Compatibility of *Metarhizium anisopliae* and *Beauveria bassiana* with insecticides and fungicides used in macadamia production in Australia

Short running title: Compatibility of fungal entomopathogens with insecticides and fungicides

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This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1002/ps.6065

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Abstract

BACKGROUND: Integrating fungal biocontrol agents into crop protection programs dominated by synthetic pesticides is an important first step towards developing an IPM program, however their successful integration relies on an understanding of how their performance may be impacted by the remaining agrochemicals deployed for managing other pests and diseases. In this study we tested ten formulated pesticides used in macadamia production at different concentrations to determine their effects on the germination, mycelial growth and sporulation of *Metarhizium anisopliae* and *Beauveria bassiana in vitro.* Further tests with laboratory grade actives of the non-compatible pesticides were conducted to determine whether any antagonistic effects were caused by the active constituent or by formulation additives.

RESULTS: At their registered concentrations, formulated trichlorfon, acephate and indoxacarb were compatible with *M. anisopliae*, whereas *B. bassiana* showed compatibility with formulated trichlorfon, acephate, indoxacarb, sulfoxaflor and spinetoram. Bioassays using laboratory grade active constituents indicated that the adverse impact of formulated beta-cyfluthrin on both fungal species and that of formulated methidathion on *B. bassiana* is probably due to components of the emulsifiable concentrate formulations rather than their active constituents. Diazinon was the only insecticidal active that showed high toxicity to both fungal species. The two fungicides, carbendazim and pyraclostrobin, were toxic to both fungal species at all tested concentrations.

CONCLUSION: Our results identify which pesticides used on macadamias in Australia are compatible and incompatible with entomopathogenic fungi. Future studies on pesticides degradation rates will help define the spray intervals required to eliminate these adverse effects.

Keywords: biological index, compatibility, entomopathogenic fungi, fungicides, insecticides

1. INTRODUCTION

Macadamias (*Macadamia integrifolia* Maiden and Betche and *M. tetraphylla* L. Johnson) are the second largest nut crop grown in Australia with a total farm-gate value of AUD 285 million and retail value of more than AUD 850 million.[1, 2] The crop is susceptible to various pests and diseases and to control them a number of insecticides and fungicides have been registered.[3] Although these agrochemicals are widely used, the Australian macadamia industry is committed to the development of an Integrated Pest and Disease Management (IPDM) program, reducing the use of broad spectrum chemicals and integrating biological control agents (BCAs) into pest management practices in order to conserve beneficial insects and protect the environment in the macadamia agro-ecosystem.[2]

The entomopathogenic fungi *Metarhizium anisopliae* (Metschn.) Sorokin and *Beauveria bassiana* (Bals. -Criv.) Vuill. are among the main fungal BCAs with cosmopolitan distributions[4, 5] and they have shown potential for controlling many economically important insect pests in horticultural crops.[6-8] However, to achieve effective control (> 90%) high inoculum rates are required to cause sufficient levels of infection within the pest population. The integration of entomopathogenic fungi with low application rates of insecticides has been shown to improve their efficacy,[9-11] and several mechanisms have been suggested for this interaction. The insecticides could be acting as a general stressor by weakening the insect cuticle,[12, 13] reducing the target pest's mobility due to paralysis caused by the insecticides, or disrupting the removal of fungal conidia via grooming behaviour[14, 15] and causing the insect to be more vulnerable to the attachment and entry of fungal entomopathogens.

Many insecticides have been recognised as compatible with entomopathogenic fungi[9-11] but some have been shown to be antagonistic.[16-18] However, most studies that have identified antagonistic interactions have been unable to identify the underlying cause of these adverse responses. Morris[19] found that components of insecticide formulations may play an important role in compatibility with bacterial entomopathogens, especially with respect to emulsifiers and similar additives. Similarly, Anderson and Roberts[20] found that emulsifiable concentration (EC) formulations of commercial insecticides had negative impacts on *B. bassiana*. Components of the EC formulations, particularly, toluene and similar aromatic solvents, were identified as toxic to *B. bassiana*.[20] In contrast to insecticides, fungicides (regardless of formulation type) are always toxic to fungal entomopathogens.[21-23]

Recent findings have demonstrated the potential of entomopathogenic fungi for controlling insect pests on macadamias (e.g. macadamia seed weevil, *Kuschelorhynchus macadamiae* Jennings and Oberprieler),[24] but as the industry still relies heavily on pesticides to minimise pest and disease problems,[3, 25] the use of entomopathogens in the field requires an understanding of the impact of each of these pesticides on the fungi. In this study, we evaluated the impact of eight common insecticides and two fungicides used in macadamia production in Australia on the germination, mycelial growth and sporulation of the entomopathogenic fungi *M. anisopliae* and *B. bassiana,* and sought to identify the cause of fungal inhibition by testing laboratory grade actives of the incompatible formulated insecticides and fungicides.

2. MATERIALS AND METHODS

2.1 Fungal isolates

Isolates of *M. anisopliae* and *B. bassiana* used in this study are listed in Table 1. The Velifer® biological insecticide (BASF Australia Ltd, Melbourne) is a commercial oil-based *B. bassiana* strain PPRI 5339 formulation containing at least 8 x 10^9 viable conidia.mL^{-1}, whereas PPRI 5339 is the *B. bassiana* fungal strain isolated from Velifer® biological insecticide. To obtain PPRI 5339, Velifer® biological insecticide was applied to macadamia seed weevils and later the conidia that emerged from cadavers were sampled and cultured on malt extract agar (MEA, 30 g malt extract (Merck Pty Ltd, Melbourne), 10 g peptone (Bio-Strategy Ltd, Melbourne), 15 g agar (Bio-Strategy Ltd, Melbourne) and 1000 mL water) media using a single conidium technique.[26]

Isolates of *M. anisopliae* were cultured on sterile Sabouraud dextrose agar (10 g peptone, 40 g dextrose (Bio-Strategy Ltd, Melbourne), 15 g agar and 1000 mL of water),[27] supplemented with 1% (w/v) yeast extract (Merck Pty Ltd, Melbourne) (SDAY) and isolates of *B. bassiana* were cultured on MEA. Malt Extract Agar and SDAY media are routinely used to grow *B. bassiana* and *M. anisopliae*[28] and in our study, isolates of *M. anisopliae* and *B. bassiana* grew best on SDAY and MEA respectively. These media were consequently used for all our cultures, ensuring that each fungal species responded appropriately to the insecticides and fungicides in the *in vitro* study while avoiding any indirect negative effects of potentially suboptimal media. All fungal isolates were incubated in the dark at 25 ± 1°C for 15 days before harvesting the conidia for experimentation.

