Fungicide control of *Gnomoniopsis smithogilvyi*, causal agent of chestnut rot in Australia

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Abstract
Chestnut rot caused by *Gnomoniopsis smithogilvyi* is considered a severe threat to the production of chestnuts from European chestnut (*Castanea sativa* Mill.) in Australia and overseas. Currently, most of the control strategies are applied post-harvest and little is known about the use of fungicides to reduce nut infection in orchards early in the season. This research evaluated the effectiveness of various fungicides against the pathogen in vitro and selected the most effective products for field trials. In vitro experiments showed that pyraclostrobin and difenoconazole-based fungicides effectively inhibited conidial germination and mycelial growth, respectively. The field trial showed that both active ingredients combined were more effective than single applications in suppressing the level of nut infection caused by *G. smithogilvyi*. Based on our results, using the selected fungicides can be an additional tool for growers to complement their current practices in the control of chestnut rot. To the best of our knowledge, this is the first study focused on the effectiveness of chemical treatments against *G. smithogilvyi*.

Keywords Disease management · Chestnut rot · Fungicide · Difenoconazole · Pyraclostrobin

Introduction
Sweet chestnut (*Castanea sativa* Mill.) is a species native to Europe and introduced by early European settlers to the Americas and Oceania (Casey and Casey 2009). In Australia, the majority of commercial chestnut orchards are located in the states of Victoria and New South Wales. More than 90% of the local chestnut production is destined to satisfy domestic demand (Horticulture Innovation Australian 2020). While most chestnut farms are profitable, their productivity is compromised by a significant deterioration of edible nut quality due to the disease, chestnut rot, caused by the fungal pathogen *Gnomoniopsis smithogilvyi* L.A. Shuttleworth, E.C.Y. Liew & D.I. Guest. There are limited options for control of the disease and currently there is no fungicides registered for its specific control in Australia.

The ascomycete *G. smithogilvyi* (syn. *Gnomoniopsis castanea*) (Gnomoniaceae, Diaporthales) was identified as the principal causal agent of chestnut rot in Australia (Shuttleworth et al. 2012) and Italy (Visentin et al. 2012). In Australia, the local industry considers this pathogen one of the main threats to maintaining a sustainable supply of fresh chestnuts to the domestic market (Horticulture Innovation Australian 2016). An exhaustive survey to determine the incidence of chestnut rot in Australian orchards found up to 70% of diseased chestnuts associated to *G. smithogilvyi* pre-harvest (Shuttleworth et al. 2013). But it is not only Australian chestnut production that is threatened by this pathogen. In Italy, for instance, studies carried out in orchards located northwest of the country found the pathogen infecting up to 93.5% of ripe nuts pre-harvest (Lione et al. 2015). Similarly, in Switzerland, some studies have reported the pathogen to be present in up to 91% of chestnuts pre-harvest (Dennert et al. 2015). Hence, *G. smithogilvyi* represents an emerging challenge to the global chestnut industry (Lione et al. 2019), and its control warrants the study of approaches to mitigate its impact.

Shuttleworth and Guest (2017) proposed that the cycle of chestnut infection begins when the *G. smithogilvyi* teleomorph releases ascospores that then infect female flowers to
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initiate the infection process. Similarly, Visentin et al. (2012) showed that chestnut flowers can be effectively infected by artificial inoculation with conidia. Following infection, symptoms of chestnut rot emerge mainly postharvest while chestnuts are in storage or displayed on shop shelves. However, rotten chestnuts can also be found while still attached to the trees (Maresi et al. 2013). Due to the severe deterioration of chestnut quality post-harvest, most strategies to manage chestnut rot have focused on controlling G. smithogilvyi post-harvest.

Some of the measures commonly used by growers post-harvest to control this pest and other fungal diseases are the sanitation of chestnuts with disinfectant and authorised fungicides during the grading and selection of nuts. Other alternatives, such as that reported by Ruocco et al. (2016), that have shown a significant reduction in the number of diseased chestnuts in storage after being treated with Trichoderma harzianum enzymatic derivatives during a hot water treatment. More recently, Vettraino et al. (2019) showed that gaseous ozone significantly reduced the growth of G. smithogilvyi colonies in vitro, suggesting that applying this gas to chestnuts post-harvest could help extend their shelf life. Nevertheless, all these strategies are palliative solutions as they do not control the disease at its origin, the orchard.

