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Evaluation of fungicides for management of *Botryosphaeriaceae* associated with dieback in Australian walnut orchards

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Summary. Dieback of fruiting spurs, stems and branches of walnut trees (*Juglans regia* L.), caused by *Botryosphaeriaceae*, is widespread in walnut orchards in Australia. Five species of *Botryosphaeriaceae* (*Diplodia seriata*, *Dothiorella omnivora*, *Neofusicoccum macroclavatum*, *N. parvum*, and *Spencermartinsia viticola*) were recovered from the Australian walnut orchards in a previous study, with *D. seriata* and *N. parvum* being the most prevalent. The present study evaluated inhibitory effects of ten fungicides on mycelium growth of those five species and on conidium germination of *D. seriata* and *N. parvum*. It investigated the preventative and curative efficacy of selected fungicides on disease incidence in glasshouse and field trials. *In vitro* experiments showed that nine of the fungicides reduced mycelium growth, and all ten inhibited conidium germination, but to varying extents. Tebuconazole, prochloraz manganese chloride, fluazinam, fludioxonil and pyraclostrobin were the most effective for inhibiting mycelium growth (EC_{50} < 0.14 µg a.i. mL^{-1}), whereas pyraclostrobin, fluxapyroxad, fluopyram, penthiopyrad and tebuconazole were the most effective for inhibiting conidium germination (EC_{50} < 2.2 µg a.i. mL^{-1}). *In planta* experiments with five fungicides confirmed that preventative treatments had greater efficacy than curative treatments. A field trial with four commercial fungicide formulations demonstrated that tebuconazole and tebuconazole + fluopyram provided protection of walnut trees for the longest period. The field trial also confirmed the efficacy of pyraclostrobin and the inhibitory effect of fluazinam. This study is the first in Australia to evaluate fungicides in different classes and with different modes of action for efficacy against *Botryosphaeriaceae* recovered from walnut orchards in Australia, and provides a wider selection of active ingredients for a fungicide rotation programme than that which is currently available to the Australian walnut industry.

Keywords. Dieback, *Diplodia seriata*, *Juglans regia*, *Neofusicoccum parvum*.

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INTRODUCTION

Australia has a young and expanding walnut industry. In recent years, yield losses caused by dieback of fruiting spurs, stems and branches has become widespread in walnut (*Juglans regia* L.) orchards, and *Botryosphaeriaceae* fungi have been implicated in the dieback syndromes. A first systematic survey conducted in 2019–2020 covered 14 orchards, representing all the major walnut-growing regions of Australia (Riverina in New South Wales, Adelaide Hills and Riverland regions in South Australia, the east coast of Tasmania, all of Victoria, and south-west Western Australia), and recovered five species of *Botryosphaeriaceae* from walnut tissues. The survey identified *Diplodia seriata* and *Neofusicoccum parvum* as the most prevalent species, constituting 93% of all isolates analysed by DNA sequencing. Pathogenicity studies confirmed the two prevalent species to be the most virulent among the five species, with *N. parvum* more virulent than *D. seriata* (Antony *et al*., 2023a). No published research has been conducted on control strategies for these pathogens in walnuts in Australia.

Symptoms of dieback, cankers, blight and fruit rot caused by *Botryosphaeriaceae* have been reported in walnut orchards in a number of countries, including Chile (Díaz *et al.,* 2018; Luna *et al.,* 2022), China (Yu *et al.,* 2015; Li *et al.,* 2016; Zhang *et al.,* 2017; Li *et al.,* 2023), the Czech Republic (Eichmeier *et al.,* 2020), Egypt (Haggag *et al.,* 2007), Greece (Rumbos, 1987), Iran (Abdollahzadeh *et al.,* 2013; Sohrabi *et al.,* 2020), Italy (Frisullo *et al.,* 1994; Gusella *et al.,* 2021), Korea (Cheon *et al.,* 2013), Spain (López-Moral *et al.,* 2020), Turkey (Kara *et al.,* 2021; Yildiz *et al.,* 2022), and the United States of America (Trouillas *et al*., 2010; Michailides *et al.,* 2012; Chen *et al.,* 2013; Chen *et al.,* 2014). Eighteen species of *Botryosphaeriaceae* from six genera have been recovered from walnut orchards in these countries. Economic losses caused by these pathogens has been estimated to be significant, although it is difficult to separate losses caused by other pathogens such as the *Diaporthe* spp. that cause similar symptoms (Moral *et al.,* 2019). *Botryosphaeriaceae* reduce yields by killing walnut tree branches, immature fruit, and wood, and by infecting nut (Hasey and Michailides, 2016). If dieback is not controlled, yield losses are likely to become more significant with successive harvests, and can lead to total crop failures (Michailides *et al.,* 2012).

Research on fungicide efficacy for control of these pathogens has not included *Botryosphaeriaceae* isolates recovered from Australia, despite well-established knowledge that plant pathogens evolve differently in different environments (van Niekerk *et al*., 2004; Slippers and Wingfield, 2007). A review of previous studies on fungicide efficacy against *Botryosphaeriaceae* in crops other than walnut and in other regions showed high variability in results, indicating that it is not possible to draw generalisations on the efficacy of the fungicides across crops and regions. Even within the same region and crop, fungicide efficacy has been found to vary between species of *Botryosphaeriaceae* (Bester *et al*., 2007; Amponsah *et al*., 2012; Pitt *et al*., 2012). Therefore, fungicide application programmes should be built on an understanding of incidence and prevalence of the target pathogens of specific hosts, in specific regions. Further research is required to assess the efficacy of fungicides for management of dieback in walnuts under Australian conditions.

No fungicides are currently registered in Australia for the control of *Botryosphaeriaceae* in walnuts, although two minor use chemical permits for formulations of pyraclostrobin [Fungicide Action Resistance Committee (FRAC) 11] and a mixture of tebuconazole (FRAC 3) plus fluopyram (FRAC 7) have been approved by the Australian Pesticides and Veterinary Medicines Authority (APVMA). This reliance on only two fungicides, with a total of three active ingredients (a.i.) that have single-site modes of action, may increase the potential risk for development of fungicide resistance among target pathogens, and is inadequate for development and implementation of a sustainable fungicide programme. Although there are no published reports of fungicide resistance developing in *Botryosphaeriaceae* associated with walnuts, reduced sensitivity of these fungi to multiple fungicides has been reported in other crops. In California, while comparing the baseline sensitivity to tebuconazole of *Botryosphaeria dothidea* populations from pistachio, Ma *et al.* (2002) identified an isolate with low sensitivity to tebuconazole. Their study also showed a shift towards greater tebuconazole EC_{50} values in orchards that had consecutive years of multiple applications of this fungicide. Similar studies in China identified emergence of tebuconazole resistance in populations of *B. dothidea* from apple orchards (Fan *et al*., 2016). This was attributed to use of consecutive multiple applications of tebuconazole for over 10 years. Likewise, resistance to pyraclostrobin has been reported to be common among *Lasiodiplodia theobromae* populations from mango in China (He *et al*., 2021; Yang *et al*., 2021). Decreased sensitivity to fluopyram has been reported in different host-pathogen systems including *Alternaria alternata* on pistachio (Avenot *et al*., 2014; Avenot *et al*., 2019) and *Botrytis cinerea* on strawberry (Amiri *et al*., 2014), after only a few years of field applications. Therefore, these resistance-prone fungicides should be used in the walnut orchards in Australia only with appropriate fungicide resistance management strategies in place.

One strategy to prevent development of fungicide resistance in pathogens is the alternation and/or combinations of contact and systemic fungicides, and inclusion of multi-site fungicides (Denman *et al*., 2004; Brent and Hollomon, 2007). Incorporating a.i. from different chemical groups with different modes of action in rotation programme, or using the fungicides in mixtures, is likely to reduce over-exposure of pathogens to only a few a.i. This could be achieved by considering the fungicides already in use in various horticultural crops to control fungal diseases, and identifying the most promising candidates that are effective against the prevalent *Botryosphaeriaceae* present in Australian walnut orchards.

