conidia had germinated at 20°C (60%; $P<0.001$) than at any other temperature, and the maximum germination tube length was 60 µm at this temperature. In contrast, at 15°C and 26°C, 40% and 41% of the conidia had germinated, respectively, and the maximum germination tube length was 30 µm and 35 µm, respectively. Only 21% of the conidia had germinated at 10°C and the maximum germination tube length was 25 µm.

After 48 h, the germination rate at 20°C and 26°C was 72% and 70%, respectively, which was significantly higher than the germination rates at the lower temperatures ($P<0.001$). However, the maximum germination tube length at 20°C was 75 µm, compared with 55 µm at 26°C. At 15°C,
the germination rate was 58% and maximum germination tube length 60 µm, while only 31% of the conidia germinated at 10°C.

**Infection of the host and re-isolation of *Elsinoe pyri***

The conidia in each of the four suspensions used to inoculate 'Royal Gala' leaves, were viable. The germination rates of the two suspensions of $2 \times 10^5$ conidia/ml and $2 \times 10^4$ conidia/ml were 85% and 75%, respectively. The PDA plates inoculated with 200 µl of the suspensions of $2 \times 10^3$ conidia/ml and $2 \times 10^2$ conidia/ml, had on average 300 and 41 *E. pyri* cultures per plate, respectively. This indicates that at least 75% of conidia in the suspension of $2 \times 10^3$ conidia/ml were viable, and 100% of the conidia in the suspension of $2 \times 10^2$ conidia/ml were viable. The plates that had been inoculated with the two higher concentrations were covered in a dense mat of *E. pyri* culture.

On leaves inoculated with the highest concentration inoculum, the first pin-prick purplish spots became visible 20 days after inoculation, and these spots developed into typical elsinoe spots 6 weeks after inoculation. On the last assessment date, 16 weeks after inoculation, 45% of the leaves inoculated with 100 µl of $2 \times 10^5$ conidia/ml had on average 1.9 elsinoe spots per leaf. The disease incidence of the leaves inoculated with $2 \times 10^4$ conidia/ml and $2 \times 10^3$ conidia/ml was 15% and 5%, respectively; these leaves all had just one lesion each. None of the leaves inoculated with $2 \times 10^2$ conidia/ml developed lesions, and neither did the control.

Pale cream-white acervuli were visible in many of the elsinoe spots, 18 weeks after inoculation (Figures 5a & 5b). Conidia from the acervuli resembled typical *E. pyri* conidia (5-6 × 2.5 µm) and grew into typical brick-red cultures of *E. pyri* on PDA. When these cultures were sub-cultured onto CMA, using a method modified from Jenkins (1932), typical *E. pyri* conidia (5-6 × 2.5 µm) were produced, fulfilling Koch's postulates.

**DISCUSSION**

The fungus *E. pyri* was isolated from over-ripe apple fruit collected from orchards in September 2008 and July 2011, but not from leaves that were collected in May 2008 and stored at 1°C for 5 months, nor from leaves without acervuli that were collected in December 2007. However, infected leaves that were collected in January 2008 did have acervuli with viable conidia. This indicates that the fungus can overwinter as acervuli on unharvested fruit on trees, but not as acervuli on leaves. However, ascomata that develop in the lesions (Woronichin 1914) may also serve as survival structures during winter. As acervuli do not appear to develop until January each year, it is likely that secondary infections may not be important until mid-summer.

When *E. pyri* was isolated, most of the cultures on the streaked plates were yeasts; only two of hundreds of isolates were *E. pyri*. It is possible that some of the yeasts that were present on the acervuli inhibited germination or growth of *E. pyri*. This could also explain the reason for the low conidial germination rate (26% after 48 h) of the field inoculum. Further research is required to determine the effect of the yeasts on *E. pyri*.

A method modified from Jenkins (1932) was developed to produce viable conidia in culture. When approximately 30 small 1-2 mm² pieces of a 2- to 6-week-old culture on PDA were sub-cultured onto a CMA plate, and incubated at 20°C in the dark for 2 days, large numbers of viable conidia were produced. When the CMA plates were incubated for shorter or longer times, fewer conidia were produced and it became more difficult to harvest the conidia from the older cultures, as they were hard to remove from the CMA. When the PDA culture was more than 6 weeks old, the viability of the conidia produced on CMA 2 days later was markedly reduced, from approximately 85% conidial germination to 12% and 3% when the PDA culture was 10 or 12 weeks old, respectively. The culture morphology of *E. pyri* on PDA also changed as the culture aged, with sectoring occurring when the cultures were 2 months old. Aging of ascomycetes has been studied in many species and is known to cause changes in growth or death of cultures (Schwartz & Osiewacz 1996; Osiewacz 2002; Simkovic et al. 2007). However, reduced conidial viability after
Figure 1 (a) A roughly circular elsinoe lesion, 3 mm in diameter, silver-grey in the centre, surrounded by a charcoal grey border and a rust-brown margin, with mature dark brown acervuli. (b) This was visible on a yellow apple, collected from an unsprayed orchard in June 2009. (c) Conidia of *Elsinoe pyri* (6 × 2.5 µm) were obtained from the acervuli.

