# The effect of fungicides on spore germination, mycelial growth and lesion development of *Phlyctema vagabunda* (syn: *Neofabraea alba*) (bull's eye rot of apples)

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**Abstract** *Phlyctema vagabunda* (syn: *Neofabraea alba*) is a plant pathogenic fungus that causes bull's eye rot on apples and pears. *Phlyctema vagabunda* fruit infections occur in orchards predominantly pre-harvest, and eventually express as a fruit rot after 4–5 months of cool storage. Twelve fungicides (captan, carbendazim, copper hydroxide, cyprodinil, difenoconazole, dithianon, dodine, isopyrazam, metiram, lime sulphur, sulphur and trifloxystrobin) were tested *in vitro* for their effects on spore germination and mycelial growth of *P. vagabunda*. Spore germination was inhibited by metiram, captan, dodine, dithianon, lime sulphur, carbendazim and isopyrazam, in order of effectiveness. Carbendazim, isopyrazam, difenoconazole and cyprodinil, in order of effectiveness, inhibited mycelial growth when used at label rates. Wettable sulphur was ineffective in both assays. On detached apple fruit, carbendazim, cyprodinil, trifloxystrobin and isopyrazam (in order of effectiveness) inhibited lesion development.

**Keywords** *Phlyctema vagabunda*, bull's eye rot, efficacy, conidia, *in vitro*, 'Sciros'/Pacific Rose™.

## INTRODUCTION

*Phlyctema vagabunda*, formerly known by the synonymous name *Neofabraea alba* (Chen 2016), is a species of fungus that causes bull's eye rot on apples and pears (Spotts 2014). It is designated a quarantine actionable pest in China (Ministry for Primary Industries 2014). Any detection of *P. vagabunda* will render all fruit from the orchard production site where it was grown ineligible for export to China. *Phlyctema vagabunda* occurs in all apple growing regions in New Zealand and was first noticed as a problematic storage rot of apples in New Zealand in 1923 (Cunningham 1925). The pathogen can infect fruit at any time of the growing season; however, apples become more susceptible near and during harvest (Edney 1974). The expression of the infection as a fruit rot usually takes place after long period of storage (4–7 months) (Creemers 2014).

Johnston et al. (2005) noted that historically bull's eye rot was a major disease problem in New Zealand until the introduction of broad spectrum dithiocarbamates; however the disease has recently increased in importance possibly due to the adoption of late-season long-storage varieties (Cameldi 2016). Minar (2006) found the fungicide trifloxystrobin to be the most effective of a range of active ingredients tested for late season control of bull's eye rot control. Henriquez et al. (2006)

found that high rates of copper sulphate (3000 mg/litre) reduced sporulation on cankers induced by *Phlyctema vagabunda*. Spotts et al. (2009) reported that the four most effective fungicides for control of bull's-eye rot caused by all species were thiabendazole, thiophanate-methyl, pyrimethanil, and pyraclostrobin + boscalid. Holmes (2011) found good activity against *P. vagabunda* from thiabendazole, iprodione and fludioxonil; however, none of these actives are registered for use on apple in New Zealand. Cameldi et al. (2016) found that the fungicide thiophanate-methyl to be the most effective in all trials with an efficacy against *P. vagabunda* of over 87%. She noted the issue of chemical residues in fruit constraining the use of many fungicides to early season; however, on a positive note she reported that an inhibitor of ethylene production (1-methylcycpropene (1-MCP)) commonly used to improve fruit storage supressed bull's eye rot expression.

The studies described in this paper were designed to identify fungicides with efficacy against bull's eye rot by screening those representing the active ingredients currently registered for apple production in New Zealand.

## MATERIALS AND METHODS

### Spore germination assay

Four *P. vagabunda* isolates (KE127, KE171, KE183 and KE892 – all collected in 2013 from conventional apple orchards in Hawke's Bay New Zealand) were grown for 3 weeks under fluorescent light at 20°C on potato dextrose agar (PDA, Merck) Petri plates containing ampicillin (50 mg/mL) and streptomycin (100 mg/mL). To produce conidia from the *P. vagabunda* isolates, a mycelial mat grown on PDA was removed from the agar, macerated and spread on corn meal agar (CMA) in Petri plates with the aid of a sterile bent-glass rod. The plates were sealed and incubated at 20°C under near ultra violet light. After 5 days, 3 mL of sterile distilled water containing 0.005% Tween®20 (Tween solution) was pipetted onto the CMA containing cultures and the surface of the agar was scraped with a sterile bent-glass rod to dislodge the conidia. The conidial suspension was removed with a pipette. This was repeated twice. The solid pieces of culture or agar were allowed to settle before the spore suspension was pipetted into a new sterile bottle. The spore concentration was determined with the aid of a haemocytometer and diluted to a concentration of 1.5 x$10^5$ spores/mL.