The use of fungicides in commercial orchards remains an essential tool in the integrated management of plant diseases. However, little is known about their effectiveness in controlling G. smithogilvyi in productive chestnut plantations. In Australia particularly, most of the information on fungicide use comes from ad hoc trials performed by growers and assays carried out by chestnut producer’s associations. Recently, a technical report showed that a series of fungicide active ingredients, including difenoconazole, cyprodinil, fludioxonil, and pyraclostrobin, effectively controlled C. cassiicola and B. cinerea in vitro (Chestnuts Australia 2018). Such fungicides have also shown effective control of other fungal pathogens of fruit crops, including Botrytis cinerea and Penicillium expansum in kiwifruit and Corynespora cassiicola and Asperisporium caricae in papaya (Vawdrey et al. 2008; Thomidis and Prodromou 2018). However, using these or other fungicides as foliar applications to control G. smithogilvyi in chestnut orchards has not yet been reported. Indeed, there is a lack of adequately designed and performed experimental trials that have evaluated the efficacy of fungicides for the control of chestnut rot. As a result, the Australian chestnut industry has no effective alternatives for managing this disease in the orchard. Moreover, although a limited number of fungicides are used in local chestnut cultivation, none of them is registered for the specific control of G. smithogilvyi (Australian Pesticides and Veterinary Medicines Authority 2021).

Therefore, the main aims of the current study were: (1) to determine the effectiveness of several fungicides that have differing modes of action for the control in vitro of G. smithogilvyi conidial germination and mycelial growth and (2) to evaluate the effectiveness of the selected fungicides or their mixture in reducing infection of artificially inoculated chestnuts in the field.

Materials and methods

G. smithogilvyi isolation, identification, and culture maintenance

Gnomoniopsis smithogilvyi isolates F1N1 and B15 were recovered from symptomatic chestnuts from two geographically distant chestnut orchards located near the towns of Fumina and Bright respectively in the state of Victoria, Australia. Isolation of the pathogen from chestnuts was performed by plating tissues onto potato dextrose agar (PDA, Difco™, New Jersey, USA), and G. smithogilvyi-like colonies were subjected to DNA extraction using a commercial kit (DNeasy Plant Minikit, Qiagen) following the manufacturer instructions. Amplification of the ITS region was carried out with primers ITS-5 (forward) and ITS-4 (reverse) according to White et al. (1990). Amplicons were Sanger-sequenced by the Australian Genome Research Facility (Melbourne, Victoria, Australia). Sequences were compared to the NCBI-GenBank database using BLASTn and there was >99.0% homology to G. smithogilvyi (accession numbers MK554867.1 and MK554866.1). For analysis of the effects of the various fungicides, or their combination, on mycelial growth isolates were grown on PDA in 9-cm-indiameter Petri plates and incubated at 23 °C in the dark. For examining the effect of the fungicides on conidia germination isolates were grown and induced to sporulate on cornmeal agar (CMA, Difco™) in 3.5 cm Petri plates at room temperature with a natural light/dark cycle. Conidia were then harvested as described below for use in the assays.

Preparation of fungicide-amended media

Six commercial fungicide formulations with different active ingredients and modes of action were evaluated in vitro to determine whether they inhibited mycelial growth and conidial germination of the G. smithogilvyi isolates (Table 1). The fungicides were filter-sterilised using 0.22 μm syringe filters (Millipore™, Massachusetts, USA), and an amount was mixed with sterile PDA at 55 °C to prepare the required concentration of the active ingredient to be evaluated.
Effect of fungicide on conidial germination