Figure 2 (a) Cultures of *Elsinoe pyri* on potato dextrose agar had a diameter of approximately 10 mm after 1 month’s growth at 20°C in the dark. (b) These started to sector and change culture morphology after 2 months of growth at 20°C in the dark.

Figure 3 (a) Apple leaf with elsinoe lesions, creamy-grey in the centre, surrounded by charcoal-grey borders and rust-brown margins, collected from an unsprayed orchard in January. (b) Mature dark brown acervuli were visible in the centre of a lesion. (c) Silver-grey elsinoe spots with rust-brown margins coalesced on a leaf collected just before leaf fall in May.

Figure 5 (a) 'Royal Gala' apple leaf, 18 weeks after inoculation with conidia of *Elsinoe pyri*, displaying typical elsinoe lesions, silver-grey in the centre, surrounded by charcoal grey borders and brown margins. (b) Pale coloured acervuli were visible in the centre of a lesion.
sub-culturing may be less common, particularly when large numbers of apparently non-viable conidia are produced. Regular rejuvenation of the culture, by placing several single conidia on fresh PDA to grow a new culture, may reduce the effects of aging of *E. pyri* cultures. Alternately, cultures may need to be re-isolated from inoculated leaves to maintain conidial viability and pathogenicity traits of the organism in culture.

The use of thirty colonies per CMA plate, spaced approximately 1 cm apart, yielded large numbers of conidia. However, thousands of tiny *E. pyri* colonies on CMA, forming an *E. pyri* mat, did not produce large numbers of conidia. Similarly, Kono et al. (2009) found that when *Elsinoe ampelina* colonies were grown at >2.5 colonies per cm², few or no conidia were produced, whereas those grown at <1 colony per cm² produced large numbers of conidia. The reason for this colony-density-dependent production of conidia was not clear, and more research is required to understand this.

The optimum temperature for conidial germination is likely to be between 20°C and 26°C. The first 24 h of conidial germination is possibly the most important time for infection to occur. Therefore, it is likely that the optimum temperature may be closer to 20°C than to 26°C, as the conidial germination rate at 20°C was significantly higher. However, it is unclear from the current data whether germination rates would decline at temperatures above 26°C. To determine the optimum temperature, conidial germination at different temperatures between 20°C and 30°C will need to be examined. Nevertheless, it is clear that temperatures greater than 20°C are optimal for conidial germination. It is, therefore, possible that infection risk during wet periods in January/February may be higher than earlier in the season, as the average temperatures during wet periods in Hawke’s Bay in September/October, November/December and January/February are approximately 10°C, 15°C and 20°C, respectively (R.M. Beresford, Plant & Food Research, personal communication). In addition, infected leaves with acervuli and viable conidia were not observed until January. This may indicate that the warmer summer months may be important
in the disease cycle, and disease management of
elsinoe spot may need to be targeted at that time
of the year. However, further research is required
to elucidate wetness duration and temperature
requirements of infection of leaves and fruit.

Apple leaves inoculated in a glasshouse
developed elsinoe lesions only when sprayed
with at least 200 conidia per leaf. When leaves
were inoculated with approximately $2 \times 10^4$
conidia per leaf, 45% of the leaves developed
symptoms with, on average, 1.9 elsinoe spots per
leaf. This indicated that a large inoculum load
was required for disease to develop.

Acervuli on the elsinoe spots on inoculated
leaves in the glasshouse were pale cream-white
instead of the dark brown colour of the mature
acervuli that were used to isolate the initial
E. pyri cultures. However, they were similar in
colour to the fawn-coloured acervuli found in
the field in summer. Acervuli in the field turn
dark brown-black as they mature. It is possible
that the acervuli in the glasshouse would have
become darker, if they had been left to mature
on the trees.

Koch’s postulates were fulfilled, conclusively
proving that E. pyri is the causal agent of elsinoe
leaf and fruit spot of apple.

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REFERENCES
Atkinson JD 1971. Diseases of tree fruits in New
Zealand. A.R. Shearer, Government Printer,
Jenkins AE 1932. Elsinoe on apple and pear. Journal
of Agricultural Research 44(9): 689-700.
Jenkins AE 1946. Elsinoe piri in France and Spain
Jenkins AE, Watson AJ 1972. Sphaceloma-
Pirinum in Association with Phyllosticta-
Pirina. Mycologia 64(2): 441-443.
Jenkins AE, Forsell MJ, Boyle LW 1945. Elsinoe
piri discovered on apple and pear in Western
Washington and Oregon. Phytopathology
Kono A, Nakaune R, Yamada M, Nakano M,
Mitani N, Ueno T 2009. Effect of culture
conditions on conidia formation by Elsinoe
ampelina, the causal organism of grapevine
Osiewacz HD 2002. Genes, mitochondria and
aging in filamentous fungi. Ageing Research
Reviews 1: 425-442.
Schwartz T, Osiewacz HD 1996. Telomere length
does not change during senescence of the
ascomycete Podospora anserina. Mutation
Research 316: 193-199.
Simkovic M, Ditte P, Chovanec P, Varecka
competence of aged Trichoderma viride
vegetative mycelia. Antonie van Leeuwenhoek
91: 407-416.
Woronichin NN 1914. Plectodiscella piri, der
Vertreter einer neuen Ascomyceten-Gruppe.