Experiments were conducted to evaluate selected fungicides from different classes and modes of action, for their abilities to: (i) reduce *in vitro* mycelium growth of five species of *Botryosphaeriaceae* recovered from walnut orchards in Australia; (ii) inhibit *in vitro* conidium germination of the two most prevalent pathogens *D. seriata* and *N. parvum*; (iii) control infections by these fungi in glasshouse experiments on detached stems and glasshouse-grown walnut plants; and (iv) evaluate effective field durations of preventative and curative action that the fungicides have against the most virulent species, *N. parvum*.

MATERIALS AND METHODS

Efficacy of fungicides against in vitro *mycelium growth of fungal isolates*

Ten active ingredients from different chemical groups (Table 1) were identified for this experiment. Fungicides banned in the European Union and not

Table 1. Fungicides tested in this study.

Active ingredient (a.i.)	Chemical class, Mode of action[b]	FRAC code, Risk of fungicide resistance development[b]	Trade name and formulation used in *in vivo* experiments and in the field
Boscalid[a]	Carboxamide SDHI, single site	FRAC 7, medium to high	Only a.i. was used *in vitro*
Captan	Phthalimide Respiration inhibitor, multi-site	M04, low	Only a.i. was used *in vitro*
Fluazinam	Pyrrole, 2,6-initroanilines Energy synthesis disruptor, multi-site	FRAC 29, low	Emblem®, Nufarm Australia, 500 g L^{-1} fluazinam
Fludioxonil	Phenylpyrrole Osmoregulation inhibitor, single site	FRAC 12, low to medium	Geoxe®, Syngenta Australia, 500 g Kg^{-1} fludioxonil
Fluopyram[a]	Pyridinyl ethyl benzamide SDHI, single site	FRAC 7, medium to high	Only a.i. was used *in vitro*
Fluxapyroxad[a]	Carboxamide SDHI, single site	FRAC 7, medium to high	Only a.i. was used *in vitro*
Penthiopyrad[a]	Carboxamide SDHI, single site	FRAC 7, medium to high	Only a.i. was used *in vitro*
Prochloraz manganese chloride	Imidazole DMI, multi-site	FRAC 3, medium	Octave®, Bayer Crop Science, Australia, 500 g Kg^{-1}
Pyraclostrobin[a]	Methoxycarbamate QoI, single site	FRAC 11, high	Cabrio®[c], BASF Australia, 250 g L^{-1} pyraclostrobin
Tebuconazole[a]	Triazole DMI, single site	FRAC 3, medium	Orius®, ADAMA Agricultural Solutions Ltd, Australia, 200 g L-1 tebuconazole
Tebuconazole + Fluopyram[a]	DMI + SDHI	FRAC 3 + FRAC 7	Luna Experience®[c], Bayer Crop Science, Australia, 200 g L^{-1} tebuconazole + 200 g L^{-1} fluopyram

[a] Used in California for control of Botryosphaeria dieback in walnuts.
[b] Source: https://www.frac.info/
[c] Minor use chemical permit approved by the Australian Pesticides and Veterinary Medicines Authority for control of Botryosphaeria dieback in walnuts.
DMI = Demethylation Inhibitors (FRAC 3).
QoI = Quinone outside Inhibitors (FRAC 11).
SDHI = Succinate Dehydrogenase Inhibitors (FRAC 7).

approved by APVMA were excluded. The concentrations of each a.i. assessed were 0, 0.001, 0.01, 0.1, 1.0, or 10 mg L^{-1}. Efficacy of these concentration/a.i. combinations were tested on nine *Botryosphaeriaceae* isolates obtained from walnut orchards in Australia during the study of Antony *et al*. (2023a). The isolates tested included three isolates each of *D. seriata* and *N. parvum,* and one isolate each of *Dothiorella omnivora, N. macroclavatum* and *Spencermartinsia viticola*. For the two prevalent species, *D. seriata* and *N. parvum*, the three most virulent isolates (DS04, DS15, and DS27 of *D. seriata*; NP03, NP05 and NP18 of *N. parvum*) were selected based on a detached stem assay conducted previously (Antony *et al*., 2023a). For the other three fungi, one isolate per species was included since the total available isolates belonging to those species was only four.

Technical grade fungicide active ingredients were used in this experiment. Since some have low solubility in water (e.g., solubility of prochloraz mc in water is 34.4 mg L^{-1}. https://www.epa.govt.nz/), acetone, classified as a solvent (https://www.ncbi.nlm.nih.gov/) was used to dissolve them, as described by Pitt *et al*. (2012). For these fungicides, each a.i. was dissolved in 100% acetone, adjusted to a 10 mg L^{-1} stock solution, and diluted to: 1.0 mg L^{-1}, 0.1 mg L^{-1}, 0.01 mg L^{-1}, or 0.001 mg L^{-1}. Experimental controls contained acetone alone. These were used to prepare potato dextrose agar (PDA; Oxoid Ltd) plates amended with each a.i. After sterilization (121°C for 20 min), the medium was allowed to cool to 50°C, and acetone containing required amounts of each a.i. was added to the PDA to produce the required fungicide concentrations. In all batches of amended media, including the control, the final acetone concentration was adjusted to 0.1% v/v. These media were mixed, then poured into 9-cm diam. Petri plates and left to cool to room temperature.

The fungicide-amended PDA plates were each inoculated with a 5 mm diam. mycelium disc taken from the margin of an actively growing 3-d-old culture of each selected isolate that was previously cultured on PDA. For each isolate/fungicide/concentration combination three replicate plates were prepared. Within each fungicide/species combination, the inoculated PDA plates were arranged in a totally randomised design, and were incubated at 25°C in the dark. After incubation for 48 h, the mycelium growth in each plate was quantified by measuring the colony across two perpendicular diameters, and calculating the average diameter. Inhibition was then calculated by subtracting mean colony diameters from those of the nil fungicide experimental controls. Percent growth inhibition relative to the control was determined using the formula:

$$\text{Inhibition (percent)} = [(C\text{-}T)/C] \times 100$$

where C = average colony diameter on the control plate, and T = average colony diameter on the fungicide-amended plate.

Inhibition data were fitted to fungicide concentrations for each isolate and fungicide. The data were normalised by logarithmic transformation for fungicide concentrations, and the probit values were used for the inhibition data. The EC_{50} value for each fungicide/isolate concentration was calculated using R Statistical Software (v4.2.2; R Core Team 2022). Means were back transformed to the original scale. The experiment was repeated twice, and the EC_{50} values obtained in the three rounds of experiments were tested for homogeneity of variance using Levene's test. Since the Levene statistics were not significant (P = 0.172, indicating equality of variances), analysis of variance (ANOVA) was applied to the EC_{50} values to determine statistically significant differences between variables. A Generalised Linear Model (GLM) univariate analysis in IBM SPSS Statistics for Windows (Version 27.0. Armonk, New York: IBM Corp) was applied, and means were separated using Tukey's HSD test at $P < 0.05$.

