A simple method for conidial production and establishing latent infections of apples by *Phlyctema vagabunda* (syn: *Neofabraea alba)*

K.R. Everett[1], I.P.S. Pushparajah[1], B.M Fisher[2] and P.N. Wood[2]

[1]*The New Zealand Institute for Plant & Food Research Limited, Mt Albert Research Centre, Private Bag 92169, Mt Albert, Auckland 1142, New Zealand*
[2]*Plant & Food Research, Havelock North Research Centre, Private Bag 1401, Havelock North, New Zealand*
Corresponding author: Kerry.Everett@plantandfood.co.nz

Abstract A method was developed to inoculate and establish infection of detached apple fruit with the bull's eye rot pathogen, *Phlyctema vagabunda* (syn: *Neofabraea alba*), without wounding. Mycelial cultures of *P. vagabunda* did not produce conidia on commonly used potato dextrose agar, or several other tested media. Growth on corn meal agar resulted in the highest conidial yield, and maximum production was achieved after 5 days. These conidia were placed on water agar, and apple fruit were placed on these spores for at least 5 days for establishment of infections. Reliable infection of 66–100% of apples required 14 days of contact. The ability to rapidly produce copious quantities of conidia and inoculate without wounding facilitates a number of other postharvest control and epidemiology studies.

Keywords Latency, quiescent infections, bull's eye rot, 'Scilate'/Envy™, rapid conidial production.

INTRODUCTION

Apple fruit in an orchard can become infected by the fungal pathogen *Phlyctema vagabunda* (syn: *Neofabraea alba*) through lenticels, wounds or at the calyx end. The resulting disease is commonly known as bull's eye rot. It causes lenticel spots and can lead to internal rot lesions that develop during long-term cool-storage (Spotts 2014). Bull's eye rot is a minor disease in New Zealand, but it is an important phytosanitary issue for some export markets.

Development of not only postharvest control treatments, such as hot water treatments, but also predictive models for informing spray decisions are important components of disease management. A simple and rapid method for conidial production and also a simple method for infecting apples with this pathogen are required as part of these developments. Existing methods for producing conidia of *P. vagabunda* require long incubation times, such as 60 days following inoculation of sterile leaves (Neri et al. 2009), at least 2 months on malt extract agar (Amiri et al. 2008) or 6 weeks on inoculated apples. Incidence of *P. vagabunda* on naturally infected apples from individual orchards in New Zealand is usually fewer than 10%, therefore, reliable inoculation methods to establish symptomless (latent) infections would reduce the number of replicates required for comparisons. Inoculations to produce latent infections are also time-consuming, for example fruit needed to be incubated at 95% relative humidity at 20°C for 7

days following spray application of spores (Neri et al. 2009).

The aims of this study were to: (i) develop a rapid method for conidial production; and (ii) develop a simple method for initiating latent infections on apple fruit for use in epidemiology and control studies. Preliminary results were published as a poster abstract (Pushparajah et al. 2016) but this paper presents a full account of the work.

MATERIALS AND METHODS

Isolate

Isolate *P. vagabunda* KE171 was used in this study, which was isolated from a rot of a stored 'Jazz' apple in September 2013. The isolate was identified on the basis of conidial and colony morphology, by amplification of DNA using the polymerase chain reaction and species-specific primers (Gariepy et al. 2003; Henriquez et al. 2004; Gariepy et al. 2005), and by DNA sequencing of the beta-tubulin gene region (de Jong et al. 2001).

Conidial production

Method 1. A 5 mm diameter agar plug from a 14-day-old colony of *P. vagabunda* grown on Difco® potato dextrose agar (PDA) was macerated and spread on: a) PDA covered with a layer of sterile cellophane; b) Difco® prune agar; and c) V8 agar (Englander & Roth 1980).

Method 2. A 'Royal Gala' apple was peeled, then surface sterilised by flaming after dipping in 95% ethanol. Pieces of the apple were placed in a Petri dish and molten PDA was added. Agar plugs from a 14-day-old colony of *P. vagabunda* were placed on the apple pieces, and some on the adjacent PDA.

Method 3. Peeled 'Royal Gala' apple tissue (100 g) was steamed for 1 hour in an autoclave. The resultant slurry was passed through a double layer of sterile cheesecloth, then 8 g dextrose and 8 g of agar were added, and deionised water to make up the volume to 400 mL. After autoclaving, the media was poured into Petri plates and a macerated agar plug of a 14-day-old *P. vagabunda* colony spread over the surface of the cooled media.