2.2 Response of *M. anisopliae* and *B. bassiana* isolates to spinetoram treated media

The formulated insecticides and fungicides used in macadamia production in Australia that we assessed are listed in Table 2. The insecticide spinetoram (Success® Neo, Dow Agrosciences

Australia Limited) was selected at random for testing the response of a number of isolates of *M. anisopliae* and *B. bassiana*, with a view to determining if all isolates were likely to respond to insecticide exposure in a uniform way. As fungicides often have severe detrimental effects on fungal entomopathogens[29, 30] these were avoided in this experiment as their use could have obscured more subtle variations in the response of different isolates.

The test method used in this study was based on established guidelines for testing the side effects of pesticides on entomopathogenic fungi.[31] Firstly, a stock suspension of spinetoram was prepared at the concentration of 50 times the full field concentration (FFC) of 24 mg $AI.L^{-1}$. The selective media for each fungus (SDAY media for *M. anisopliae* and MEA media for *B. bassiana*) was sterilized (121°C for 15 min) and cooled to 45 – 55°C. Spinetoram stock suspension was then added at either 1/50 or 1/100 the total volume of media in order to create toxic media at 100% and 50% of the FFC, respectively. The liquid media was gently inverted for 20 sec then poured into 90 mm diameter sterile Petri dishes.

To test the response of *M. anisopliae* and *B. bassiana* isolates to spinetoram, the germination, mycelial growth and sporulation of each fungal isolate was measured. Spinetoram at concentrations of 100%, 50% and 0% (control) of its FFC were used to evaluate the response of 12 fungal isolates (six of each fungal species). This experiment was replicated five times at 24 h intervals. For each replicate, the conidial suspension of each isolate was prepared independently from one of five separate fungal plates.

Prior to inoculation of each replicate, the fungal conidia were harvested from sporulated cultures by scraping the surface of the agar plates with sterile spatulas and dispersing the conidia in sterile water containing 0.05% v/v Tween®20 (Sigma-Aldrich, Sydney). Each suspension was homogenised by vortexing for 5 min and the conidial concentration was calculated using a

haemocytometer (Laboroptik Ltd, Lancing, UK) and an Olympus BX53 compound microscope (400x) equipped with a digital camera (DP74, Olympus Australia, Melbourne). The suspensions were adjusted to 1 x 10^4 conidia.mL^{-1} by dilution with Tween®20 (0.05% v/v).

For conidia germination, 20 µL of conidial suspension at a concentration of 1 x 10^4 conidia.mL^{-1} was spread evenly on a block (4 cm^2) of SDAY or MEA toxic media on a sterile glass slide. The slides were placed inside sterile Petri dishes lined with filter paper dampened with sterile distilled water and incubated at 25 ± 1°C in the dark. After 18 h of incubation, percentage conidial germination was determined from 100 – 200 conidial counts per slide using an Olympus BX53 compound microscope (400x). The conidia were considered to have germinated if the germ-tubes were twice the diameter of the propagule.[27]

For mycelial growth, 10 µL of conidial suspension at the concentration of 1 x 10^4 conidia.mL^{-1} was inoculated in the centre of SDAY or MEA toxic media, double sealed with Parafilm® and incubated at 25 ± 1°C for 15 days. Radial growth of the colony was measured on days 5, 10 and 15 after inoculation.

To determine sporulation levels, the mycelial mat was harvested 15 days after inoculation by scraping the entire surface of the colony with a sterile spatula and suspending the dislodged conidia in 10 mL of sterile Tween®20 (0.05% v/v) and homogenised by vortexing for 5 min. The conidial concentration was determined using a haemocytometer as described previously.

2.3 Response of QS155 and B50 to media containing pesticides registered for use on macadamia in Australia

Metarhizium anisopliae QS155 and *B. bassiana* B50 were selected for this experiment because they showed similar responses to spinetoram when compared to the other tested *M.*

anisopliae and *B. bassiana* isolates. In this experiment, 10 pesticides (8 insecticides and 2 fungicides; Table 2) at concentrations of 100%, 50%, 25%, 12.5%, 6.25% and 0% (control) of their FFCs were used to check the response of the two fungal species. Conidial germination, mycelial growth and sporulation assessments were conducted as described in section 2.2. Sabouraud dextrose agar with yeast was used for *M. anisopliae* QS155 and MEA media was used for *B. bassiana* B50. This experiment was replicated five times at 24 h intervals. For each replicate the conidial suspension of each isolate was prepared independently from one of five separate fungal plates.

The toxic media containing pesticides at different concentrations was prepared as described in section 2.2. Stock suspensions of pesticides were prepared at concentrations 50 times that of each FFC and added to the warm media (45 – 55˚C) at 1/50, 1/100, 1/200, 1/400 and 1/800 times the total volume of the media in order to achieve toxic media at 100%, 50%, 25%, 12.5% and 6.25% of each FFC, respectively.

2.4 Response of QS155 and B50 to acetone treated media

As commercial formulations of methidathion, diazinon, beta-cyfluthrin, carbendazim and pyraclostrobin were not compatible with either fungal species, the laboratory grade active constituents of these pesticides were used to verify that their antagonistic effects were due to the active ingredients and no other formulation components. These actives needed to be dissolved in acetone to be dispersed into the culture media, and as 3% (v/v) acetone is known to have a negative impact on *B. bassiana*,[20] acetone at lower concentrations was tested for its effects on the fungal cultures prior to the evaluation of laboratory grade actives. Acetone (HPLC grade, ≥ 99.8%, Sigma-Aldrich) was added to sterile distilled water to achieve a 25% v/v acetone stock solution, which was added to warm media at 1/12.5, 1/25 and 1/50 of the total volume of media in order to achieve 2%,

1% and 0.5% v/v acetone media, respectively. Media without acetone was used in the control treatment. Five conidial suspensions of each isolate were prepared independently from five fungal plates and one was used per replicate. Five Petri dishes (replicates) were used per concentration. The germination observations were performed as described in section 2.2. For mycelial growth, the process was similar to that described in section 2.2, except that the sterile Petri dishes had a diameter of 60 mm and mycelial growth was measured three times at 4, 8- and 12-day post-inoculation. For sporulation, the mycelial mat was harvested 12 days after inoculation by scraping the entire sporulation surface with a sterile spatula and the conidia were counted as described in section 2.2.