Effects of fungicides on in vitro *conidium germination*

The same ten a.i. evaluated for the mycelium growth inhibition were tested for their effects on conidium germination of *D. seriata* and *N. parvum*. The same isolates of *D. seriata* and *N. parvum* used in the experiment described above were used to prepare conidium suspensions (10^4 conidia mL^{-1}) of the two species. To prepare the mixed isolate conidium suspension, an equal volume of filtered conidium suspension from each of the three selected isolates was combined in a sterile tube, as described by Antony *et al*. (2023b). Each a.i. was dissolved in acetone (as described above), and was then mixed with sterile distilled water (SDW) at twice the designated concentration so that, when mixed with an equal volume of conidium suspension or SDW, the resultant solution would achieve the designated concentration. For each germination evaluation, 100 µL of the mixed isolate conidium suspension was added to 100 µL of each a.i. solution, or 100 µL of SDW for the experimental control treatments. To establish the germination test for each fungicide/concentration/conidium suspension combination, three 50 µL drops were placed onto each of three replicate glass slides. Each slide was placed on a water moistened filter paper in a Petri plate, which was then sealed with parafilm to maintain high humidity. The plates were then arranged in a completely randomised

design, and incubated at 25°C in the dark. After 24 h of incubation, germination of 100 conidia was assessed within randomly selected microscope fields of view (×40 magnification) from each droplet. A conidium was considered to have germinated if the length of the germ tube was greater than half the length of the conidium. The percent germination inhibition data relative to the controls were calculated for each microscope slide, and the values were used to determine the EC_{50} for each fungicide and species, as described above for inhibition of mycelium growth. The experiment was repeated twice, and the EC_{50} values were investigated as described above.

Evaluation of fungicides: detached stem assays

Based on the efficacy of the a.i. for *in vitro* inhibition of mycelium growth and conidium germination, and considering their chemical classes and modes of action, six a.i. were selected for further assessment of their preventative and curative effects against *D. seriata* and *N. parvum* infections (Table 1). For five chemicals, (tebuconazole, pyraclostrobin, prochloraz mc, fluazinam and fludioxonil), commercial formulations that each had the individual a.i. as the only active constituent were used. The sixth fungicide was a mixture of two a.i., tebuconazole and fluopyram, and has a minor use permit approved by the APVMA for use in management of Botryosphaeria dieback in walnuts in Australia (Permit Number – PER91994. https://portal.apvma.gov.au/permits).

Asymptomatic 1-year-old dormant stems of walnut trees 'Chandler' were collected in winter from an orchard in Victoria, Australia. The stems were each pruned at the apical end and separately placed into 125 mL capacity plastic tubes filled with water. Two mixed isolate conidium suspensions were prepared with the three isolates each of *D. seriata* and *N. parvum* described above. The fungicides were mixed at label rates according to manufacturer recommendations. Since the commercial formulations contained additive required for dispersion in water, they were directly mixed with SDW, and then applied using 125 mL capacity spray bottles fitted with calibrated nozzles. For the preventative treatments, the stems were 'pruned and treated' immediately with 200 µL of the selected fungicide, and SDW was applied for the control treatment. On day 1, 3, or 7 post treatment, wounds of the stems were moistened by spraying with SDW and then each inoculated with 20 µL of a mixed isolate conidium suspension containing ~500 conidia of either *D. seriata* or *N. parvum*, using a micropipette. For the curative treatments, the stems were 'pruned and inoculated' immediately with a mixed isolate conidium suspension of either *D. seriata* or *N. parvum*. The inoculated pruning wounds were then sprayed with the selected fungicides at label rates, on either day 1, 3, or 7 post inoculation. For experimental control treatments, both positive (inoculated + no fungicide, designated "IC") and negative (non-inoculated + no fungicide, designated "NIC") controls were established without any fungicide application.

Each treatment was allocated to five walnut stems per replicate, and the experiment was set up with five replicates in a randomised complete block experimental design (RCBD), which was maintained in a glasshouse at ambient temperature of 17–25°C. The stems were assessed for lesion development 6 weeks after inoculation. The bark of each stem was peeled, and any internal and external lesions measured using a digital calliper. Wood segments excised from lesion margins were surface sterilised by soaking in 2% (v/v) sodium hypochlorite for 2 min, followed by rinsing twice in SDW. Fungal re-isolations were made by plating these wood segments onto PDA, to confirm Koch's postulates. After confirming homogeneity of variances, lesion lengths were analysed using univariate analysis as for the experiments described above.

Evaluation of fungicides using glasshouse plants

Of the six fungicides evaluated on detached walnut stems, five (tebuconazole, tebuconazole + fluopyram, pyraclostrobin, prochloraz mc, and fluazinam) were also tested on green walnut shoots, and four (tebuconazole, tebuconazole + fluopyram, pyraclostrobin, and fluazinam) were tested on 1-year-old stems of potted walnut plants 'Chandler', grown under glasshouse conditions. Both the preventative and curative efficacy of the fungicides against *D. seriata* and *N. parvum* were assessed on 1-year-old host stems; however, due to the limited availability of plants and considering industry requirements at that time for curative protection of young trees post-pruning for form training, only curative effects of five fungicides were assessed on green walnut shoots.

In the green shoot experiment, each treatment was assigned to three replicate plants in a RCBD. Three green shoots of similar thickness were identified on each plant. The shoots were tip pruned at ~12–15 cm from the basal ends, and were each immediately inoculated with 20 µL of a mixed isolate conidium suspension containing ~500 conidia of either *N. parvum* or *D. seriata*. Following inoculation, each treated shoot was covered with a transparent plastic bag for 24 h to reduce evaporation of the conidium suspension. Twenty-four hours after pruning and inoculation, wounds were sprayed with the selected fungicides mixed at label rates. Positive and negative

control stems were not given any fungicide spray. The plants were maintained in the glasshouse at ambient temperature of 17–25°C. The shoots were assessed for lesion development as described (above) for the detached stem assay 8 months after inoculation. Fungal re-isolations from lesion margins and analysis of lesion lengths were carried out as described above.

The experiment on 1-year-old stems was set up with four replicate potted walnut plants 'Chandler', grown under glasshouse conditions. Two stems of similar size were identified on each plant, one for a preventive treatment and the other for the corresponding curative treatment. For the curative treatments, inoculations and treatments were applied as described (above) in the green shoot experiment. For the preventive treatments, the stems were 'pruned and immediately treated' with the selected fungicides. The control stems were treated with SDW. Twenty-four hours after pruning and treatment, inoculations were carried out with mixed isolate conidium suspensions of *N. parvum* and *D. seriata* as described (above) for the detached stem assays. The inoculated plants were maintained in a glasshouse at ambient temperature of 17–25°C. The stems were assessed for lesion development 12 months after inoculation, as described (above) in the green shoot experiment. Fungal re-isolations from lesion margins and analyses of lesion lengths were carried out as described above. Fungal re-isolations were also carried out from 10 mm beyond each lesion margin, at 5 mm above the base of the closest side shoot and 5 mm below the node of the closest side shoot, to monitor pathogen progression beyond each dieback.

Field evaluation of fungicides

A field trial was established to evaluate the four most promising fungicides against *N. parvum* in walnut, as indicated from the *in vitro* and *in vivo* experiments, and to assess the duration of fungicide efficacy from preventative and curative treatments. The field trial was conducted from June 2022 to May 2023 in a commercial walnut orchard located in New South Wales (NSW), Australia. Efficacy of the fungicide treatments tebuconazole, tebuconazole + fluopyram, pyraclostrobin, or fluazinam were compared to an experimental control treatment of SDW, for providing preventative and curative protection, with six application timings, three of which were preventative and three were curative. The trial was established as a RCBD, with six replications, in 20-year-old walnut trees 'Chandler' with no observable disease symptoms. Within a replicate, each treatment was allotted to a tree, and in each tree, six 1-year-old stems were tagged for the allotted treatment.

For the preventative treatments, as in the glasshouse experiments, the identified stems were pruned and the treatments were immediately applied to the pruning wounds, using a paintbrush. A 20 µL mixed isolate conidium suspension of *N. parvum* (~500 conidia) was applied to each wound with a micropipette at either day 1, 7 or 14 post 'pruning and treatment'. For the experimental control treatment, stems were treated with SDW immediately after pruning, and then inoculated as described for the other preventative treatments. For the curative treatments, immediately after pruning the stems, the wounds were each inoculated with a 20 µL mixed isolate conidium suspension of *N. parvum* containing ~500 conidia. The fungicide treatments were applied either at day 1, 3 or 7 post 'pruning and inoculation'. For the positive experimental control (IC), stems were inoculated with conidium suspension of *N. parvum,* and no fungicide treatment was applied post inoculation. For the negative experimental control (NIC), stems were inoculated with SDW, and no further fungicide treatment was applied. Twelve months after inoculation, the treated stems were harvested for assessment of lesion development. Lesion lengths were measured, and fungal re-isolations were attempted from the margins of the lesions to confirm Koch's postulates.