Twelve fungicides representative of the mode of action groups currently used in New Zealand apple orchards were tested: captan, carbendazim, copper hydroxide, cyprodinil, difenoconazole, dithianon, dodine, isopyrazam, metiram, lime sulphur, sulphur and trifloxystrobin (Table 1). Fungicides were tested at five concentrations (0, 1, 10, 100 and 1000 ppm a.i.). *Phlyctema vagabunda* conidia from each of the four isolates were separately added to each concentration of fungicide, resulting in a concentration of 1 x $10^5$ conidia/mL. Two 70 µL aliquots for each isolate and fungicide combination were placed on a glass microscope slide, followed by a cover slip. Slides were placed on a raised mesh in a humid chamber at 20°C. After 24 h, germination was stopped by pipetting lacto aniline blue stain onto the slides. The stain was pipetted next to the cover slips, and mixed with the spore suspension through diffusion. Percent spore germination was determined by examining 100 conidia in at least nine fields of view. Each of the four *P. vagabunda* isolates was a replicate.

### Mycelial growth assay

Mycelial plugs (2 $mm^2$) were cut from the margin of colonies grown on PDA for 6 weeks, using a sterilised scalpel and placed on antibiotic PDA amended with the same 12 fungicides (Table 1) used in the spore germination assay at concentrations of 0, 1, 10, 100 and 1000 ppm. Ampicillin (50 mg/mL) and streptomycin (100 mg/mL) were also added to the fungicide amended PDA.

Mycelial diameter was marked on the base of the plates after 5, 9, 15 and 22 days. Day 15 was the linear phase of the fungal growth on nil fungicide so radial growth was measured (mm) using the 15 day markings. The four *P. vagabunda* isolates used in the germination assay were used as replicates for each fungicide rate.

**Table 1** Fungicidal treatments used for the *Phlyctema vagabunda* spore germination assay.

| Active ingredient (ai) | Product | Percent ai | Chemical Group[1] | Batch # | Date of Manufacture[2] |
|---|---|---|---|---|---|
| captan | Crop Care Captan 900WG | 90 | pthalimide | 161037 | September 2013 |
| carbendazim | Protek® | 50 | MBC | 7916 | 18/06/2013 |
| copper hydroxide | Champ™ DP | 37.5 | inorganic copper | unknown | Purchased 2014 |
| cyprodinil | Chorus® | 50 | AP | unknown | Purchased 2014 |
| difenoconazole | Score® 10WG | 10 | DMI | SMO4D0015 | 11/04/2014 |
| dithianon | Delan® WG | 70 | quinone | unknown | Purchased 2013 |
| dodine | Dodine 400 | 40 | guanidine | 8433 | 30/05/2014 |
| isopyrazam | Seguris Flexi® | 12.5 | SDHI | unknown | 27/03/2014 |
| lime sulphur | Yates Lime Sulfur | 20 | calcium polysulphides | 182505 | November 2013 |
| metiram | Polyram® DF | 70 | dithio-carbamate | unknown | Purchased 2014 |
| sulphur | Kumulus® DF | 80 | inorganic sulphur | L162299 | 30/05/2013 |
| trifloxystrobin | Protiva® | 50 | QoI | PROTIVA001SVT | 25/09/2012 |

[1] MBC = methyl benzimidazole carbamate, AP = anilinopyrimidine, DMI = DeMethylation Inhibitors, SDHI succinate dehydrogenase inhibitors and QoI = Quinone outside inhibitors are chemical groups.
[2] Where date of manufacture is unknown the purchase date is listed.

**Detached fruit assay**

'Sciros'/Pacific Rose™ fruit from an organic apple orchard in Hawke's Bay were bagged for 2 months prior to harvest to exclude orchard sprays and prevent *P. vagabunda* infection. These fruit were picked on 16 April 2015 when mature. Fruit were held at ambient temperature until inoculation. On 20 April 2015, 560 fruit were injured to a depth of 4-5mm (with a florist's 66 pin flower holder (each pin 1 mm dia.) following the method of Holmes (2011), Spotts (2009) and Tate (personal communication).

Immediately after injury, 40 fruit per treatment were dipped for 30 s in a 15 litre bucket containing one of the 12 fungicides at label rates (Table 2) or with water for the untreated controls. The adjuvant Du-Wett® was added at 0.5 mL per litre to all treatment dips except the water control and the dipped apples were agitated to ensure uniform coverage of the injured apple tissues. Treated fruit were then placed on fibreboard trays with the wounds upwards.