Method 4. A macerated agar plug from a 14-day-old colony of *P. vagabunda* was spread on either modified Matsushima agar (Matsushima 1961; Scheper et al. 2014) or Difco® corn meal agar (CMA).

For all methods, conidia were harvested in 1 mL of deionised water by scraping the surface of the Petri plate with a sterile bent glass rod and counting the conidia with the aid of a haemocytometer.

Preparation of conidia for inoculations

Conidia were prepared by spreading CMA with a macerated agar plug from a 14-day-old culture of *P. vagabunda* grown on PDA. After 7–10 days growth at 20°C under fluorescent tubes on a 12:12 hour light:dark cycle, conidia were harvested in sterile deionised water (SDW) and the concentration was adjusted within 2 hours of inoculation.

Investigation of infection period and conidial number on establishment of infections

To determine the minimum contact time required for infection (infection period), inoculation of 'Royal Gala' apples was conducted with the previously prepared conidial suspensions adjusted to 10^5, 10^6 and 10^7 conidia per mL. An aliquot of 100 µL was placed in the centre of a Petri plate containing water agar. The cheeks of three 'Royal Gala' apple fruit for each time/concentration combination were secured with masking tape to the surface of a Petri plate containing water agar and a droplet of conidia and held at ambient temperature (c. 20°C). Fruit were removed after 2, 5, 7, 10 and 14 days, incubated at 20°C under humid conditions (in a plastic container with a moist paper towel) and symptoms recorded after 31 days.

To verify results obtained with 'Royal Gala' apples, inoculation tests were also conducted on three 'Scilate' and Envy™ apples for each time/concentration combination using 10^5 and 10^7 conidia per mL as described above. Fruit were removed from the Petri plates after 5, 7, 14, 21 and 28 days and incubated at c. 20°C under humid conditions. Symptoms were recorded after 39 days.

To determine the optimal time for assessment of lesions on 'Scilate'/Envy™ apple fruit after inoculation, fruit were inoculated with 10^6 conidia/mL as described above. Fruit were taped on Petri plates containing conidia for 7, 14 and 21 days. There were 10 fruit for each inoculation time period and 10 fruit were not inoculated. Lesions were assessed after 0, 7, 14, 28 and 42 days incubation at c. 20°C under humid conditions.

RESULTS

Production of conidia

Phlyctema vagabunda did not produce conidia on PDA, prune agar, V8 agar, or apple medium. A few conidia formed on apple pieces immersed in PDA, but this medium was difficult to prepare. *Phlyctema vagabunda* conidia formed on Matsushima media and CMA. From these, 10-fold more were produced on CMA (3.3–3.9 x 10^6 conidia/mL) than on Matsushima media (1.5–2.8 x 10^5 conidia/mL, Figure 1).

Investigation of contact time and conidial concentration on establishment of infections

Inoculation of 'Royal Gala' apples with a 7-day contact time, or longer, and 10^7 conidia/mL, resulted in lesions on 33% of inoculated apples (Table 1). Inoculation with a conidial concentration of 10^5 conidia/mL or 10^6 conidia/mL was not successful for a 2- and 5-day contact time or for a 2- and 10-day contact time, respectively.

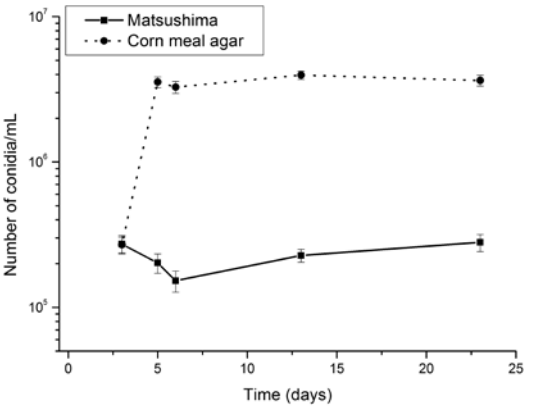

Figure 1 *Phlyctema vagabunda* conidia harvested from Matsushima agar or Difco® corn meal agar over 23 days. Conidia were harvested by washing with 1 mL of sterile deionised water. Values are means of three replicates ± standard errors.

Table 1 Mean number of lesions per fruit and number of fruit infected out of three 'Royal Gala' apples inoculated with three conidial concentrations of *Phlyctema vagabunda* and incubated for six different times. Apples were assessed after 31 days in a plastic container at c. 20°C under humid conditions.