Levene's test of equal variances applied to lesion length data confirmed homogeneity of variances. Differences in treatment effects were then investigated by ANOVA of lesion lengths, by applying a univariate analysis in IBM SPSS Statistics for Windows, as for the experiments described above. For the three preventative treatments, disease inhibition was calculated by subtracting mean lesion lengths of the fungicide treatments from those of the SDW treatments, at the corresponding inoculation times. The mean percent control (MPC) was then calculated using the formula:

$$\text{MPC on day X inoculation} = [(IC_X\text{-}T_X)/IC_X] \times 100$$

where X = 1, 7, or 14; IC_X = lesion length corresponding to day X inoculation post SDW treatment; T_X = lesion length corresponding to day X inoculation post fungicide treatment.

For the curative treatments, MPC was calculated using the formula:

$$\text{MPC of day X treatment} = [(IC\text{-}T_X)/IC] \times 100$$

where X = 1, 3, or 7; IC = lesion length of the inoculated control; T_X = lesion length corresponding to day X treatment with fungicide.

RESULTS

Efficacy of fungicides against in vitro *mycelium growth of fungal isolates*

The fungicides varied in their effectiveness for inhibiting mycelium growth of the five fungi tested, with mean EC_{50} values for the species ranging from 0.004 to 154.6 mg a.i. L^{-1}. Captan gave the greatest mean EC_{50}, which was ~15 times greater than the maximum concentration of 10 mg L^{-1} used in this experiment. To remove the influence of this value for assessing the efficacy of the other fungicides, captan was not included in the statistical analysis presented in Table 2. The EC_{50} values for the individual treatments that were obtained in the three rounds of the experiment did not differ significantly ($F = 1.952$, $DF = 2$, $P = 0.146$), so the data were combined. Logarithmic transformation was applied to normalise the data for further analyses of variance, and the results were back transformed to original scale. Mean effects of the fungicides across pathogen species were calculated to evaluate the relative efficacy of the fungicides on the species present in the Australian walnut orchards. Similarly, pathogen species means across fungicides were calculated to assess the relative sensitivity of each pathogen to the fungicides tested.

An ANOVA of the EC_{50} values indicated that five fungicides (fluazinam, tebuconazole, fludioxonil, prochloraz, and pyraclostrobin) were the most effective, and they were similar in their inhibitory effects on mycelium growth, forming a homogenous group according to Tukey's HSD test at $P < 0.05$ (Table 2). Fluopyram and penthiopyrad were next in their effectiveness, followed by fluxapyroxad. Boscalid was moderately effective, as shown by the dose required for effective inhibi-

Table 2. Mean EC_{50} values for selected technical grade fungicide active ingredients (a.i.) for inhibition of *in vitro* mycelium growth of five *Botryosphaeriaceae* species.

Fungicide	Mean EC_{50} (mg a.i. L^{-1}) values[a] and standard error within parentheses					
	Botryosphaeriaceae species					Fungicide mean effect[b]
	Diplodia seriata	*Dothiorella omnivora*	*Neofusicoccum macroclavatum*	*N. parvum*	*Spencermartinsia viticola*	
Boscalid	11.378	0.627	29.128	28.325	0.106	13.913 d
	(0.44)	(0.02)	(0.69)	(0.48)	(0.07)	(3.41)
Fluazinam	0.005	0.002	0.007	0.007	0.001	0.004 a
	(0.0004)	(0.0001)	(0.001)	(0.0003)	(0.001)	(0.001)
Fludioxonil	0.013	0.032	0.016	0.011	0.001	0.015 a
	(0.0004)	(0.0002)	(0.001)	(0.0001)	(0.001)	(0.01)
Fluopyram	3.092	0.017	2.681	2.214	0.021	1.609 b
	(0.06)	(0.001)	(0.06)	(0.10)	(0.001)	(0.35)
Fluxapyroxad	4.711	0.023	5.586	4.252	0.02	2.915 c
	(0.32)	(0.01)	(0.05)	(0.67)	(0.002)	(0.65)
Penthiopyrad	4.238	0.051	2.181	2.033	0.024	1.705 b
	(0.22)	(0.02)	(0.27)	(0.24)	(0.004)	(0.43)
Prochloraz	0.015	0.030	0.030	0.005	0.0002	0.016 a
	(0.002)	(0.0001)	(0.01)	(0.0001)	(0.004)	(0.004)
Pyraclostrobin	0.105	0.072	0.082	0.385	0.008	0.131 a
	(0.01)	(0.002)	(0.01)	(0.01)	(0.01)	(0.04)
Tebuconazole	0.004	0.028	0.009	0.005	0.001	0.009 a
	(0.0001)	(0.0001)	(0.001)	(0.001)	(0.001)	(0.003)
Species mean[c]	2.616 y	0.096 x	4.413 z	4.142 z	0.020 x	
	(0.72)	(0.01)	(0.75)	(0.71)	(0.04)	

[a] EC_{50} = mean concentration (mg L^{-1}) of fungicide at which mycelium growth is inhibited by 50%.
[b] Data pooled across species, to provide mean EC_{50} values for fungicide efficacy.
[c] Data pooled across fungicides to provide mean EC_{50} values for species sensitivity.
Fungicide effects on the prevalent species *D. seriata* (A, B, C and D) and *N. parvum* (P, Q, R and S) were significantly different ($P < 0.001$). Fungicide mean effects (a, b, c and d) were significant ($P < 0.001$). For species, the mean effects (x, y and z) were significant ($P < 0.001$). Values within each row or column followed by the same letter are not significantly different ($P > 0.05$), according to Tukey's HSD test.

tion of mycelium growth. Intra-species variation was not significant for *D. seriata* or *N. parvum*, so only the species means are presented in Table 2. The species effect was significant ($P < 0.001$), with the five species falling into three groups. *Spencermartinsia viticola* (EC_{50} = 0.020 mg L^{-1}) and *Do. omnivora* (EC_{50} = 0.096 mg L^{-1}) were similar (P=0.724), and were the most sensitive to the fungicides, followed by *D. seriata* (EC_{50} = 2.616 mg L^{-1}). *Neofusicoccum macroclavatum* (EC_{50} = 4.413) and *N. parvum* (EC_{50} = 4.142) had similar sensitivities to the fungicides (P=0.208), and *Neofusicoccum* spp. had lower sensitivities to the fungicides than *D. seriata*.

Effects of fungicides on in vitro *conidium germination*

The *in vitro* experiment assessing conidium germination was repeated twice, and the EC_{50} values obtained in the three rounds did not differ significantly (P=0.79), so the data were combined for further analyses. The fungicides varied in their effectiveness for inhibiting conidium germination of *D. seriata* and *N. parvum*, with their mean fungicide EC_{50} values ranging from 0.21 to 9.00 mg a.i. L^{-1} (Table 3).

Pyraclostrobin was the most effective fungicide ($P < 0.001$), giving the lowest mean EC_{50} of 0.21 mg L^{-1}, followed by fluopyram, fluxapyroxad, penthiopyrad and tebuconazole with mean EC_{50}s between 1.26 and 2.15 mg a.i. L^{-1}. Boscalid (EC_{50} = 5.53 mg L^{-1}) and captan (EC_{50} = 6.33 mg L^{-1}) were similar (P=0.052), and next in their effectiveness. Prochloraz mc, fluazinam and fludioxonil were similar (P=0.983), and had greater mean EC_{50}s, between 8.76 and 9.00 mg a.i. L^{-1}, indicating lower efficacy compared to the other fungicides. The fungi differed in their sensitivities to the fungicides, with *N. parvum* showing lower sensitivity than *D. seriata* ($P < 0.001$) (Table 3).