2.5 Response of QS155 and B50 to media containing laboratory grade active ingredients of incompatible pesticides

Laboratory grade analytical standards of methidathion, diazinon, beta-cyfluthrin, carbendazim and pyraclostrobin were used in this experiment. All five compounds were obtained from Sigma-Aldrich and had purity levels between 96.3 and 99.9%. Five pesticides (three insecticides and two fungicides) at concentrations of 100%, 50% and 25% of their respective FFCs and a control (no pesticide and 0.5% v/v acetone) were used to evaluate the response of *M. anisopliae* QS155 and *B. bassiana* B50. This experiment was replicated five times at 24 h intervals. For each replicate the conidial suspension of each isolate was prepared independently from one of five separate fungal plates.

A stock solution of each pesticide was prepared by dissolving the laboratory grade active in acetone in order to achieve 200 times its FFC. This stock solution was then diluted with sterile distilled water to 50 times its FFC. This was in turn added to the warm media at 1/50, 1/100 and 1/200 time of the total volume of the media in order to provide toxic media at concentrations of 100%, 50%

and 25% of the FFC, respectively. As the toxic media at 50% and 25% of FFC contained acetone at only 0.25% and 0.125%, respectively, 25% acetone stock solution was added to the media to achieve a consistent acetone concentration of 0.5% in each treatment. The observations for conidia germination, mycelial growth and sporulation were made as described in section 2.4.

2.6 Statistical analysis

To determine the compatibility of entomopathogenic fungi with formulated commercial pesticides (section 2.2 and 2.3), acetone (section 2.4) and laboratory grade pesticides (section 2.5), the biological index (BI) proposed by Rossi-Zalaf *et al.*[32] as cited in Alves *et al.*[16] and others[33, 34] was used, calculated as

$$BI = \frac{(47 * VG)+(43 * SP)+(10 * GER)}{100}$$

where *VG* is the percentage of vegetative growth of fungal colony, *SP* is the percentage of colony sporulation and *GER* is the percentage of conidia germination relative to the control. The value of *BI* indicates the level of compatibility where a *BI* value of 0 to 41 = toxic, 42 to 66 = moderately toxic, and more than 66 = compatible. All subsequent analyses were performed in Rstudio[35] Version 1.2.1335 built on R[36] Version 3.5.2.

2.6.1 Analyses of the biological index for M. anisopliae

For the experiments in section 2.2 and 2.4, the Shapiro-Wilk Test[37] for normality and Levene's test for homogeneity of variance using the CAR[38] (Companion to Applied Regression) Version 3.0-3 package were applied, and as data conformed to the assumption of normality, two-way analysis of variance (ANOVA) was used for experiments in section 2.2 and one-way ANOVA was used for experiments in section 2.4. Significant differences between treatment means were identified

with a Tukey adjustment for multiple comparisons using the Lsmeans[39] (Least-Squares means) Version 2.30-0 package.

For the experiments in section 2.3 and 2.5, the assumption of normality was not met, so we used generalised linear mixed models (GLMMs) in order to accommodate data with mixed and random effects.[40, 41] We evaluated the effects of pesticides, concentrations, and their interactions (fixed factors) and replicates (as a random factor) on the biological index of *M. anisopliae* QS155. GLMMs with beta binomial distribution and log-link function were used (following Akaike's Information Criterion) with the glmmTMB[42] (Generalised Linear Mixed Models using Template Model Builder) Version 0.2.3 and BRMS[43, 44] (Bayesian Regression Models using Stan) Version 2.9.0 packages; means were compared with a Tukey adjustment for multiple comparisons using Lsmeans[39] Version 2.30-0 package. As the values for pyraclostrobin were zero for all concentrations it was excluded from the analyses.

2.6.2 Analyses of the biological index for B. bassiana

In the experiments of section 2.2, 2.3 and 2.5, the assumption of data normality was not met, so GLMMs were used in order to accommodate data with mixed and random effects.[40, 41] We assessed main effects and interactions of spinetoram concentrations and fungal isolates (fixed factors) in the experiment in section 2.2, and pesticides and concentrations (fixed factors) in the experiments of sections 2.3 and 2.5. Replicates were treated as random factors in all analyses. Analyses were conducted with beta binomial distributions and log-link functions using the same protocols and analysis packages used for analysis of the *M. anisopliae* data.

In the section 2.4 experiment biological index was analysed using a non-parametric one-way ANOVA (Kruskal-Wallis test) followed by Dunn's post-hoc test using the FSA[45] (Fisheries Stock

Analysis) Version 0.8.25 package with a Bonferroni correction for multiple comparisons, since the data did not fulfil the assumption for an analysis of variance even after transformation.

3. RESULTS

3.1 Response of *M. anisopliae* and *B. bassiana* isolates to spinetoram treated media

There were significant differences in the response of *M. anisopliae* isolates to spinetoram at 50% of FFC (12 mg AI.L^{-1}), with the BI of all isolates between 67 and 81 and showing that formulated spinetoram at this concentration is compatible with *M. anisopliae* ($p < 0.05$, Table 3). At 100% of FFC (24 mg AI.L^{-1}) the BI values fell to between 50 and 62, indicating incompatibility at this concentration. No significant differences were observed between isolates ($p > 0.05$) at this concentration. However, significant differences between means were observed due to spinetoram concentrations ($p < 0.05$).

In contrast, *B. bassiana* isolates showed more variability in their response to spinetoram at the two test concentrations. B48 was not compatible to spinetoram at either 50% or 100% of the FFC (12 and 24 mg AI.L^{-1} respectively), whereas B49 was not compatible with spinetoram only at the higher rate (Table 3). B27, B50, B60 and PPRI 5339 had BI values above 66, showing that they were compatible to spinetoram at both concentrations. B50 showed no significant differences from any of the other isolates at 50% of FFC, except B48 ($p < 0.05$), or from any of the isolates at 100% of FFC ($p > 0.05$). Again, significant differences between means were only observed on PPRI 5339, B27, B49 and B50 isolates due to spinetoram concentrations ($p < 0.05$).