Two hours later, the air-dried apples were micro-spray inoculated (~0.5 mL of 1 x$10^5$ conidia/mL per apple injury site) using a mist bottle. The four *P. vagabunda* isolates (KE127, KE171, KE183 and KE892) were used as replicates. Conidial suspensions were prepared as described previously.

These fruit (10 per plot) were then placed at 20°C in sealed plastic bags to induce conditions of high humidity. After 3 weeks, any lesions that developed were assessed for typical bull's eye rot symptoms and measured. A small tissue sample was aseptically removed from the margins of lesions on 25 apples with bull's eye rot symptoms and placed on PDA to confirm visual diagnosis.

Data from all three experiments were analysed using GenStat Release 17.1.0.14713. Disease incidence (% fruit with bull's eye rot) and individual apple infection severity (% of the injured area that was rotten) were calculated for each plot. Percent *P. vagabunda* germination, bull's eye rot incidence and severity data were subjected to angular

**Table 2** Fungicidal treatments and rates used for the *Phlyctema vagabunda* assay on detached 'Sciros'/ Pacific Rose™ fruit.

| Active ingredient (ai) | Product | Percent ai | Product label rate per 100 litres | Product label rate ppm ai |
|---|---|---|---|---|
| captan | Crop Care Captan 900WG | 90 | 110 g | 990 |
| carbendazim | Protek® | 50 | 25 mL | 125 |
| copper hydroxide | Champ™ DP | 37.5 | 32 g | 120 |
| cyprodinil | Chorus® | 50 | 30 g | 150 |
| difenoconazole | Score® 10WG | 10 | 25 g | 25 |
| dithianon | Delan® WG | 70 | 18 g | 126 |
| dodine | Dodine 400 | 40 | 80 mL | 320 |
| isopyrazam | Seguris Flexi® | 12.5 | 80 mL | 100 |
| lime sulphur | Yates Lime Sulfur | 20 | 1000 mL | 2000 |
| metiram | Polyram® DF | 70 | 150 g | 1050 |
| sulphur | Kumulus® DF | 80 | 200 g | 1600 |
| trifloxystrobin | Protiva® | 50 | 10 mL | 50 |

Note; see Table 1 for product batch numbers and date of manufacture.

transformation. Analysis of Variance (ANOVA) and Fisher's Protected Least Significant Differences of Means (LSD, α=0.05) were used to determine statistical differences between treatments. The untransformed means are reported in the results.

## RESULTS

### Spore germination assay

Metiram, captan, dodine, dithianon, lime sulphur, carbendazim and isopyrazam were the most effective fungicides when tested at label rates, Table 3. Copper hydroxide also inhibited spore germination when used at 1000 ppm, but much less so at concentrations used by organic apple growers during the growing season. Treatment with sulphur at all rates resulted in a germination rate significantly higher than the untreated controls. At the lowest rate (1 ppm) copper hydroxide and dodine had similar percentage germination to sulphur; at higher rates (10+ ppm) germination was significantly lower than the untreated controls.

### Mycelial growth assay

Colony growth on the fungicide-amended plates showed that carbendazim, isopyrazam, difenoconazole and cyprodinil were the most effective fungicides when tested at, or near, label rates. Dodine, copper hydroxide and trifloxystrobin prevented mycelial growth when used at 1000 ppm, but not at label concentrations (Table 4).

Although all the effects and interactions were significantly larger than the three-way (isolate x treatment x rate) interaction, the main effects (isolate, treatment and rate) were by far the largest. Similarly the germination assay where the *P. vagabunda* isolate used formed the replicate, the difference in treatment response to different isolates was similar to the replicate to replicate (isolate) variability of the untreated control, i.e. all isolates followed similar patterns in their response to the treatments.

### Detached fruit assay

Three weeks after injured and treated apples were inoculated with one of four *P. vagabunda* isolates and incubated at 20°C under high humidity there were sufficient typical bull's eye rot symptoms at the time of assessment (Table 5). The most effective

**Table 3** Effect of different concentrations of 12 different fungicides at four different rates on the germination rate of *Phlyctema vagabunda* conidia on glass slides after 24 h at 20°C.