Evaluation of fungicides: detached stem assay

All the fungicide treatments, both preventative and curative, were effective for reducing mean lesion lengths compared to the experimental controls. The fungi differed significantly in their sensitivities to the fungicides, with *N. parvum* having lower sensitivity than *D. seriata* ($P < 0.001$). All the preventative fungicide treatments reduced lesion lengths ($P < 0.001$) compared to the corresponding inoculated controls treated with SDW. All six fungicides reduced the lesion length on day 1 post treatment, to sizes similar to the NIC (Figure 1a). The later inoculations on day 7 post treatment resulted in lesions that were longer than the NIC, and the two tebuconazole formulations and prochloraz mc gave greater

Table 3. Mean EC_{50}s for selected technical grade fungicide active ingredients (a.i.) for *in vitro* inhibition of conidium germination of *Diplodia seriata* and *Neofusicoccum parvum*.

Fungicide	Mean EC_{50} (mg a.i. L^{-1}) values[a] and standard error (SE)					
	Diplodia seriata		*Neofusicoccum parvum*		Fungicide mean effect[b]	
	Mean and SE		Mean and SE		Mean and SE	
Boscalid	4.78 C	0.21	6.28 S	0.24	5.53 d	0.37
Captan	5.40 C	0.23	7.04 S	0.18	6.22 d	0.39
Fluazinam	7.69 D	0.18	9.86 T	0.20	8.76 e	0.50
Fludioxonil	8.11 D	0.23	9.64 T	0.22	8.88 e	0.37
Fluopyram	1.18 AB	0.23	1.35 Q	0.24	1.26 b	0.15
Fluxapyroxad	1.22 B	0.22	1.50 Q	0.13	1.36 b	0.13
Penthiopyrad	1.29 B	0.23	1.85 QR	0.24	1.57 bc	0.19
Prochloraz mc	8.67 D	0.21	9.33 T	0.24	9.00 e	0.20
Pyraclostrobin	0.17 A	0.12	0.24 P	0.12	0.21 a	0.08
Tebuconazole	1.43 B	0.22	2.87 R	0.20	2.15 c	0.35
Species mean effect[c]	3.99 x	0.59	5.00 y	0.68		

[a] EC_{50} = mean concentration (mg L^{-1}) of fungicide at which conidium germination was inhibited by 50%.
[b] Data pooled across species to provide mean EC_{50} values for fungicide efficacy.
[c] Data pooled across fungicides to provide mean EC_{50} values for species sensitivity.
Fungicide effects on *D. seriata* (A, B, C and D) and *N. parvum* (P, Q, R, S and T) were significant ($P < 0.001$). Fungicide mean effects (a, b, c, d and e) were significant ($P < 0.001$). For species, the mean effects (x and y) were significant ($P < 0.001$). Values within each row or column followed by the same letter are not significantly different ($P < 0.05$) according to Tukey's HSD test.

efficacy for lesion size reduction than the other three fungicides (Figure 1a).

Among the curative treatments, as for the preventative treatments, application of the fungicides 1 d post inoculation reduced lesion lengths that were similar ($P > 0.05$) to those from the NIC (Figure 1b). The two tebuconazole formulations applied at day 3 post inoculation gave similar efficacy, and reduced the lesion lengths to those similar to the NIC. Treatments on day 7 post inoculation, however, resulted in lesions that were longer ($P < 0.05$) than the NIC (Figure 1b). The other four fungicides gave various levels of efficacy when applied on days 3 or 7 post inoculation. Fludioxonil and fluazinam applied on day 7 resulted in the longest lesions compared to the corresponding treatments of the other four fungicides. However, those lesions were smaller ($P < 0.05$) than those from the inoculated control.

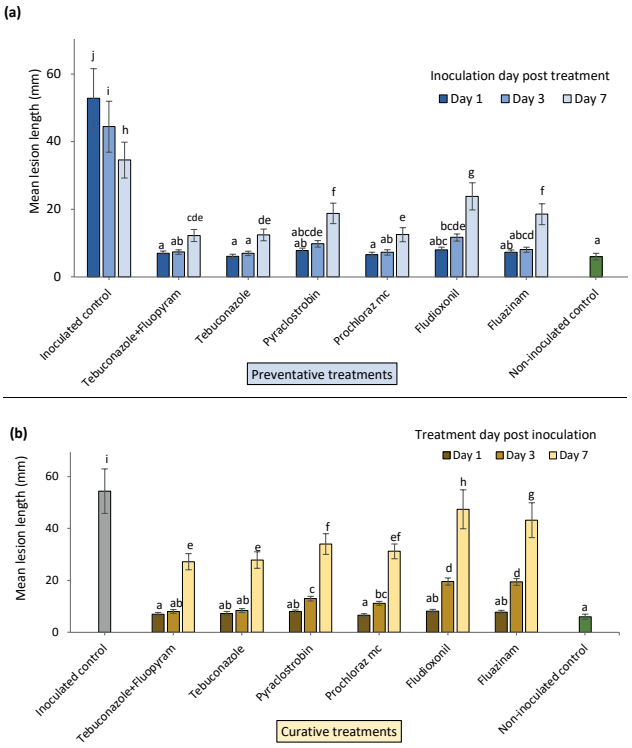

Figure 1. Mean lesion lengths caused by Botryosphaeria dieback pathogens on 1-year-old detached walnut stems after applications of six different fungicide products. (a) Preventative treatments with the fungicides, with inoculation of either *Diplodia seriata* or *Neofusicoccum parvum* at 1, 3 or 7 d after fungicide treatment. (b) Curative treatments with the fungicides, applied at 1, 3 or 7 d post inoculation with *D. seriata* and *N. parvum*. The error bars indicate standard errors of the means. Different letters associated with the means indicate differences ($P < 0.05$), according to Tukey's HSD test.

Evaluation of fungicides on glasshouse-grown plants

On green shoots of glasshouse plants, all five fungicides applied as curative treatments reduced the lesion lengths for both pathogen species, compared to the inoculated controls. The two tebuconazole formulations gave greater efficacy ($P < 0.05$) than pyraclostrobin (Figure 2). Prochloraz mc and fluazinam were not different ($P > 0.05$) from the other three fungicides for reducing lesion lengths (Figure 2). However, none of the treatments reduced the lesion lengths to the size of the NIC. Pathogen recovery from lesion margins was 17% from the fluazinam treatment and zero for the other four fungicide treatments.

On 1-year-old stems of glasshouse plants, the effects of fungicides as preventative treatments for reducing the lesion lengths were statistically significant ($P < 0.001$)