3.2 Response of QS155 and B50 to media containing pesticides registered for use on macadamia in Australia

Significant differences were observed when *M. anisopliae* QS155 was exposed to different pesticides and concentrations ($p < 0.05$, Table 4). Trichlorfon, acephate and indoxacarb at all concentrations were compatible with QS155 whereas beta-cyfluthrin and spinetoram were compatible with QS155 at 50% of their FFCs or lower. Sulfoxaflor was compatible with QS155 at 25% of FFC or lower. However, methidathion and diazinon were moderately toxic at 6.25% and 12.5% of FFC and very toxic to QS155 at higher concentrations. Increasing the concentration of insecticides in the media from 6.25% to 100% of their respective FFCs significantly reduced BI values ($p < 0.05$) for all insecticides except trichlorfon and indoxacarb. Both fungicides (carbendazim and pyraclostrobin) were very toxic to QS155 even at the lowest concentration, 6.25% of FFC.

Differences between the BI values for *B. bassiana* B50 exposed to different pesticides and concentrations were also statistically significant ($p < 0.05$, Table 4). Trichlorfon, acephate, indoxacarb, sulfoxaflor and spinetoram were compatible with B50 at all concentrations, whereas beta-cyfluthrin and methidathion were compatible with B50 only at 25% of their FFCs or lower. Diazinon was compatible with B50 only at 12.5% of its FFC or below. Increasing the concentrations of insecticides in the media from 6.25% to 100% of their respective FFCs significantly reduced the BI values of all insecticides ($p < 0.05$). Both fungicides at all tested concentrations were highly toxic to B50.

3.3 Response of QS155 and B50 to acetone treated media

Acetone at 2% showed a strong toxic effect on both fungal species with the BI values of *M. anisopliae* QS155 and *B. bassiana* B50 decreasing to 44 and 46, respectively. At 1% acetone B50

responded positively with the BI increasing to 84 but QS155 was still quite sensitive (BI = 65). At 0.5% acetone, both fungal species were compatible (BI values 80 – 94). At 2% acetone, BI values were significantly ($p < 0.05$) reduced relative to the 0.5% concentration for both fungal isolates (Table 5).

3.4 Response of QS155 and B50 to media containing laboratory grade active ingredients of incompatible pesticides

The BI of *M. anisopliae* QS155 exposed to different laboratory grade pesticides and concentrations varied significantly ($p < 0.05$, Table 6). Laboratory grade beta-cyfluthrin at all concentrations was compatible with QS155 whereas methidathion showed compatibility with QS155 at 50% of FFC or lower. However, laboratory grade diazinon was toxic to QS155 at all tested concentrations. Both laboratory grade fungicides (carbendazim and pyraclostrobin) were toxic to QS155 even at the lowest concentration (25% of FFC). BI values for beta-cyfluthrin and methidathion were significantly reduced by higher toxicant concentrations ($p < 0.05$).

Significant differences were observed when *B. bassiana* B50 was exposed to different laboratory grade pesticides and concentrations ($p < 0.05$, Table 6). In contrast to the results from QS155, beta-cyfluthrin and methidathion showed compatibility with B50 at all concentrations, diazinon was compatible with B50 only at 25% of its FFC. However, all laboratory grade fungicides at all concentrations were still toxic to B50. The BI values of all insecticides were significantly reduced by higher test concentrations ($p < 0.05$).

4. DISCUSSION

There were very few differences between the BI values of different *M. anisopliae* isolates or *B. bassiana* isolates to formulated spinetoram at 12 and 24 mg AI.L^{-1}, demonstrating the general similarity of responses to this representative agrochemical across isolates. In a study on *B. bassiana* isolates from diverse geographic areas, formulated piperonyl butoxide and permethrin adversely affected all isolates, whilst formulated carbaryl and oxamyl had no adverse impact on any of them.[20] Similarly, Duarte *et al.*[46] found that four *B. bassiana* isolates responded similarly to five pesticides (neem, acephate, thiamethoxam, deltamethrin and methomyl) and Pires *et al.*[47] found that two isolates of *M. anisopliae* showed similar responses to neem, indoxacarb and spinosad, supporting our decision to use single isolates of each fungal species for further testing.

The results of all compatibility tests using *M. anisopliae* QS155 and *B. bassiana* B50 are summarised in Table 7 and show that the tested chemicals fit into several clear categories. The fungicides carbendazim and pyraclostrobin were, perhaps unsurprisingly, highly toxic to both fungal species at all rates tested down to 6.25% of FFC levels (15.6 and 6.2 mg AI.L^{-1} respectively). Relative to their field rates, carbendazim appeared more active than pyraclostrobin against *B. bassiana*, whilst the opposite response occurred with *M. anisopliae*. Pyraclostrobin reduced the BI of *M. anisopliae* to zero at all concentrations tested. Our results agreed with the findings of Moorhouse *et al.*[48] and others[49-51] who found that carbendazim had post-germination fungicidal effects on both species. They found that the conidia of *M. anisopliae* and *B. bassiana* normally germinated in 24 h when they were cultured on media containing carbendazim at concentrations between 55 and 5,500 mg AI.L^{-1} but that mycelial growth was totally inhibited. This agreed with our observation that the conidia produced abnormal, distorted, swollen and stunted germlings after exposure to carbendazim at < 50% of FFC for 48 h, but the cell-walls ruptured after exposure to carbendazim at 100% of the FFC for the same

period. In contrast, studies on pyraclostrobin have shown fungistatic effects on both species at 67 – 600 mg AI.L^{-1} with no conidia germinating in the first 24 h but some poor mycelial growth occuring.[29, 30] Again, this conforms with our observations in which *B. bassiana* conidia enlarged and germinated after exposure to pyraclostrobin at any tested concentrations for 72 h or longer, but that mycelial growth remained stunted after 5 days incubation. The detrimental effect of fungicides was also observed on other fungal taxa pathogenic to invertebrate pests including *Isaria fumosorosea* Wize, *Isaria farinosa* (Holmsk.) Fr. and *Paecilomyces lilacinus* (Thom) Samson.[52-54]