Conidia of each isolate were harvested from two ten-day-old colonies grown on CMA by adding 5 mL of sterile water to one plate and then scraping conidia into the water using a sterile microscope slide, the liquid containing conidia was then removed by pipette and dispensed into the secondary Petri plate. Following further agitation of the plate using the same microscope slide, the resulting conidial suspension was removed by pipette and was dispensed into a 5 mL pipette tip containing a folded sterile miracloth (Merck, USA). Conidial suspension was force-filtered by centrifugation for 2 min at 5000 rpm (Eppendorf™ 5804R, Hamburg, Germany) into a 50 mL conical tube. The conidial concentration was then determined using a hemocytometer (Hirschmann, UE) and the suspension then adjusted to $1.3 \times 10^6$ conidia/mL. The conidial suspension (10 µL) of each isolate was pipetted onto two square (5 mm x 5 mm), sterile cellophane membranes that were in place on the amended agar medium within the Petri plates and then incubated at 23 °C in the dark. After 19 h of incubation, the cellophane squares with conidia were stained with 5 µL of 0.4% trypan blue per
square and observed under a microscope (Zeiss Axioscope M2, Zeiss, Oberkochen, Germany). Conidia were considered to have germinated when the germ tube had reached twice the size of their long axis. The percentage of conidia that had germinated was calculated by randomly counting one hundred conidia per cellophane membrane. Conidial germination inhibition (CGI) for each fungicide and for each concentration tested was then calculated using the formula: 

\[ \text{CGI} (\%) = \frac{(G_{\text{control}} - G_{\text{treatment}})}{G_{\text{control}}} \times 100, \]

where \( G_{\text{control}} \) is the percentage of germination in the control treatment and \( G_{\text{treatment}} \) is the percentage of germination in fungicide treatments. Each combination of isolate and fungicide concentration was performed in triplicate, and the experiment was repeated three times.

**Effect of fungicide on mycelial growth and EC\textsubscript{50} determination**

To examine the effect of each fungicide, or their combination, on mycelial growth in vitro a 0.6 cm-in-diameter plug taken from a five-day-old colony of either *G. smithogilvyi* F1N1 or B15 isolates was placed on the centre of a fungicide-amended PDA plate. Plates without the addition of fungicide served as the control. The plates were then incubated at 23 °C in the dark for six days. Three measurements of radial growth were taken perpendicularly to each other from the edge of the plug to the edge of the colony. Each combination of isolate, fungicide type and fungicide concentration was performed in triplicate, and the experiment was repeated three times. The percentage of mycelial growth inhibition (MGI) for each fungicide and each concentration was calculated using the formula: 

\[ \text{MGI} (\%) = \frac{(MG_{\text{control}} - MG_{\text{treatment}})}{MG_{\text{control}}} \times 100, \]

where \( MG \) is the mean mycelial radial growth (mm) in control and fungicide treatment, respectively. The half-maximum effective concentration (EC\textsubscript{50}) for each fungicide was determined by comparing the mycelial growth inhibition of the isolates against the Log-transformed fungicide concentration.

**Evaluation of fungicides under field conditions**

**Field trial location and experimental design**

Based on the results for the effect of each fungicide tested on mycelial growth and conidial germination in vitro, two fungicides, pyraclostrobin and difenoconazole, were selected for field evaluation. Field trial studies were performed in a privately owned chestnut orchard at Fumina, Victoria, Australia (coordinates 37°54′38.3″S; 146°06′56.9″E) during the growing season from December 2020 to March 2021. Twelve four-year-old chestnut trees cultivar ‘Premium Delight’ were evaluated using a completely randomised block design. Each block contained four trees assigned randomly to each treatment: water control, pyraclostrobin, difenoconazole and treatment with a combination of both pyraclostrobin and difenoconazole.

**Fungicide application, flower inoculation and sample collection**

Before fungicide application and at the start of the season, fifty chestnut flowers per tree were randomly selected and tagged to enable further identification. Fungicides and water control treatments were then applied using a battery-powered backpack sprayer (AEG, Frankfurt, Germany) equipped with a flat-spray nozzle at a rate of 1.4 L/min. Trees were sprayed early in the morning around the canopy until run-off. Twenty-four hours after fungicide treatment the tagged flowers were each inoculated with 10 µL of a conidial suspension (2.0 × 10⁶ conidia/mL) of isolate F1N1, which was isolated from diseased chestnut tissues from the same property. Fungicide application was repeated at 30 and 60 days after inoculation at the concentrations shown in Table 1. Ten tagged flowers or developing burrs per treated tree were collected randomly and just prior to spraying the trees with fungicide at 30 and 60 days post inoculation (dpi) to examine the level of chestnut infection. At 90 and 110 dpi the remaining burrs were collected directly from the treated trees before mature nuts were shed. Samples were placed in transparent polyethylene resealable bags, transported in portable coolers and maintained at 4 °C (+/-2 °C) until processing in the laboratory.