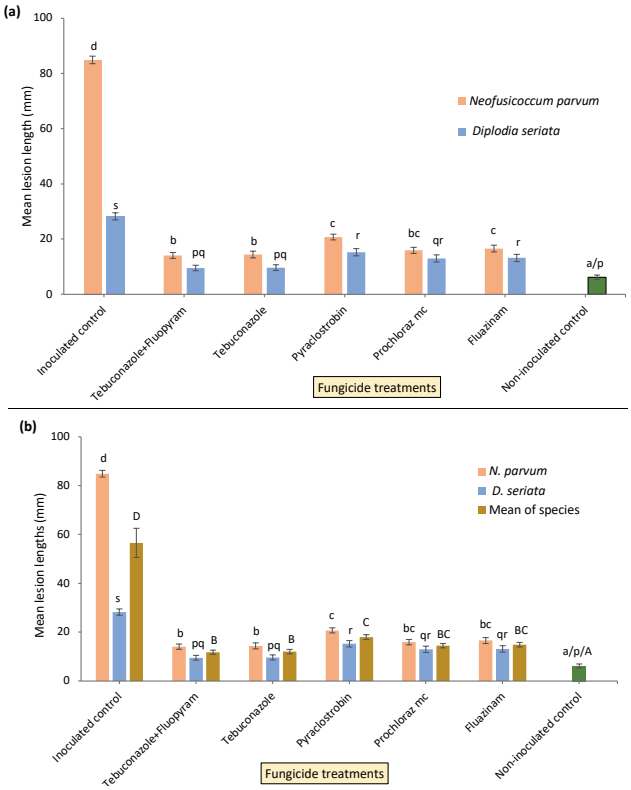

Figure 2. Mean lesion lengths caused by Botryosphaeria dieback pathogens on green shoots of glasshouse plants after curative fungicide treatments, applied 24 h after inoculations with conidium suspensions of *Neofusicoccum parvum* and *Diplodia seriata*. Figure 2a presents the effects of curative treatments on *N. parvum* and *D. seriata*. Figure 2b includes the mean effects of curative treatments on infections caused by the two pathogens. Error bars indicate standard errors of the means. Different letters on the bars (a, b, c and d for *N. parvum*; p, q, r and s for *D. seriata*; A, B, C and D for means of species) indicate differences ($P < 0.05$), according to Tukey's HSD test.

when compared to the controls inoculated with both pathogen species. The tebuconazole and fluazinam formulations had the greatest preventative efficacy, reducing lesion lengths to those similar from the NIC (Figure 3). Mean pathogen recovery from the lesion margins was zero for all the four preventative fungicide treatments.

The curative effects of the fungicides, applied 24 h post inoculation, were also statistically significant ($P < 0.001$), reducing the lengths of lesions resulting from inoculations with both fungi compared with the inoculated controls (Figure 3b). Although the four fungicides had similar curative effects ($P < 0.05$), none of the treatments reduced mean lesion lengths to those from the NIC. Mean pathogen recovery from the lesion margins was zero for all four fungicide treatments. For both the preventative and curative applications, fungal re-isolations from the three sites beyond lesion margins indicated that pathogen recovery from plant side shoots was zero, which indicates fungicide efficacy, but these isolations varied between zero and 25% from the main stems. Due to small sample sizes, these data were not further statistically analysed.

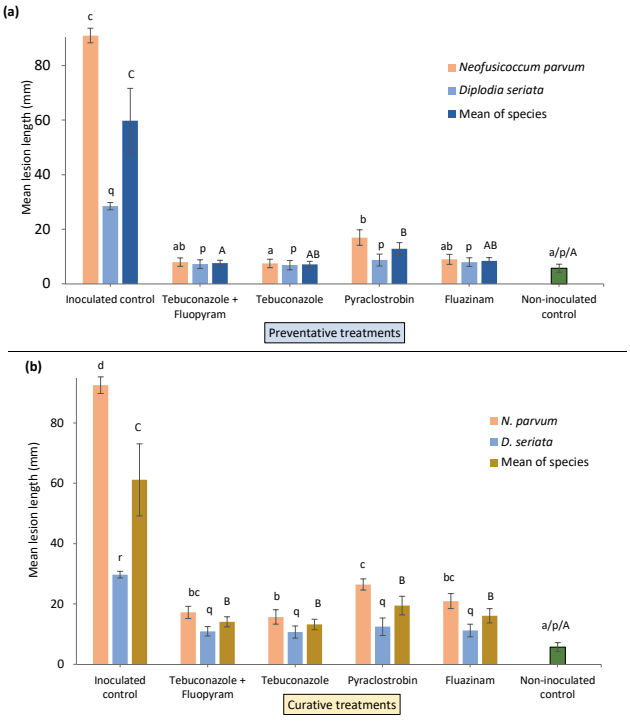

Figure 3. Mean lesion lengths caused by Botryosphaeria dieback pathogens on 1-year-old stems of glasshouse plants. (a) Preventative treatments with the fungicides, applied 24 h before inoculations with conidium suspensions of either *Diplodia seriata* or *Neofusicoccum parvum*. (b) Curative treatments with the fungicides, applied 24 h post inoculation with *D. seriata* and *N. parvum*. The error bars indicate standard errors of the means. Different letters associated with the means (a, b, c and d for *N. parvum*; p, q and r for *D. seriata*; A, B and C for means of species) indicate differences ($P < 0.05$), according to Tukey's HSD test.

Field evaluation of fungicides

All four fungicides provided preventative and curative protection of walnut trees against *N. parvum*, but for different periods and with different efficacies. Among the preventative treatments, the fungicides gave similar inhibitory effects ($P > 0.05$) on lesions caused by day 1 and day 7 inoculations (Figure 4a). The two tebuconazole formulations and pyraclostrobin were more effective for reducing lesions caused by day 14 inocula-

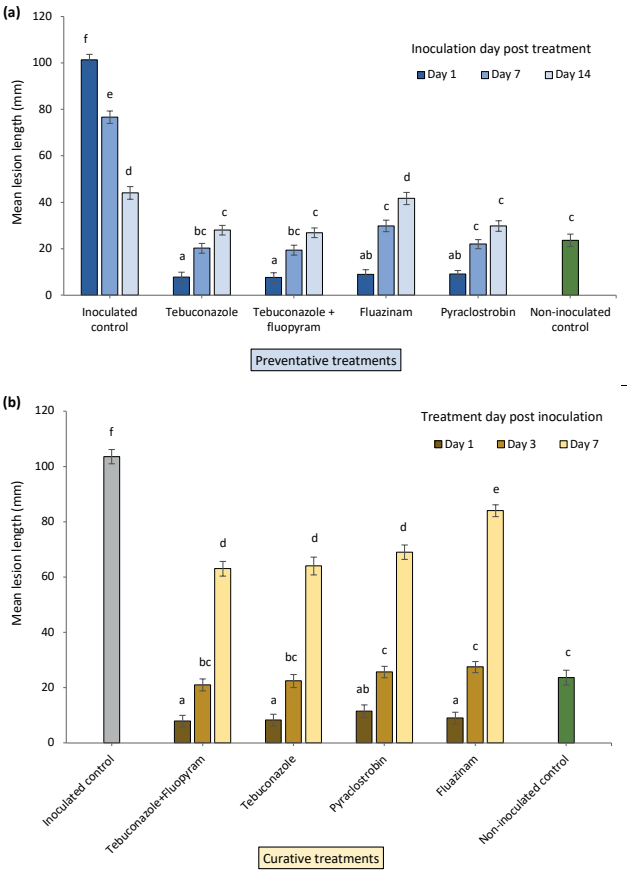

Figure 4. Mean lesion lengths caused by Botryosphaeria dieback pathogens on 1-year-old attached walnut stems in the field, after applications of four different fungicide products. (a) Preventative treatments with the fungicides, applied at 1, 7 or 14 d before inoculation with *Neofusicoccum parvum*. (b) Curative treatments with the fungicides, applied at 1, 3 or 7 d post inoculation with *N. parvum*. The error bars indicate standard errors of the means. Different letters associated with the means indicate differences ($P < 0.05$), according to Tukey's HSD test.

Table 4. Mean percent reductions in walnut tree dieback lesion lengths after conidium inoculations with *Neofusicoccum parvum* on pruned stems treated with different preventative or curative fungicide applications.