The second category of compounds are those where the formulated products were compatible (BI $\geq$ 66) with the fungi at rates up to 100% of their full field concentrations. These included acephate, trichlorfon and indoxacarb for both species, and sulfoxaflor and spinetoram for *B. bassiana* only. Our results are in accordance with the results of Saito[55] who found that acephate was not toxic to *B. bassiana* even at 1,000 mg AI.L^{-1}. Akbar *et al.*[18] and others[47, 51, 56] found indoxacarb was compatible with *M. anisopliae* and *I. fumosorosea*. To our knowledge, no literature is available on the direct effect of sulfoxaflor and spinetoram on *M. anisopliae* or *B. bassiana*, although Wari *et al.* [57] have conducted bioassays assessing the impact of spinetoram alone and in combination with *B. bassiana* strain GHA against the whitefly *Bemisia tabaci* (Gennadius). Other studies have found that spinosad, which belongs to the same insecticide group as spinetoram, is not toxic to *M. anisopliae*[18] or *B. bassiana*.[47, 58]

The compatibility of trichlorfon with both fungal species that we found in this study contrasts with the findings of other workers. Saito[55] found that trichlorfon at 1,000 mg AI.L^{-1} reduced mycelial growth of *B. bassiana* by 43% and Ayala-Zermeño *et al.*[59] found that trichlorfon at 5,000 mg AI.L^{-1} reduced mycelial growth of *M. anisopliae* and *Paecilomyces fumosoroseus* (Wize) A.H.S. Br. & G. Sm. by 27% and 38%, respectively. At a higher concentration of 8,750 mg AI.L^{-1} trichlorfon

decreased the mycelial growth of *M. anisopliae* by 41% and *P. fumosoroseus* by 70%.[59] Our results showed that trichlorfon at 500 mg $AI.L^{-1}$ reduced the mycelial growth of *B. bassiana* B50 and *M. anisopliae* QS155 by only 14% and 6% respectively, however this relatively minor impact could reflect the relatively low concentrations we tested compared to those evaluated by other workers, particularly Ayala-Zermeño *et al.*[59]

Compounds in the third category are those in emulsifiable concentrate (EC) formulations where the formulated products were moderately to highly toxic at 100% of their FFC levels but showed reduced toxicity when the laboratory grade materials were tested alone. These included beta-cyfluthrin, methidathion and diazinon. There is extensive evidence to show that EC pesticide formulations can have adverse impacts on entomopathogenic fungi. Anderson and Roberts[20] found that EC formulations of permethrin and piperonyl butoxide had negative impacts on six isolates of *B. bassiana* sourced from three separate countries. These formulations were found to contain toluene and similar aromatic solvents, which were toxic to the fungal entomopathogens. Similar negative effects of commercial EC insecticide formulations (chlorpyrifos, indoxacarb, emamectin benzoate, lufenuron, prophenophos, abamectin, diazinon, L-cyhalothrin, cypermethrin and methidathion) were also observed on *M. anisopliae* by Asi *et al.*[17] and a number of other authors[34, 49, 58, 60]. The adverse effects of EC formulation components on entomopathogens is not confined to those used with insecticides. Emulsifiable concentrate formulations of acaricides (amitraz, pyridaphenthion and pyridine) are very toxic to *B. bassiana*,[61] and herbicides formulated as ECs (e.g. flurochloridone and pendimethalin) are also antagonistic to this species.[21, 34]

Integrating entomopathogens into the pest management plan for any crop requires an understanding of the potential adverse effects of agrochemicals that may be applied before, after, or with the entomopathogen. It is also important to understand whether these adverse effects are caused

by the active ingredient (and are therefore probably intractable), or whether they are associated with other components of the formulation and have the potential to be reduced or eliminated through the substitution of particular additives or the development of alternative formulation types. This study has shown that the fungicides carbendazim and pyraclostrobin are inherently detrimental to *M. anisopliae* and *B. bassiana* and their application to control fungal diseases of macadamias will largely eliminate these entomopathogens if they have been previously applied or are present naturally. Residual concentrations of these compounds on plant surfaces will need to fall by well over 93% before the application of entomopathogens will be likely to provide reasonable levels of insect infection, and field studies on residue dynamics will be needed to determine the time periods required to achieve these levels of chemical breakdown.

Whilst our bioassays have reinforced earlier findings that components of EC formulations can have adverse impacts on entomopathogenic fungi, these components are used for specific reasons such as enhancing product efficacy and surface wetting, and for providing uniform spray mixtures with active ingredients that often have very low water solubilities. The use of formulated products is therefore unavoidable, however our data provides the basis for selecting formulated products that, when timed appropriately, can be used to target various macadamia pests without compromising the benefits derived from *M. anisopliae* and *B. bassiana* applications.

Our data shows that EC formulations of methidathion and diazinon remain moderately toxic to *M. anisopliae* even at 6.25% of their FFC values. In contrast, formulated acephate, indoxacarb and trichlorfon are compatible with *M. anisopliae* at rates up to and including their FFCs of 75 and 500 mg AI.L^{-1} respectively, and could be applied at the same time as *M. anisopliae*, although further work would be required to determine their compatibility in tank mixes. Applications of the remaining insecticides (beta-cyfluthrin, sulfoxaflor and spinetoram) will be likely to need a buffer period for at

least partial breakdown before *M. anisopliae* is applied unless the fungus can be formulated in a way that provides the conidia with some protection.

Beauveria bassiana was generally less affected by the formulated insecticides than *M. anisopliae*; however, diazinon remains problematic for both fungal species due to the toxicity of the active and the high spray concentration routinely used against macadamia pests in Australia. Five insecticides, acephate, trichlorfon, indoxacarb, sulfoxaflor and spinetoram, were all compatible with *B. bassiana* at 100% of their full field concentrations.