**Assessment of infection level**

Twenty chestnuts were randomly selected from the collected burrs to quantify the infection level. Chestnuts were surface sterilised in 85% ethanol (1 min), rinsed in sterile water (30 s), followed by 2.5% NaOCl (5 min), rinsed twice with sterile water (1 min/each), and dried within a laminar flow cabinet. Samples were then sectioned with a sterile scalpel into three separate tissue types, namely stigma, style and nut. Whole stigmas, styles and nuts were placed onto PDA and incubated at 23 °C for 5 days in the dark. Colonies of *G. smithogilvyi* isolate F1N1 that grown from each tissue were morphologically confirmed and twenty colonies were subjected to DNA extraction for further identification with a specific multiplex-PCR assay (unpublished data). The level of chestnut infection following each treatment at each time point was calculated by using a scoring scale from 0 to 100% where each *Gnomoniopsis*-infected tissue (stigma, style or nut) contributed 33.33% to the total. On the other hand, the percentage of infection for each tissue type was calculated.
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strongly inhibited across the broad range of concentrations tested. Although pyraclostrobin effectiveness was slightly less at a concentration of $1 \times 10^{-2}$ µg/mL, suppression of germination remained very high. No statistical difference ($P > 0.05$) in fungicide susceptibility was observed between isolates B15 and F1N1. Difenoconazole was also effective in suppressing conidial germination (Fig. 1b). The inhibitory effect of difenoconazole at $1.25 \times 10^{2}$ µg/mL on both isolates was $>90\%$ with no statistical differences ($P > 0.05$) in susceptibility of the two isolates. However, isolate F1N1 was significantly more tolerant than B15 to difenoconazole at concentrations between $1.25 \times 10^{-2}$ µg/mL and $1.25 \times 10^{1}$ µg/mL. Similarly, isolate F1N1 was significantly ($P > 0.05$) more tolerant than B15 to prochloraz, fludioxonil and cyprodinil + fludioxonil. Iprodione was the least effective fungicide against both isolates (Fig. 1e). Suppression of conidial germination of both isolates was $\leq 25\%$ with iprodione at the highest concentration tested of $2.5 \times 10^{2}$ µg/mL. However, F1N1 was significantly more tolerant than B15 to this concentration of iprodione.

**Results**

**Effect of fungicides on conidial germination**

The six fungicides examined differed in their effectiveness to inhibit *G. smithogilvyi* conidial germination (Fig. 1a-f). Pyraclostrobin was the most effective fungicide at all concentrations tested (Figs. 1a and 2) and germination was as the proportion of a specific infected tissue relative to the total of the three tissues analysed under the same treatment.

**Statistical analysis**

Data for mycelial growth, conidial germination, level of infection and percentage of infection per tissue type were arcsine $\sqrt{(y/100)}$ transformed to fit normality before analysis by two-way ANOVA. Fisher's least significant difference (LSD) test was used to determine significant differences at a 5% of significance level ($\alpha = 0.05$). Data is presented untransformed for clarity. The EC$_{50}$ for each fungicide was determined by fitting a sigmoidal dose-response curve of the relative mycelium growth inhibition (%) against the Log-transformed fungicide concentration. Statistical analysis and graphical representation were performed with a commercial software package (GraphPad Prism 8, version 8.0.0 for Windows, GraphPad Software, San Diego, California, USA).