| Treatments[1] | Product label rates ppm ai | Germination rates of *Phlyctema vagabunda* conidia (%) | | | | |
|---|---|---|---|---|---|---|
| | | 0 ppm[2] | 1 ppm | 10 ppm[3] | 100 ppm | 1000 ppm |
| untreated | | 80 | | | | |
| captan | 990 | | 71 | 13 | 11 | **3** |
| carbendazim | 125 | | 52 | 53 | **12** | 7 |
| copper hydroxide | 120 | | 91 | 65 | **30** | 5 |
| cyprodinil | 150 | | 74 | 68 | **64** | 77 |
| difenoconazole | 25 | | 81 | **84** | 47 | 43 |
| dithianon | 126 | | 20 | 22 | **12** | 1 |
| dodine | 320 | | 91 | 16 | **10** | 2 |
| isopyrazam | 100 | | 53 | 25 | **14** | 13 |
| lime sulphur | 2000 | | 79 | 44 | 10 | **5** |
| metiram | 1050 | | 83 | 10 | 7 | **2** |
| sulphur | 1600 | | 91 | 92 | 92 | **91** |
| trifloxystrobin | 50 | | 68 | 72 | **61** | 37 |

[1] See Table 1 for full description of treatments.
[2] Germination (%) of *Phlyctema vagabunda* conidia on glass slides after 24 h at 20°C.
[3] *Phlyctema vagabunda* conidial germination values bolded and underlined to indicate closest fungicide rate tested for each fungicide label rate. (LSD = 6.5%, $\alpha = 0.05$).

**Table 4** Effect of different concentrations of 12 different fungicides at four different rates (fungicide amended agar) on the growth of *Phlyctema vagabunda* mycelial plugs on fungicide amended agar after 15 days at 20°C.

| Treatments[1] | Product label rates ppm ai | Diameter of *Phlyctema vagabunda* mycelial colonies (mm) | | | | |
|---|---|---|---|---|---|---|
| | | 0 ppm[2] | 1 ppm | 10 ppm[3] | 100 ppm | 1000 ppm |
| untreated | | 6.0 | | | | |
| captan | 990 | | 4.3 | 2.1 | 1.8 | **1.7** |
| carbendazim | 125 | | 0.0 | 0.0 | **0.0** | 0.0 |
| copper hydroxide | 120 | | 6.0 | 5.5 | **2.5** | 0.0 |
| cyprodinil | 150 | | 4.7 | 1.6 | **0.3** | 0.0 |
| difenoconazole | 25 | | 1.8 | **1.1** | 0.4 | 0.0 |
| dithianon | 126 | | 5.1 | 4.5 | **4.0** | 3.3 |
| dodine | 320 | | 4.9 | 4.1 | **2.0** | 0.0 |
| isopyrazam | 100 | | 2.0 | 0.3 | **0.0** | 0.0 |
| lime sulphur | 2000 | | 6.2 | 7.4 | 8.2 | **6.2** |
| metiram | 1050 | | 5.8 | 5.5 | 4.9 | **2.5** |
| sulphur | 1600 | | 5.8 | 6.3 | 6.3 | **5.1** |
| trifloxystrobin | 50 | | 2.8 | **2.6** | **2.1** | 0.4 |

[1] See Table 1 for full description of treatments.
[2] Growth of *Phlyctema vagabunda* mycelia plugs on fungicide amended agar after 15 days at 20°C.
[3] *Phlyctema vagabunda* growth bolded and underlined to indicate closest fungicide rate assayed for each fungicide label rate. (LSD = 1.4 mm, $\alpha = 0.05$).

**Table 5** Effect of different fungicide treatments applied as a 30 s dip to injured mature 'Sciros'/Pacific Rose™ fruit 2 h before inoculation of the wound sites on *Phlyctema vagabunda* incidence (expressed as mean percentage of apples with bull's eye rot) and bull's eye rot fruit severity (expressed as mean percentage of wound rotted by area) compared with the uninoculated untreated and inoculated untreated controls. bull's eye rot assessments were performed 3 weeks after inoculation on 20 April 2015.

| Treatment name (Product applied or control)[1] | Bull's eye rot incidence (% fruit infected) | | Bull's eye rot severity (% injured area rotted) | |
|---|---|---|---|---|
| uninoculated untreated | 0 | a | 0.0 | a |
| carbendazim | 0 | a | 0.0 | a |
| cyprodinil | 3 | ab | 0.1 | a |
| trifloxystrobin | 13 | abc | 1.2 | a |
| isopyrazam | 18 | bc | 2.7 | ab |
| dithianon | 45 | cd | 14.6 | bc |
| sulphur | 48 | de | 25.6 | cde |
| difenoconazole | 53 | de | 27.9 | cde |
| metiram | 53 | de | 27.4 | cde |
| dodine | 59 | de | 35.3 | de |
| inoculated untreated | 60 | de | 19.5 | cd |
| captan | 68 | de | 36.1 | de |
| copper hydroxide | 70 | de | 29.0 | cde |
| lime sulphur | 73 | e | 44.1 | e |