If there is sufficient market incentive the substitution of EC formulation additives with alternative emulsifiers and adjuvants may lower the impact of formulated products on entomopathogenic fungi. However, formulation changes can be made by manufacturers for other reasons and initiate the reverse effect, effectively making a formulation more toxic to an entomopathogen rather than reducing its toxicity. There is generally only limited disclosure of formulation components on product labels, and in many jurisdictions, there is no requirement to advise end-users of a change in formulation constituents other than those involving the active ingredient. As a consequence, industries integrating entomopathogens into crop protection programs need to monitor potential adverse pesticide impacts on BCAs and develop crop protection calendars that reflect both the interactions between biological and chemical control agents and the weathering profiles of chemicals under field conditions. Our data and the published literature indicate that emulsifiable concentrate formulations, insecticides applied at high application rates with actives inherently detrimental to fungal germination and growth (such as diazinon), and particularly fungicides pose the greatest risk to successfully introducing entomopathogenic fungi into crop protection programs dominated by agrochemicals.

5. CONCLUSION

This study has identified the crop protection compounds that can be safely applied to Australian macadamia orchards where the entomopathogens *M. anisopliae* and/or *B. bassiana* are active, either as natural populations or as a consequence of deliberate application. Some treatments were identified as antagonistic to these fungi, and residue breakdown studies need to be conducted to determine the necessary periods between the application of these treatments and any subsequent entomopathogen applications. With this information it will be possible to conduct more detailed studies on the response of pests such as the macadamia seed weevil to sequential or combination treatments of insecticides and entomopathogens that have the potential to reduce total insecticide inputs and delay or prevent the development of insecticide resistance.

6. ACKNOWLEDGEMENT

Craig Maddox of Wollongbar Primary Industries Institute, NSW Department of Primary Industries is thanked for providing us with pesticide samples. Rachel King, USQ statistician, is thanked for assistance with RStudio. This study was supported by a postgraduate scholarship to the first author from the University of Southern Queensland and was partly funded by Hort Innovation, Australia with the Australian macadamia industry's research and development levy and contributions from the Australian Government.

7. CONFLICT OF INTEREST DECLARATION

The authors declare no conflict of interest.

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Table 1: Fungal isolates used in this study and screened against formulated spinetoram. Known collection localities are all in Australia

Fungal species	Isolate / Accession[†]	Origin / References	Collection Locality	Year	Collector /Provider
Metarhizium anisopliae	B4A1 /BRIP 70268	Soil	Bundaberg	2017	B. Wilson
	DA1 /BRIP 70271	Soil	Bundaberg	2017	B. Wilson
	ECF1 /BRIP 70270	Soil	Rockhampton	2017	B. Wilson
	ECS1 /BRIP 70272	Soil	Rockhampton	2017	B. Wilson
	M81 /BRIP 70266	[62, 63]	Yeerongpilly	2007	D. Leemon
	QS155 /DAR 82480	[64]	Mapuru	2015	R. Dotaona
Beauveria bassiana	B27 /BRIP 70267	*Bovicola ovis*	Yeerongpilly	2005	D. Leemon
	B48 /BRIP 70269	*Kuschelorhynchus macadamiae*	Alstonville	2016	C. Maddox
	B49 /BRIP 70274	*Paropsisterna tigrina*	Lismore	2015	C. Maddox
	B50 /BRIP 70276	*Kuschelorhynchus macadamiae*	Binna Burra	2017	J. Coates
	B60 /BRIP 70275	Unknown	Dutton Park	2017	D. Leemon
	PPRI 5339	Isolated from Velifer® biological insecticide			

[†] BRIP, lodged in the Queensland Plant Pathology Herbarium, Queensland Department of Agriculture and Fisheries, Brisbane; DAR, lodged in the New South Wales Plant Pathology Herbarium, NSW Department of Primary Industries, Orange.

Table 2: Agrochemical treatments used on macadamias in Australia[3] and evaluated in this study

Pesticides	Trade name and formulation type[†]	IRAC[‡] or FRAC[§] codes	Active ingredient (AI)	Application rate (amount.100L^{-1})	Spray concentration (FFC[¶], mg AI.L^{-1})	Manufacturer
Insecticides	Lancer® GR	1B	Acephate 970 g.kg^{-1}	80 g	776	UPL Australia Limited
	Diazinon® EC	1B	Diazinon 800 g.L^{-1}	125 mL	1000	Amgrow Pty Ltd
	Suprathion® EC	1B	Methidathion 400 g.L^{-1}	125 mL	500	Adama Australia Pty Limited
	Tyranex® SL	1B	Trichlorfon 500 g.L^{-1}	100 mL	500	Imtrade Australia Pty Ltd
	Bulldock® EC	3A	Beta-cyfluthrin 25 g.L^{-1}	50 mL	12.5	Bayer Crop Science Pty Ltd
	Transform® SC	4C	Sulfoxaflor 240 g.L^{-1}	40 mL	96	Dow Agrosciences Australia Limited
	Success® Neo SC	5	Spinetoram 120 g.L^{-1}	20 mL	24	Dow Agrosciences Australia Limited
	Avatar® WG	22A	Indoxacarb 300 g.kg^{-1}	25 g	75	FMC Australia Pty Ltd
Fungicides	Howzat® SC	1	Carbendazim 500 g.L^{-1}	50 mL	250	Adama Australia Pty Limited
	Cabrio® EC	11	Pyraclostrobin 250 g.L^{-1}	40 mL	100	BASF Australia Ltd

[†] GR, granular; EC, emulsifiable concentrate; SL, suspension liquid; SC, suspension concentrate; WG, wettable granule.

[‡] IRAC, Insecticides Resistance Action Committee.

[§] FRAC, Fungicide Resistance Action Committee.

[¶] FFC, full field concentration.

Table 3: Biological index (BI) of the response of *Metarhizium anisopliae* and *Beauveria bassiana* to spinetoram at 50% and 100% of full field concentration (FFC) (12 and 24 mg AI.L^{-1} respectively)

		Spinetoram concentrations	
Fungal species	Isolate / Accession	50% of FFC (%) ± SE	100% of FFC (%) ± SE
Metarhizium anisopliae[†]	QS155 /DAR 82480	81.34 ± 5.25 Aa	61.38 ± 3.63 Ab
	B4A1 /BRIP 70268	78.55 ± 2.85 ABa	62.48 ± 4.75 Ab
	ECS1 /BRIP 70272	76.74 ± 1.32 ABa	54.33 ± 1.20 Ab
	ECF1 /BRIP 70270	71.41 ± 1.42 ABa	60.07 ± 2.60 Ab
	M81 /BRIP 70266	67.08 ± 2.71 Ba	54.37 ± 1.39 Ab
	DA1 /BRIP 70271	67.50 ± 4.56 Ba	50.79 ± 2.76 Ab
Beauveria bassiana[‡]	PPRI 5339	94.30 ± 1.69 Aa	82.77 ± 3.68 Ab
	B49 /BRIP 70274	86.91 ± 5.54 Aa	64.12 ± 4.81 Bb
	B50 /BRIP 70276	85.55 ± 5.44 Aa	69.00 ± 0.94 ABb
	B60 /BRIP 70275	82.75 ± 4.34 Aa	78.23 ± 5.26 ABa
	B27 /BRIP 70267	80.62 ± 4.90 ABa	68.13 ± 2.19 ABb
	B48 /BRIP 70269	64.31 ± 3.37 Ba	60.93 ± 2.96 Ba