**Effect of fungicides on mycelial growth**

The mean EC$_{50}$ of each fungicide against isolates B15 and F1N1 is presented in Table 2. The EC$_{50}$ for this group of fungicides ranged from 0.018 µg/mL for difenoconazole against B15 to 12.04 µg/mL for the combination of cyprodinil + fludioxonil against both isolates. All six fungicides suppressed the mycelial growth of both isolates by 100%...
growth. This fungicide suppressed growth by 100% in the range of $1.25 \times 10^{-1}$ and $1.25 \times 10^2 \mu g/mL$ (Figs. 3b and 4). Regarding susceptibility, isolate F1N1 was significantly more tolerant than B15 to difenoconazole only at $1.25 \times 10^{-3} \mu g/mL$. Fludioxonil was the second most effective fungicide, which inhibited the growth of both isolates more than 80% from $2.99 \times 10^{-1} \mu g/mL$ onwards (Fig. 3d). A significant difference between isolate susceptibility to fludioxonil was observed only at $2.99 \times 10^0 \mu g/mL$. Pyraclostrobin was significantly more effective in inhibiting mycelial growth of isolate B15 than isolate F1N1 at concentrations between $1.0 \times 10^{-2}$ and $1.0 \times 10^1 \mu g/mL$. Prochloraz effectiveness against both isolates was statistically similar at all concentrations, and the fungicide iprodione differed in terms of effectiveness only at $2.5 \times 10^{-2} \mu g/mL$. The fungicide composed of the active ingredients cyprodinil + fludioxonil was the least effective product evaluated (Fig. 3f). Its effectiveness was reduced to only 15% at $3.0 \times 10^0 \mu g/mL$. The susceptibility of both isolates to cyprodinil + fludioxonil was statistically similar for the concentrations tested.

Table 2 The mean of EC$_{50}$ (µg/mL) for mycelial growth inhibition of G. smithogilvyi isolates

| Isolate | Pyraclostrobin | Prochloraz | Iprodione | Fludioxonil | Difenoconazole | Cyprodinil + Fludioxonil |
|---------|----------------|------------|-----------|-------------|----------------|-------------------------|
| F1N1    | 1.276 (0.710–2.394) | 1.815 (1.381–2.378) | 3.335 (2.585–4.312) | 0.076 (0.063–0.092) | 0.020 (0.016–0.025) | 12.040 (9.460–15.290) |
| B15     | 0.469 (0.278–0.739) | 1.455 (1.104–1.915) | 3.150 (2.395–4.154) | 0.081 (0.058–0.113) | 0.018 (0.015–0.022) | 12.040 (8.785–16.460) |

*Values in brackets correspond to the 95% confident intervals of the EC$_{50}$ mean

Fig. 2 Effect of the lowest two concentrations tested of pyraclostrobin on conidial germination of isolates B15 and F1N1. Conidia were induced to germinate on cellophane membranes laid on PDA amended plates in the dark at 23 °C for 19 h. Scale bar = 20 µm

at the highest concentration tested (Fig. 3a-f). Difenoconazole was the most effective fungicide in inhibiting mycelial growth. This fungicide suppressed growth by 100% in the range of $1.25 \times 10^{-1}$ and $1.25 \times 10^2 \mu g/mL$ (Figs. 3b and 4). Regarding susceptibility, isolate F1N1 was significantly more tolerant than B15 to difenoconazole only at $1.25 \times 10^{-3} \mu g/mL$. Fludioxonil was the second most effective fungicide, which inhibited the growth of both isolates more than 80% from $2.99 \times 10^{-1} \mu g/mL$ onwards (Fig. 3d). A significant difference between isolate susceptibility to fludioxonil was observed only at $2.99 \times 10^0 \mu g/mL$. Pyraclostrobin was significantly more effective in inhibiting mycelial growth of isolate B15 than isolate F1N1 at concentrations between $1.0 \times 10^{-2}$ and $1.0 \times 10^1 \mu g/mL$. Prochloraz effectiveness against both isolates was statistically similar at all concentrations, and the fungicide iprodione differed in terms of effectiveness only at $2.5 \times 10^{-2} \mu g/mL$. The fungicide composed of the active ingredients cyprodinil + fludioxonil was the least effective product evaluated (Fig. 3f). Its effectiveness was reduced to only 15% at $3.0 \times 10^0 \mu g/mL$. The susceptibility of both isolates to cyprodinil + fludioxonil was statistically similar for the concentrations tested.