Contact time (days)	Concentration of inoculum (conidia/mL)							
	0		10^5		10^6		10^7	
	No. of infected fruit	No. of lesions	No. of infected fruit	No. of lesions	No. of infected fruit	No. of lesions	No. of infected fruit	No. of lesions
2	0	0	0	0	0	0	0	0
5	0	0	0	0	1	1.3 ± 1.3	0	0
7	0	0	2	5.7 ± 5.2	1	0.3 ± 0.3	1	0.3 ± 0.3
10	0	0	1	1.7 ± 1.2	0	0	1	0.3 ± 0.3
14	0	0	0	0	2	0.7 ± 0.3	1	0.3 ± 0.3

Note: not all visible lesions developed into rots.

For 'Scilate'/Envy™ apples, lesions were produced by inoculating with 10^5 or 10^7 conidia/mL with a contact time of 7 days or greater (Table 2). A higher number of 'Scilate'/Envy™ than 'Royal Gala' (66–100% cf. 33%) apples became infected.

Inoculations of 'Scilate'/Envy™ apples with 10^6 conidia/mL resulted in 30% infection when a 7-day contact time was used, and 70–80% infection when contact time was longer than 7 days (Table 3). Symptoms were expressed on inoculated apples 7 days after removal from the Petri plates, and very few new lesions developed during a further 35 days of incubation (Figure 2). No lesions were apparent on uninoculated apples.

DISCUSSION

Conidial production by isolate KE 171 of *Phlyctema vagabunda* was more prolific on CMA than on any other medium used in this study, and conidia were produced more rapidly than by any published methods (Gariepy et al. 2005; Amiri et al. 2008; Neri et al. 2009). The ability to rapidly produce copious quantities of conidia facilitates a number of other studies such as treatment with hot water to control the disease and determination of temperatures required for infection to occur.

The minimum contact time that resulted in lesions was 7 days, and was independent of the concentration of conidia, or the apple variety, with one exception. One lesion on one 'Royal Gala' fruit resulted from inoculation with 10^6 conidia/mL and an incubation of 5 days. However, for reliable infection, 14 days was the minimum incubation time that was required for both 'Scilate'/Envy™ and 'Royal Gala' apples. A maximum of two out of the three 'Royal Gala'

Table 2 Mean number of lesions per fruit and mean number of fruit infected out three 'Scilate'/Envy™ apples inoculated with two conidial concentrations of *Phlyctema vagabunda* and incubated for six different times. Apples were assessed after 39 days in a plastic container at c. 20°C under humid conditions.

Incubation time (days)	Concentration of inoculum (conidia/mL)					
	0		10^5		10^7	
	No. of lesions	No. of infected fruit	No. of lesions	No. of infected fruit	No. of lesions	No. of infected fruit
0	0	0	0	0	0	0
5	0	0	0	0	0	0
7	0	0	3.3 ± 2.4	2	5.3 ± 3.2	2
14	0	0	6.0 ± 4.2	2	8.7 ± 2.3	3
21	0	0	0	0	14.0 ± 7.2	2
28	0	0	1.7 ± 0.9	2	8.0 ± 4.0	3

Table 3 Mean number of lesions per fruit and mean number of fruit infected out 10 'Scilate'/Envy™ apples inoculated with 10^6 conidia/mL of *Phlyctema vagabunda* and incubated for three different times. Apples were assessed after 42 days in a plastic container at c. 20°C under humid conditions.

	Uninoculated fruit		Inoculated fruit	
Incubation time (days)	No. of lesions	No. infected fruit	No. of lesions	No. infected fruit
7	0	0	2.9 ± 2.2	3
14	0	0	5.5 ± 2.8	8
21	0	0	13.2 ± 5.2	7

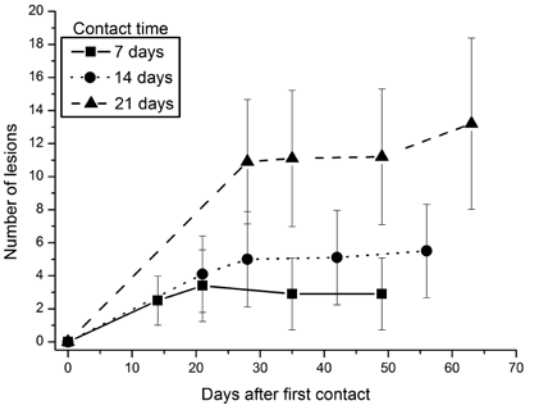

Figure 2 Development of symptoms on 'Scilate'/Envy™ apples inoculated with 10^6 conidia/mL of *Phlyctema vagabunda* and in contact with conidia on water agar in Petri plates (contact times) for 7, 14 or 21 days. Apples were held in a plastic container at c. 20°C under humid conditions and were assessed for 42 days. Values are means of 10 replicates ± standard errors.

apples per time/concentration combination became infected even when an incubation of 21 days was used, whereas for 'Scilate'/Envy™ the minimum number of apples that were infected after an incubation of more than 7 days was two of three and seven of 10. This suggests that 'Scilate'/Envy™ was more susceptible to *P. vagabunda* than 'Royal Gala', but this requires more evidence before any conclusions can be made.