[†] $F_{(5, 48)} = 4.86$, $p < 0.01$ (for isolate factor), $F_{(1, 48)} = 81.32$, $p < 0.001$ (for concentration factor), $F_{(5, 48)} = 0.87$, $p > 0.05$ (for interaction). Means followed by different upper-case letters in columns and lower-case letters in rows indicate significant different (LSMEANS test with Tukey adjustment, $\alpha = 0.05$).

‡ Wald $\chi^2 = 39.67$, df = 5, $p < 0.01$ (for isolate factor), Wald $\chi^2 = 5.47$, df = 1, $p < 0.05$ (for concentration factor), Wald $\chi^2 = 13.8$, df = 5, $p < 0.05$ (for interaction). Means followed by different upper-case letters in columns and lower-case letters in rows indicate significant different (LSMEANS test with Tukey adjustment, $\alpha = 0.05$).

Table 4: Biological index of the response of *Metarhizium anisopliae* QS155 and *Beauveria bassiana* B50 in response to pesticides at 6.25%, 12.5%, 25%, 50% and 100% of their respective full field concentrations (FFCs).

Isolate	Pesticide	Pesticide concentrations				
		6.25% of FFC ± SE	12.5% of FFC ± SE	25% of FFC ± SE	50% of FFC ± SE	100% of FFC ± SE
Metarhizium anisopliae QS155[†]	Trichlorfon	91.24 ± 2.04 Aa	90.23 ± 1.40 ABa	87.75 ± 1.18 Aa	88.52 ± 2.98 Aa	87.47 ± 3.21 Aa
	Acephate	93.28 ± 1.94 Aa	91.30 ± 1.64 Aab	86.35 ± 2.68 Abc	82.40 ± 2.57 Ac	80.94 ± 2.07 Ac
	Indoxacarb	86.03 ± 1.15 Aa	82.93 ± 2.95 BCa	81.74 ± 2.32 Aa	81.16 ± 2.54 ABa	79.90 ± 1.48 Aa
	Beta-cyfluthrin	89.92 ± 1.95 Aa	85.34 ± 2.60 ABCab	78.99 ± 1.48 ABbc	69.97 ± 1.80 CDcd	62.07 ± 4.86 Bd
	Spinetoram	89.94 ± 2.13 Aa	84.47 ± 1.82 ABCab	80.38 ± 2.59 Ab	71.33 ± 2.34 BCc	55.38 ± 4.77 BCd
	Sulfoxaflor	87.57 ± 3.26 Aa	78.42 ± 3.03 Cb	68.84 ± 3.24 Bc	59.45 ± 4.71 Dcd	49.48 ± 6.12 Cd
	Methidathion	64.79 ± 2.32 Ba	48.67 ± 2.49 Db	37.93 ± 1.84 Cc	24.99 ± 1.06 Ed	20.61 ± 1.48 Dd
	Diazinon	63.68 ± 1.83 Ba	48.41 ± 1.82 Db	33.74 ± 1.05 Cc	22.20 ± 3.13 Ed	17.91 ± 0.12 Dd
	Carbendazim	8.26 ± 0.53 Ca	8.21 ± 0.43 Ea	7.59 ± 0.33 Da	7.16 ± 0.40 Fa	5.52 ± 0.20 Ea
	Pyraclostrobin	0.00	0.00	0.00	0.00	0.00
Beauveria bassiana B50[‡]	Trichlorfon	92.52 ± 1.91 ABa	86.57 ± 1.72 ABb	85.87 ± 2.00 Ab	83.49 ± 1.33 ABb	80.66 ± 1.55 Ab
	Acephate	95.22 ± 0.98 Aa	90.84 ± 1.80 Aab	90.18 ± 1.07 Ab	86.56 ± 1.66 Abc	80.53 ± 2.72 Ac
	Indoxacarb	93.40 ± 2.62 Aa	88.36 ± 1.22 ABab	87.88 ± 1.34 Ab	81.24 ± 1.21 ABc	79.15 ± 2.18 ABc
	Sulfoxaflor	94.65 ± 1.30 Aa	91.75 ± 1.62 Aab	88.47 ± 1.77 Abc	84.15 ± 2.41 ABc	76.58 ± 1.67 ABd
	Spinetoram	92.51 ± 1.93 ABa	87.81 ± 2.40 ABab	83.89 ± 1.15 Abc	77.75 ± 2.83 Bcd	70.14 ± 2.30 Bd
	Beta-cyfluthrin	94.88 ± 0.95 Aa	90.59 ± 2.55 Aa	83.03 ± 2.32 Ab	64.03 ± 2.72 Cc	50.48 ± 4.94 Cd
	Methidathion	87.10 ± 1.66 BCa	81.12 ± 0.88 BCa	68.54 ± 1.45 Bb	56.90 ± 0.79 Cc	45.33 ± 3.21 Cd
	Diazinon	81.89 ± 4.11 Ca	73.21 ± 4.22 Cb	57.13 ± 1.90 Cc	38.36 ± 1.89 Dd	29.00 ± 0.81 De
	Pyraclostrobin	21.76 ± 0.41 Da	20.96 ± 0.40 Da	20.83 ± 0.46 Da	17.65 ± 0.78 Ea	8.88 ± 2.23 Eb
	Carbendazim	9.37 ± 0.16 Ea	9.42 ± 0.12 Ea	9.07 ± 0.15 Ea	8.98 ± 0.08 Fa	8.62 ± 0.17 Ea

† Wald χ^2 = 582.59, df = 8, $p < 0.01$ (for pesticide factor), Wald χ^2 = 33.46, df = 4, $p < 0.01$ (for concentration factor), Wald χ^2 = 137.94, df = 32, $p < 0.01$ (for interaction). Means followed by different upper-case letters in columns and lower-case letters in rows indicate significant different (LSMEANS test with a Tukey adjustment, $\alpha = 0.05$).