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Fungicide control of *Gnomoniopsis smithogilvyi*, causal agent of chestnut rot in Australia

The best two performing fungicides in vitro, pyraclostrobin (PYR) and difenoconazole (DIF) and their combination pyraclostrobin + difenoconazole (PYR + DIF), were tested under field conditions for control of chestnut infection after female flowers were artificially inoculated (Fig. 5 and Supplementary Table 1). At 30 dpi results showed that the first fungicide application (A1 in Fig. 5a) carried out 24 h before inoculation did not preclude the initial infection of chestnuts. As a result, the level of infection following each fungicidal treatment was not statistically different to the control \((P>0.05)\) at 30 dpi. However, fungicide treatment showed a tendency to reduce the level of chestnut infection, where treatment PYR + DIF showed the lowest level of infection. The results at 60 dpi showed the effect of the second fungicide application (A2), which was carried out at 30 dpi. At this time point PYR + DIF was the most effective treatment in reducing chestnut infection down to 23.3% compared to 65.5% for the control treatment \((P<0.05)\). On the other hand, levels of infection following treatment with PYR and DIF alone did not differ statistically from the control \((P>0.05)\) at 30 dpi. Similarly, the following two applications of DIF (A2 and A3) lowered the percentage of infection observed at 60 dpi and 90 dpi. On the other hand, the first applications of PYR and PYR + DIF were not effective in reducing the percentage of chestnut infection observed at 30 dpi compared to the control. However, treatment PYR + DIF showed the first effect on the percentage of infection at 60 dpi and remained effective at 90 dpi and 110 dpi. Following the third application (A3) performed at 60 dpi reduced significantly \((P<0.05)\) mean nut infection observed at 90 dpi in all fungicide-treated chestnuts compared to the control. Chestnuts treated with PYR + DIF had the lowest level of infection (24.9%), followed by DIF (34.4%) and PYR (41.6%), whereas the level of infection in the control treatment was 61.1% at this time point. The mean level of chestnut infection increased dramatically for the three fungicidal treatments at 110 dpi (50 days after A3). PYR and DIF alone recorded 67.8% and 56.7%, respectively, whereas the level of infection following fungicide treatment PYR + DIF reached 41.6%. None of the treatments differed statistically \((P>0.05)\) from the control (70.5%).

**Effect of selected fungicides on the percentage of tissue infection**

The percentage of infection per tissue was quantified by calculating the proportion of each infected tissue type (stigmas, styles and nuts) relative to the total number of tissues analysed (Fig. 6a and Supplementary Table 2). Tissues were considered infected when *G. smithogilvyi* isolate F1N1 was recovered on PDA after five days of incubation at 23°C.

**Percentage of infected stigmas**

Fungicide treatments had a significant \((P<0.05)\) effect on the percentage of infected stigmas (Fig. 6b). The first application (A1) of DIF reduced the percentage of infected stigmas observed at 30 dpi. Similarly, the following two applications of DIF (A2 and A3) lowered the percentage of infection observed at 60 dpi and 90 dpi. On the other hand, the first applications of PYR and PYR + DIF were not effective in reducing the percentage of infection observed at 30 dpi compared to the control. However, treatment with PYR + DIF showed the first effect on the percentage of infection at 60 dpi after the second application (A2) and remained effective at 90 dpi and 110 dpi. Following the third application (A3) of PYR a reduced percentage of infection was observed at 90 dpi. However, the effect of PYR was negligible at 110 dpi.

**Percentage of infected styles**

The percentage of infected styles was noticeably higher than the stigmas and nuts across the experiment (Fig. 6c).
The first application of PYR + DIF was the only effective treatment in reducing significantly \((P<0.05)\) style infections observed at 30 dpi. Although none of the treatments was statistically different to the control at 60 dpi, 90 dpi and 110 dpi, the mean infection under treatment with PYR + DIF tended to be lower than the other treatments at these time points.

**Percentage of infected nuts**

The infection of nuts was consistently lower than the other two tissues. However, the mean infection percentage of nuts showed substantial variation across the experiment (Fig. 4d). The first application of DIF (A1) suppressed significantly nut infection observed at 30 dpi. However, the effectiveness of this fungicide in the following applications (A2 and A3) became negligible at 60 dpi, 90 dpi and 110 dpi. Applications of PYR did not significantly suppress nut infection at all time points evaluated. On the other hand, after the second application of PYR + DIF there was a significant reduction \((P<0.05)\) in infection compared with the other treatments and the control at 60 dpi.