Fungicides	Reduction in lesion length compared to the control (%) Mean Percent Control and Standard Error (SE)											
	Preventative treatment						Curative treatment					
	Day 1		Day 7		Day 14		Day 1		Day 3		Day 7	
	Mean and SE		Mean and SE		Mean and SE		Mean and SE		Mean and SE		Mean and SE	
Fluazinam	91 a	2.09	61 b	3.23	05 d	3.99	91 a	2.07	73 c	2.00	19 e	2.12
Pyraclostrobin	91 a	1.62	71 b	2.58	32 c	4.79	89 ab	2.25	75 c	2.05	33 d	2.60
Tebuconazole	92 a	2.13	74 b	2.75	36 c	3.01	92 a	2.12	78 bc	2.35	38 d	3.20
Tebuconazole+fluopyram	93 a	2.08	75 b	2.80	39 c	3.85	92 a	2.08	80 bc	2.15	39 d	2.64

tions compared to fluazinam, which resulted in lesions similar ($P > 0.05$) to those from SDW treatment (Figure 4a). This indicates that fluazinam did not have any preventative effects on *N. parvum* at day 14. Among the curative treatments, the four fungicides were again similar in their effects when applied on day 1 or 3, and reduced the lesion lengths ($P < 0.05$) compared to the inoculated control (Figure 4b). The tebuconazole and pyraclostrobin formulations applied as curative treatments on day 7 were similar in their effects for reducing lesion lengths, and were more effective than fluazinam ($P < 0.05$; Figure 4b).

The preventative and curative treatments of the four fungicides were similarly effective, reducing mean lesion lengths by 89 to 93%, when time between treatment and inoculation was 1 d (Table 4). With increased time between treatment and inoculation, efficacy of the curative treatments reduced sooner than from the preventative treatments. Differences in efficacy of the fungicides also became apparent. The two tebuconazole formulations and the pyraclostrobin formulation had greater efficacy for a longer period than fluazinam. The preventative treatments were more effective for longer periods than the curative treatments, irrespective of the fungicide.

Neofusicoccum parvum was re-isolated from all inoculated controls treated with SDW. From the non-inoculated controls 20% pathogen recovery was recorded, which indicated presence of background *N. parvum* infections in the field. Pathogen recovery from lesion margins was zero for all preventative and curative treatments, when the time between treatment and inoculation was 24 h. When this time increased, pathogen recovery also increased, with curative treatments showing greater pathogen recovery than the preventative treatments. For fluazinam, pathogen recovery for later inoculations and later treatments was greatest, and varied between 35 and 90% compared to the corresponding results from treatments with the other three fungicides, that varied between 15 and 60%.

DISCUSSION

This study identified potential fungicides from different chemical classes and modes of action that could be incorporated into sustainable disease management for Botryosphaeria dieback in walnuts. In determining the efficacy of the selected fungicides, the study considered their inhibitory effects on both mycelium growth and conidium germination of the pathogen. Although natural field infections are instigated from conidia, inhibiting mycelium growth is also essential for effective disease control. Tebuconazole, fluopyram, pyraclostrobin, fluazinam, fludioxonil, fluxapyroxad, penthiopyrad, and prochloraz were effective for controlling mycelium growth and conidium germination of the assessed walnut pathogens, with EC_{50} values between 0.004 and 2.15 mg a.i. L^{-1}. These fungicides belong to seven different chemical classes, have five different modes of action, including single-site and multi-site inhibitors, and thus provide a wider selection for a fungicide rotation programme than that currently available to the Australian walnut industry. These fungicides were also among those tested and endorsed for efficacy in apple (Song *et al*., 2018), almond (Olmo *et al*., 2017), avocado (Twizeyimana *et al*., 2013), blueberry (Latorre *et al*., 2013; Tennakoon *et al*., 2019), and grapevine (Savocchia *et al*., 2005; Bester *et al*., 2007, Amponsah *et al*., 2012; Pitt *et al*., 2012).

Captan was also identified in this study as a possible candidate for management of Botryosphaeria dieback of walnuts. As a multi-site contact fungicide, this compound is at low risk for the development of fungicide resistance. Although captan *in vitro* inhibited mycelium growth at a relatively high concentration, it effectively

inhibited conidium germination of *D. seriata* (EC_{50} = 5.40 mg L^{-1}) and *N. parvum* (EC_{50} = 7.04 mg L^{-1}) with a mean EC_{50} of 6.22 mg L^{-1}. This result is consistent with those from previous studies on almond and blueberry that have reported high EC_{50} values for captan on *Botryosphaeriaceae* mycelium inhibition but lower values for inhibition of conidium germination (Olmo *et al*., 2017; Tennakoon *et al*., 2019). In Australia, captan is approved for use in pistachios for control of anthracnose (caused by *Colletotrichum acutatum*), and in almonds for control of anthracnose, blossom blight (*Monolinia laxa*), shot hole (*Wilsonomyces carpophilum*), and nut scab (*Cladosporium carpophilum*). Therefore, captan may provide disease management options when *Botryosphaeriaceae* spore populations and/or spore dispersal are high within nut orchards. Furthermore, incorporating a moderately effective fungicide in fungicide rotations is an anti-resistance strategy, since it reduces selection pressure on fungal pathogen populations (Brent and Hollomon, 2007).

The fungicides tested in the present study varied in their inhibitory effects on mycelium growth and conidium germination of pathogens causing Botryosphaeria dieback. Tebuconazole was the most effective for inhibiting mycelium growth of the pathogens, whereas its efficacy was less (ranked fifth of the ten fungicides studied) for reducing conidium germination. In contrast, pyraclostrobin was the most effective against conidium germination, but only fifth for reducing mycelium growth. Previous studies on management of *Botryosphaeriaceae* species in grapevine and blueberries in New Zealand have also reported that fungicides effective in reducing mycelium growth of pathogens generally did not perform well as inhibitors of conidium germination, and *vice versa* (Amponsah *et al*., 2012; Tennakoon *et al*., 2019). Although there was variation in the levels of efficacy among the fungicides, both pyraclostrobin and tebuconazole were among the top five fungicides for inhibition of both conidium germination and mycelium growth, confirming their important potential roles in Botryosphaeria dieback management programmes for walnut.

Tebuconazole and prochloraz, the two DMI fungicides assessed in this study, were among the top three compounds for inhibition of pathogen mycelium growth, but had less efficacy against conidium germination. Torres *et al*. (2013) reported that mycelia were more sensitive to DMIs than were conidia, in *Diplodia* and *Neofusicoccum* spp. associated with Botryosphaeria canker of grapevine. This response is attributed to the generic mode of action of the DMI fungicides, that inhibit sterol biosynthesis in fungi in the formation of cell membranes. This mode of action may be more effective against mycelium extension than for inhibition of conidium germination (Tennakoon *et al*., 2019).

The *in vitro* assessments of conidium germination showed that *N. parvum* was less sensitive than *D. seriata* to all the ten assessed fungicides. This result indicates a risk to the walnut industry, since *N. parvum* is one of the two most prevalent pathogens recovered from the Australian walnut orchards. This fungus caused lesions on green shoots and 1-year-old stems of glasshouse plants that were three times larger than those caused by *D. seriata*. This is similar to results from studies in major walnut-growing countries including Chile (Luna *et al*., 2022), China (Yu *et al*., 2015), Spain (López-Moral *et al*., 2020), and the United States of America (Chen *et al*., 2014), where *N. parvum* is considered one of the most aggressive pathogens causing walnut tree cankers and dieback. However, *N. parvum* was isolated only from two walnut-growing regions of Australia, New South Wales and Victoria both in southeastern Australia (Antony *et al*., 2023a). Furthermore, *N. macroclavatum*, isolated from a walnut orchard in southwestern Western Australia (Antony *et al*., 2023a), was similar to *N. parvum* in its sensitivity to these fungicides. Disease management programmes in these regions should take account of this occurrence, and ongoing monitoring is necessary for presence and/or absence of *Neofusicoccum* spp. in all walnut-growing regions of Australia.