‡ Wald χ^2 = 1354.75, df = 9, $p < 0.01$ (for pesticide factor), Wald χ^2 = 50.72, df = 4, $p < 0.01$ (for concentration factor), Wald χ^2 = 253.44, df = 36, $p < 0.01$ (for interaction). Means followed by different upper-case letters in columns and lower-case letters in rows indicate significant different (LSMEANS test with a Tukey adjustment, $\alpha = 0.05$).

Table 5: Biological index of the response of *Metarhizium anisopliae* QS155 and *Beauveria bassiana* B50 to media impregnated with acetone at 0.5%, 1% and 2%.

	Acetone concentrations (v/v)		
Fungal species	0.5% (%) ± SE	1% (%) ± SE	2% (%) ± SE
Metarhizium anisopliae QS155[†]	80.19 ± 3.25 a	65.98 ± 1.32 b	44.48 ± 1.65 c
Beauveria bassiana B50[‡]	94.10 ± 1.03 a	84.71 ± 1.78 ab	46.13 ± 0.57 b

[†] $F_{(2, 12)} = 64.63$, $p < 0.001$. Means followed by different lowercase letters are significant different (LSMEANS test with Tukey adjustment, $\alpha = 0.05$).

[‡] Kruskal-Wallis test; $\chi^2=12.5$, df=2, $p < 0.01$. Means followed by different lowercase letters are significant different (Dunn's post hoc test with Bonferroni correction, $p < 0.05$).

Table 6: Biological index measuring the response of *Metarhizium anisopliae* QS155 and *Beauveria bassiana* B50 to laboratory grade pesticides at 25%, 50% and 100% of their respective FFC (full field concentration) values.

		Pesticide concentrations (laboratory grade)		
Isolate	Pesticide	25% of FFC ± SE	50% of FFC ± SE	100% of FFC ± SE
Metarhizium	Beta-cyfluthrin	92.61 ± 2.92 Aa	90.57 ± 3.46 Aab	85.53 ± 2.49 Ab
anisopliae	Methidathion	93.27 ± 2.99 Aa	82.86 ± 5.05 Ab	55.23 ± 1.15 Bc
QS155[†]	Diazinon	41.00 ± 0.80 Ba	39.96 ± 1.26 Ba	35.60 ± 1.17 Ca
	Carbendazim	9.37 ± 0.07 Ca	8.62 ± 0.26 Ca	8.18 ± 0.35 Da
	Pyraclostrobin	0.00	0.00	0.00
Beauveria	Beta-cyfluthrin	98.40 ± 0.35 Aa	91.74 ± 0.69 Ab	87.30 ± 0.94 Ab
bassiana	Methidathion	91.76 ± 0.55 Ba	88.37 ± 0.68 Aa	81.19 ± 1.15 Ab
B50[‡]	Diazinon	78.21 ± 1.43 Ca	59.79 ± 1.15 Bb	45.39 ± 1.64 Bc
	Pyraclostrobin	14.06 ± 0.25 Da	13.99 ± 0.16 Ca	12.87 ± 0.11 Ca
	Carbendazim	9.29 ± 0.07 Da	8.53 ± 0.23 Ca	8.03 ± 0.15 Ca

[†] Wald $\chi^2 = 291.78$, df = 3, $p < 0.01$ (for pesticide factor), Wald $\chi^2 = 7.91$, df = 2, $p < 0.05$ (for concentration factor), Wald $\chi^2 = 49.43$, df = 6, $p < 0.01$ (for interaction). Means followed by different upper-case letters in columns and lower-case letters in rows indicate significant different (LSMEANS test with a Tukey adjustment, $\alpha = 0.05$).

‡ Wald χ^2 = 906.04, df = 4, $p < 0.01$ (for pesticide factor), Wald χ^2 = 34.1, df = 2, $p < 0.01$ (for concentration factor), Wald χ^2 = 58.36, df = 8, $p < 0.01$ (for interaction). Means followed by different upper-case letters in columns and lower-case letters in rows indicate significant different (LSMEANS test with a Tukey adjustment, $\alpha = 0.05$).

Table 7: Summary of the responses of *Metarhizium anisopliae* QS155 and *Beauveria bassiana* B50 to formulated and laboratory grade pesticides used for macadamia crop protection in Australia. Data are biological index (BI) values. FFC, full field concentration. Orange cells, highly toxic (BI ≤ 41); yellow cells, moderately toxic (BI 42 – 66); green cells, compatible (BI ≥ 66).

Active	FFC (mg AI.L^{-1})	Commercial formulations (see table 2)					Laboratory grade material		
		6.25% FFC	12.5% FFC	25% FFC	50% FFC	100% FFC	25% FFC	50% FFC	100% FFC
		Metarhizium anisopliae QS155							
Acephate	776	93	91	86	82	81			
Methidiathion	500	65	49	38	25	21	93	83	55
Diazinon	1000	64	48	34	22	18	41	40	36
Trichlorfon	500	91	90	88	89	87			
Indoxacarb	75	86	83	82	81	80			
Beta-cyfluthrin	12.5	90	85	79	70	62	93	91	86
Sulfoxaflor	96	88	78	69	59	49			
Spinetoram	24	90	84	80	71	55			
Carbendazim	250	8	8	8	7	6	9	9	8
Pyraclostrobin	100	0	0	0	0	0	0	0	0
		Beauveria bassiana B50							
Acephate	776	95	91	90	87	81			
Methidiathion	500	87	81	69	57	45	92	88	81
Diazinon	1000	82	73	57	38	29	78	60	45
Trichlorfon	500	93	87	86	83	81			
Indoxacarb	75	93	88	88	81	79			
Beta-cyfluthrin	12.5	95	91	83	64	50	98	92	87
Sulfoxaflor	96	95	92	88	84	77			

Spinetoram	24	93	88	84	78	70			
Carbendazim	250	9	9	9	9	9	9	9	8
Pyraclostrobin	100	22	21	21	18	9	14	14	13