**Effectiveness of fungicides over time**

The effectiveness of fungicides over time was analysed by comparing the percentage of infection at 30 dpi for each treatment with the percentage of infection of the same treatment at 60 dpi 90 dpi and 110 dpi. Firstly, it was evident that the infection rate for the stigmas increased markedly from...
Discussion

This research investigated the effectiveness of six fungicides for inhibition of growth of *G. smithogilvyi* in vitro. We then tested the two most effective in suppression of the pathogen under field conditions. We found that pyraclostrobin and difenoconazole effectively inhibited conidal germination and mycelial growth in vitro, respectively. Under field conditions, our results showed that the combination of pyraclostrobin (100 µg/mL) with difenoconazole...
(125 µg/mL) outperformed both fungicides when applied individually in suppressing the level of chestnut infection up to 90 days after inoculation. In addition, the mixture of both fungicides tended to reduce the proportion of infected stigmas, styles and nuts. The use of these two fungicides, especially in combination, should be considered for control of G. smithogilvyi in Australia.

Of particular interest for the in vitro studies was the significant difference found between the two tested G. smithogilvyi isolates, in terms of their susceptibility to several of the fungicides tested. In our experiments the isolate B15 was significantly more susceptible to the fungicides than was FIN1. Differences in the response of different isolates of a fungal pathogen to fungicides is well recognised (Massi et al. 2021; Gleason et al. 2021) and needs to be considered when decisions about which fungicide is used for effective pathogen control.

We found that the effectiveness of the tested fungicides in suppressing conidial germination in vitro varied greatly. Nevertheless, our results showed that pyraclostrobin was strongly effective at inhibiting conidial germination at all concentrations tested. Bartlett et al. (2002) suggested that the effectiveness of respiratory chain inhibitors such as pyraclostrobin is due to their role in blocking the energy supply during conidial germination. The superior effectiveness of pyraclostrobin over other active ingredients in suppressing conidial germination in vitro has also been shown with other pathogenic ascomycetes including Colletotrichum acutatum (chilli anthracnose) and C. fructicola (apple leaf spot) (Gao et al. 2017; Jiang et al. 2021). All fungicides tested showed strong inhibition of mycelial growth at the higher concentrations evaluated in vitro. Nevertheless, our results showed that difenoconazole outperformed the other fungicides as it inhibited mycelial growth even at a relatively low concentration. The remarkable effectiveness of difenoconazole in inhibiting mycelial growth may be explained by its role in blocking the biosynthesis of ergosterol, one of the most abundant sterols in the fungal cell membrane (Douglas and Konopka 2014; Rodrigues 2018). Difenoconazole is a demethylation inhibitor that acts on the enzyme C14-demethylase which converts lanosterol into ergosterol. Disruption of this enzymatic process leads to cell death due to the accumulation of toxic methylsterols (ergosterol precursors) and a shortage of ergosterols essential to maintain fungal cell membrane integrity (Ghannoun and Rice 1999; Klink et al. 2021). The effectiveness of difenoconazole in inhibiting mycelial growth has also been shown in other plant-pathogen systems. For example, Dai et al. (2017) found that difenoconazole was significantly superior to iprodione and prochloraz that were also used in our study, for inhibition of mycelial growth in vitro of three Botryosphaeria dothidea isolates, the causal agent of trunk canker in Chinese hickory (Carya cathayensis). Similarly, mycelial growth in vitro of Colletotrichum godetiae and C. nymphaeae that are linked to olive anthracnose and C. gloeosporioides that is responsible for walnut anthracnose were significantly suppressed by difenoconazole (Moral et al. 2018; Wang et al. 2020). Although the effectiveness of pyraclostrobin and difenoconazole applied alone was only significant at 90 dpi (that is after three applications) in field conditions, they tended to suppress the level of infection across the experiment. Both fungicides have shown significant effects in controlling other ascomycetes under Australian field conditions. For example, pyraclostrobin and difenoconazole significantly reduced the incidence of husk spot disease in macadamia caused by Pseudocercospora macadamia (Akinsanmi et al. 2008). Similarly, the use of pyraclostrobin and difenoconazole reduced significantly the severity of the disease caused by Corynespora cassiicola (brown spot) and Asperisporium caricae (black spot) in papaya (Vawdrey et al. 2008). It was notable that by 110 dpi that infection tended to increase and was found to be not significantly different to the control, however there was a clear trend that mirrored the suppression of infection found at the earlier time points. This level of infection at 110 dpi is likely due to the reduced efficacy of the applied fungicides and may indicate that a further application of fungicides is required at 90 dpi. In contrast to the efficacy of pyraclostrobin and difenoconazole applied alone, their combination provided significant disease suppression under field conditions. Our study showed that two applications of pyraclostrobin + difenoconazole reduced significantly the level of nut infection 30 days earlier than pyraclostrobin and difenoconazole applied individually. This suggests that the mode of action of both fungicides could act synergistically. More importantly, the early control activity as shown by this fungicide combination offers growers an alternative for the early management of chestnut rot onset. The effectiveness of the mixture pyraclostrobin + difenoconazole has also been shown in other plant-pathogen systems. For example, Shi et al. (2021) reported that the mixture of both fungicides was significantly better than when used individually in reducing anthracnose incidence caused by Colletotrichum scovillei and C. fructicola in chilli under greenhouse conditions. Nevertheless, this fungicide mixture should be used judiciously and in rotation with other fungicides to avoid inducing resistance in G. smithogilvyi or other fungal populations. In this regard, further studies should be carried out to evaluate single or combined fungicide applications and thus provide growers with a more complete program for the control of chestnut rot.