The fungicides assessed in this study included one QoI (pyraclostrobin), two DMIs (prochloraz, tebuconazole) and four SDHIs (boscalid, fluopyram, fluxapyroxad, penthiopyrad), chemical classes that have been implicated in widespread development of fungicide resistance among target organisms. For instance, a study during 2010 to 2012 in strawberry fields in Florida found that 87% of the *B. cinerea* isolates tested were resistant to pyraclostrobin (Amiri *et al*., 2013). Resistance at this high level would mean complete failure of disease control and complete crop loss. In Australia, pyraclostrobin (QoI) and tebuconazole (DMI) were first approved by the APVMA in 2017 for disease management in walnut orchards (Lang and Simpson, 2018), pyraclostrobin for controlling Botryosphaeria dieback and tebuconazole for managing apical necrosis (*Alternaria* spp. and *Fusarium* spp.). Since then, the Australian walnut industry has used multiple applications of these two fungicides in each growing season for 5 years, until a mixture of fluopyram and tebuconazole was approved in 2022. Resistances to QoI and DMI fungicides have been reported in other crops following field applications over 2 years for QoIs and 7 years for DMIs (Brent and Hollomon, 2007). Therefore, field application of pyraclostrobin and tebuconazole for more than 7 years warrants careful monitoring for any changes

in pathogen sensitivity to these fungicides. The present study did not determine whether reduced efficacy of pyraclostrobin in the glasshouse and field experiments, compared to the tebuconazole formulations, was due to multiple applications of pyraclostrobin in commercial walnut orchards over the previous 6 years. Establishing baseline sensitivity data of the *Botryosphaeriaceae* spp. recovered from Australian walnut orchards, for fungicides to be included in rotation programmes, is an aspect requiring further research.

This study found that efficacy of the mixture of fluopyram (SDHI) and tebuconazole gave similar efficacy to formulation containing only tebuconazole. After a comprehensive review of experimental and modelling evidence, van den Bosch *et al*. (2014) supported use of fungicide mixtures containing chemicals with different modes of action as a resistance management strategy, even if the individual components belonged to at-risk categories for resistance development. However, use of mixtures with SDHIs requires careful monitoring for emergence of cross-resistance to other SDHIs. Cross-resistance patterns between boscalid and other SDHIs including fluopyram have been reported in other crops, and a fluopyram-resistant isolate of *A. alternata* showing cross resistance to other SDHIs was detected in pistachios (Avenot *et al*., 2014; Avenot *et al*., 2019). Shifts in sensitivity to SDHI constituents of fungicide mixtures should be monitored. Even with constituents other than SDHIs, it is possible that applications over successive years may lead to the survival of *Botryosphaeriaceae* isolates with high levels of resistance to the constituent fungicides. Therefore, strategies beyond using mixtures of at-risk fungicides are important for maintaining effective fungicide application programmes.

The detached stem assays, glasshouse experiments and field trial outlined here consistently showed that all the fungicides assessed were more effective when applied as preventative treatments than as curative treatments. This is similar to the results of Díaz and Latorre (2013), who found that regardless of the fungicide and application method, the treatments applied 24 h before inoculations with grapevine trunk disease pathogens gave better protection than treatments applied 24 h post-inoculation. The field trial also showed that the tebuconazole, tebuconazole + fluopyram and pyraclostrobin formulations gave high preventative efficacy for 7 d, and high curative efficacy for 3 d. The preventative efficacy was moderate for 14 d and curative efficacies were also moderate but only for 7 d. The present study used artificial inoculations with high numbers (~500) of conidia in the field experiment. In the natural inoculation processes, host wounds may not be exposed to this amount of inoculum, so under field conditions with natural inoculation in commercial orchards, tebuconazole, tebuconazole + fluopyram and pyraclostrobin may have better preventative and curative efficacy for longer periods (e.g. 2 weeks of preventative protection and 1 week of curative protection).

Periods of preventative and curative efficacy should be considered in light of host wound susceptibility periods, as has been reported to be greatest during the first week following wounding (Antony *et al*., 2023b). Therefore, an application of one of the three fungicides within 3 to 7 d following wounding may provide protection against *Botryosphaeriaceae* pathogens for 2 weeks. Similarly, for the multi-site contact fungicide fluazinam, an application within 3 d post wounding may provide effective protection for 1 week. These conclusions align with those from studies on grapevines (Sosnowski *et al*., 2017; Ayres *et al*., 2022). In field trials in California, Michailides *et al*. (2016) confirmed that most of the fungicides tested had long-term effects for reducing Botryosphaeria dieback in walnuts. However, the long-term effect may depend upon whether the fungicide has systemic or contact activity, and on the prevailing weather conditions such as rainfall at the time of fungicide application that may dilute or wash the fungicides off plant surfaces. These factors should be considered when identifying times for host pruning and fungicide applications.

This study has shown that the fungicides assessed as preventative and curative treatments in the glasshouse experiment prevented pathogen progression beyond the host lesion margins. Colonisation of *Botryosphaeriaceae* beyond lesion edges has been reported in previous studies in walnut, leading to recommendations that, to ensure the complete removal of infected wood, at least approx. 5 cm of the wood beyond the lesion edges should be removed (Michailides *et al*., 2016; Moral *et al*., 2019). For the isolates of *N. parvum* used in the present study, Antony *et al*. (2023b) have previously shown that in glasshouse plants, colonisation distances during 12 months on 1-year-old stems were up to 44 mm beyond lesion edges. In the present study, zero pathogen re-isolation at 5 mm and 10 mm beyond lesion edges 12 months after inoculations indicates that, when the time between treatment and pathogen exposure is less than 24 h, the assessed fungicides prevented further *Botryosphaeriaceae* colonisation, indicating efficacy of the treatments as wound protectants.

Although all the ten active ingredients assessed in this study were effective for inhibiting conidium germination of the pathogens, only four (tebuconazole, tebuconazole + fluopyram, pyraclostrobin, fluazinam) were tested under field conditions, and variations were

observed in duration of efficacy between fluazinam and the other fungicides. Developing an effective fungicide rotation programme will require establishment of the protective and curative efficacy periods for the other fungicides that were assessed in the present study.

While this study evaluated efficacy of fungicides for inhibition of *in vitro* mycelium growth for all five fungi recovered from Australian walnut orchards, further experiments on effects on conidium germination, detached stems, and glasshouse plants targeted only the two prevalent species, *D. seriata* and *N. parvum*. These two fungi were also the most virulent among the species recovered from Australian walnut orchards, with *N. parvum* more virulent than *D. seriata,* so these fungi were the focus of the study. Considering the intra-species variation in virulence among the isolates of these species on various types of walnut tissue (Antony *et al*., 2023b), to ensure the validity of the results for informing fungicide management strategies for the Australian walnut industry, three isolates were used for each prevailing pathogen. However, for the field experiment, *N. parvum* was selected because of its aggressiveness and potential for damaging productivity of the Australian walnut industry in Victoria and New South Wales. While the national survey recovered *N. parvum* from two states, *D. seriata* was recovered from all the walnut-growing regions in five Australian states. Further field trials are required, particularly for *D. seriata* and in walnut-growing regions of South Australia, where all the isolates recovered were identified as *D. seriata* (Antony *et al*., 2023a).

This study investigated efficacy of fungicides for reducing disease incidence on wounds created by tip pruning of walnut stems, since infections by the *Botryosphaeriaceae* are primarily through host wounds (Slippers and Wingfield, 2007; Adaskaveg *et al*., 2022). The present study has provided useful recommendations for protection of walnut trees from Botryosphaeria dieback when specific orchard activities (e.g., hedge pruning, tree training, canopy management) are employed that cause injuries to trees. There are also other factors that could contribute to disease pressure in walnut orchards. These include: latent infections by *Botryosphaeriaceae* (Slippers and Wingfield, 2007), internal infections from previous season's infected fruits that lead to invasion of pathogens into host spurs through peduncles (Michailides *et al*., 2014; Adaskaveg *et al*., 2017), and ability of *N. parvum* to penetrate non-wounded green tissues (Antony *et al.,* 2023a). To address these issues, development of integrated disease management is warranted, which incorporates rotational fungicide programmes with biological control agents, along with cultural and crop management strategies, including orchard hygiene, appropriately timed tree training practices and selection of disease-resistant cultivars.

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