[1]See Tables 1 and 2 for full description of treatments
Within each column, means followed by the same letter are not significantly different (LSD, $\alpha = 0.05$).

fungicides on injured detached fruit compared with the untreated inoculated controls were carbendazim, cyprodinil, trifloxystrobin and isopyrazam. These fungicides have possible *P. vagabunda* curative activity based on the results of the mycelial growth tests. Protectant fungicides, such as metiram, captan and dithianon, did not significantly reduce lesion incidence or severity in this assay, despite performing well in spore germination assay. Wounding the skin before the fungicide dip should not have negated the mode of action of the protectant fungicides. This is because the super-wetter adjuvant Du-Wett® was added to all treatment dips and the dipped apples were agitated to ensure uniform coverage of the injured apple tissues.

## DISCUSSION

Twelve fungicides were tested at a number of different rates (1, 10, 100, 1000 ppm) on four isolates of *P. vagabunda*. The combination of these three factors resulted in a multitude of treatments so the four isolates were replicates in the analysis to keep the study manageable. Therefore, it was encouraging that the difference in treatment response to different isolates was similar to the replicate to replicate (isolate) variability of the untreated control. This result suggests that although the isolates had different germination percentages (Isolate main effect, $P<0.001$), they followed similar patterns in their response to the treatments. The treatments differed significantly in their average percent germination (Treatment main effect, $P<0.001$); within the treatments there was a general tendency for the percent

germination to drop as rate increased (Rate main effect, P<0.001), but there were also some differences in the patterns between treatments (Treatment x Rate interaction, P<0.001).

There is little literature clearly stating which fungicides available in New Zealand are recommended for the control of *P. vagabunda*. This lack of knowledge for bull's eye rot is further complicated by differences in fungicide response between the *Neofabraea* species (Spotts 2009) and historic difficulties in correctly identifying the causal organism of bull's eye rot. *Phlyctema vagabunda* rots were considerably reduced by applying captan in the orchard in July, August and September for two seasons, and in August and September for one season in England (Moore & Edney 1959). This equates to applications in January, February and March in New Zealand. Captan is the preferred pre-harvest bulls eye rot fungicide for the New Zealand apple industry due to its relatively short (14-day) pre-harvest interval (Ministry for Primary Industries 2016).

Slides amended with captan, dodine, metiram and dithianon had low germination, but did not inhibit growth as much as some other fungicides, whereas difenoconazole, cyprodinil and trifloxystrobin all inhibited growth well, but did not reduce germination. Results from the fungicide-amended slide spore germination assay indicated that metiram, captan, dodine, dithianon, lime sulphur, carbendazim and isopyrazam (in order of effectiveness) were the most effective fungicides when tested at label rates. For organic products, results showed that sulphur was ineffective against *P. vagabunda*. Copper hydroxide inhibited spore germination when used at 1000 ppm, but not at concentrations used by commercial apple growers in season so it appears that lime sulphur is their best choice of fungicide from those tested here. The best in-vitro combined performers were isopyrazam and carbendazim, which resulted in low growth and low germination of *P. vagabunda*. Use of captan, dodine, metiram or dithianon led to low germination of *P. vagabunda*, but did not inhibit growth as much as other fungicides tested. Difenoconazole, cyprodinil and trifloxystrobin all inhibited *P. vagabunda* growth well, but did not reduce germination as much as other fungicides tested. The most effective fungicides (in order of efficacy) on injured detached fruit compared with the untreated inoculated controls were carbendazim, cyprodinil, trifloxystrobin and isopyrazam however they have long pre-harvest intervals (PHIs) of 80 d PHI, petal fall, 70 d PHI and petal fall respectively.

The research team is now focusing on determining *P. vagabunda* sensitivity baselines for key 'at-risk of resistance' fungicide groups and the efficacy of novel nil-residue treatments for application near and during harvest.

## ACKNOWLEDGEMENTS

Thanks to Dr Kerry Everett for supply of the *Phlyctema vagabunda* isolates. We thank Dr Duncan Hedderley for the statistical analyses. Thanks to Dr Kerry Everett, Dr Reiny Scheper and Tim Herman for comments on the draft manuscript. We also thank Catherine Langford and Dr Suvi Viljanen for their valuable editorial suggestions. This research was funded by Pipfruit New Zealand and MBIE Partnership Fund (Apple Futures II). The use of trade names in this paper does not imply endorsement of products named.

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