We have shown that under the fungicide application regime used in the field trial, fungicides arrested the development of the pathogen within chestnut tissues rather than
eradicated it. This was clear after the consistent re-isolation of *G. smithogilvyi*, even at 110 dpi. Moreover, we found that the percentage of infection was notoriously higher in the styles than the other two tissues, suggesting that the styles might be the primary pathogen reservoir. The continuous presence of the pathogen in the styles very likely resulted in the pathogen breaking out into stigmatic and nut tissues by 110 dpi and therefore accounting for the higher level of overall infection. Therefore, further studies are warranted to determine the role of the styles in pathogen persistence and their role in chestnut rot and perhaps establish strategies that include ways that the styles could be separately treated or even removed before chestnuts are stored.

Individual fungicides showed differences in their effect on the rate of infection in different nut tissues. Based on our observations, pyraclostrobin and difenoconazole were consistently more effective at reducing infection of the stigmas than of styles and nuts. The stigmas are continuously exposed to the external environment, which facilitates their contact with the fungicides, whereas styles and nuts are rapidly enclosed by the burr (30 dpi in our experiment). The physical barrier of the burrs suggests that a critical time for fungicide application to hamper *G. smithogilvyi* infection is before the burr encloses the styles and nuts. However, further studies should be performed to clarify this and determine if fungicide applications delivered between flower anthesis, and burr enclosure reduce nut infection and thus chestnut rot.

In conclusion, we have reported the effectiveness of fungicides in controlling *G. smithogilvyi* under both in vitro and field conditions. We showed that pyraclostrobin and difenoconazole were the most effective fungicides of those tested for controlling conidial germination and mycelial growth, respectively. Moreover, the mixture of pyraclostrobin and difenoconazole was shown to be more effective than single fungicide applications for the control of chestnut infection in the field. Our research is complementary to the majority of studies that have focused on the control of *G. smithogilvyi* post-harvest, thus providing growers an additional tool for the effective management of chestnut rot. However, further studies should be performed to evaluate the effectiveness of other active ingredients, including those with multi-site activity in single or combined applications. This approach provides growers sufficient fungicide alternatives to rotate applications during the season, thus reducing the risk of inducing resistance of *G. smithogilvyi* or other fungal species. Additionally, further research should also focus on defining an appropriate application regime for fungicides that consider burr development and the persistence of *G. smithogilvyi* in the tissues analysed in this study. Finally, it has been suggested (Lione et al. 2021) that within an integrated disease management programme, an appropriate application regime should also consider both climatic factors and the availability throughout the year of *G. smithogilvyi* inoculum.

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**Data Availability** The datasets generated during and/or analysed during the current study are not publicly available due to commercial confidentiality but are available from the corresponding author on reasonable request.

**Declarations**

**Conflict of interest** The authors declare no conflict of interest.

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