The time required for successful inoculation of 'Royal Gala' and 'Scilate'/Envy™ apple fruit by *P. vagabunda* in the current study was similar to that required for infection of 'Golden Delicious' and 'Pink Lady' apples, which required high humidity (95% RH) for 7 days (Neri et al. 2009). However, for reliable inoculations, a 14-day incubation would be preferable to a 7-day incubation. Despite the length of time required for successful infection, the method developed here did not require specialised equipment, and resulted in symptomless (latent) infection of 66–80% of the inoculated 'Scilate'/Envy™ apples. Although apples in our study were incubated after inoculation in enclosed plastic boxes above damp paper towels, there were very few new infections after the initial disease expression 7 days after inoculation.

The ability to rapidly produce copious amounts of conidia facilitates further study of the bull's eye rot fungus, including studies of infection timing in the field and the effect of fungicides and environmental factors on spore germination. The ability to reliably inoculate fruit without causing symptoms that express immediately also facilitates testing postharvest treatments on symptomless (latent) infections. Substituting CMA for water agar may hasten infection from inoculation without wounding and needs to be investigated.

ACKNOWLEDGEMENTS

Thanks to Pipfruit New Zealand and the Ministry of Business Innovation and Enterprise for funding. Thanks to apple growers and packers (Mr Apple, Turners and Growers, Compass) for ready access to fruit for our laboratory studies.

REFERENCES

Amiri A, Dugas R, Pichot AL, Bompeix G 2008. In vitro and in vivo activity of eugenol oil (*Eugenia caryophylata*) against four important postharvest apple pathogens. International Journal of Food Microbiology 126: 13-19.

de Jong SN, Levesque CA, Verkley GJM, Abeln ECA, Rahe JE, Braun PG 2001. Phylogenetic relationships among *Neofabraea* species causing tree cankers and bull's-eye rot of apple based on DNA sequencing of ITS nuclear rDNA, mitochondrial rDNA, and the beta-tubulin gene. Mycological Research 105: 658-669.

Englander L, Roth LF 1980. Interaction of light and sterol on sporangium and chlamydospore production by *Phytophthora lateralis*. Phytopathology 70: 650-654.

Gariepy TD, Levesque CA, de Jong SN, Rahe JE 2003. Species specific identification of the *Neofabraea* pathogen complex associated wtih pome fruits using PCR and multiplex DNA amplification. Mycological Research 107: 528-536.

Gariepy TD, Rahe JE, Levesque CA, Spotts RA, Sugar DL, Henriquez JL 2005. *Neofabraea* species associated with bull's-eye rot and cankers of apple and pear in the Pacific Northwest. Canadian Journal of Plant Pathology 27: 118-124.

Henriquez JL, Sugar D, Spotts RA 2004. Etiology of bull's eye rot of pear caused by *Neofabraea* spp. in Oregon, Washington, and California. Plant Disease 88: 1134-1138.

Matsushima T 1961. A new medium for identification of hyphomycetes. Transactions of the Mycological Society of Japan 2: 118-120.

Neri F, Mari M, Brigati S, Bertolini P 2009. Control of *Neofabraea alba* by plant volatile compounds and hot water. Postharvest Biology and Technology 51: 425-430.

Pushparajah IPS, Fisher BM, Wood PN, Everett KR 2016. A simple method for conidial production and for inoculating apples with *Neofabraea alba*. [Poster]. New Zealand Plant Protection 69: 328.

Scheper RWA, Fisher BM, Amponsah NT, Walter M 2014. Effect of culture medium, light and air circulation on sporulation of *Neonectria ditissima*. New Zealand Plant Protection 67: 123-132.

Spotts RA 2014. Postharvest diseases: Bull's-Eye Rot. In: Sutton TB, Aldwinckle HS, Agnello AM, Walgenbach JF eds. Compendium of Apple and Pear Diseases. 2nd edition. American Phytopathological Society, St. Paul, MN. Pp. 78-79.