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Virulence Screen of Beauveria Bassiana Isolates for Australian Carpophilus (Coleoptera: Nitidulidae) Beetle Biocontrol

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Abstract: *Carpophilus* beetles are serious pests of Australian fruit and nut crops, causing significant damage through adult and larval feeding and vectoring plant diseases. Six strains of the entomopathogenic fungus *Beauveria bassiana* ((Balsamo) Vuillemin; Hypocreales: Cordycipitaceae), isolated from a range of hosts in Australia, together with one commercial strain, were screened for virulence to adult and larval stages of *Carpophilus* attacking stone fruits (*C. davidsoni* (Dobson)) and almonds (*C. truncatus* (Murray)) under laboratory conditions. The two species differed significantly in their susceptibility to the *B. bassiana* isolates. In the adult beetle assay, *C. truncatus* had a maximum Abbott's control corrected mortality of 19% when treated with the most effective isolate, B54, compared to 52% for *C. davidsoni*. In larval bioassays, mortality rates for the two species were generally higher than adults: four isolates caused greater than 80% mortality in *C. davidsoni*; while only one isolate was considered effective against *C. truncatus* (causing 73% mortality), all other isolates caused less than 40% mortality. The results indicate promising potential for *B. bassiana* to be applied as a biopesticide as part of an integrated pest management strategy, which might take the form of a soil application against larvae or an autodissemination program using adult beetles.

Keywords: *Carpophilus* beetle; entomopathogenic fungi; Beauveria Bassiana; biological control

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1. Introduction

Almonds and stone fruits are high value crops in Australian domestic and international markets. In the 2019/20 season, Australia produced 104,437 tonnes of almond kernels, with an export value of A$772.6 million [1], in the 2018/19 season 23,013 tonnes of stone fruit worth over A$88 million [2]. *Carpophilus* beetles can cause significant levels of economic damage to fruit and nut produce, both through direct adult and larval feeding and through vectoring bacterial and fungal rot [3]. *Carpophilus truncatus* has recently emerged as the primary insect pest of Australian almonds, with reports of up to 10% loss of kernels and loss to revenue that can exceed A$20 million annually [4]. *Carpophilus davidsoni* is an Australian pest of stone fruit crops that can cause considerable loss of revenue through pre-harvest damage to ripening fruits and increased post-harvest spoilage [5,6].

The control of *Carpophilus* beetles in orchards utilises integrated pest management (IPM), employing a toolkit of complimentary management practices that work in combination to lower population levels to below target thresholds for economic damage [7,8]. Population suppression of *C. truncatus* follows an IPM strategy that relies on orchard hygiene, with labour intensive destruction of residual "mummy" nuts (nuts that remain on the tree after harvest) as one of the most important factors [4].

As these beetles live inside mummy nuts and also in new nuts (where they feed on the almond kernel), chemical insecticide sprays are unable to reach areas where the beetles are feeding, and are thus largely ineffective. Similarly for *C. davidsoni*, orchard hygiene practices are important, and include collection of fallen fruit and treatment of fruit dumps. Attract-and-kill traps have been shown to be effective in *C. davidsoni* pre-harvest population suppression, but these mass trapping strategies decrease in effectiveness as the season progresses [5]. *Carpophilus davidsoni* are more susceptible to chemical insecticide sprays, however pre-harvest intervals of between 7 to 28 days for many insecticides preclude the use of sprays closer to fruit ripening, when the risk of pest outbreak is high [5,9]. Frequent use of broad-spectrum chemical insecticides can also trigger secondary pest outbreaks such as mites, which are otherwise naturally controlled by invertebrate predators [10]. The limitations and environmental concerns associated with chemical insecticides, together with benefits associated with "clean and green" organic production, have driven interest in more sustainable control technologies; in particular biocontrol strategies, which utilise and enhance natural predators and diseases of target pests and fit within an IPM strategy to provide greater control [11].

Entomopathogenic fungi (EPF) are an effective and environmentally safe alternative to chemical insecticides that have been successfully adopted in a range of agricultural IPM systems [12]; including coffee berry borer, where the pests have similarly cryptic habitats to *Carpophilus* spp.; and a range of pests of glasshouse crops, where the requirement for human safety is paramount due to the increased exposure risk [13,14]. The role of EPF in IPM systems is supported by their compatibility with other beneficial organisms; such as bees, which are crucial for the pollination of stone fruit and almond crops [15]. Off-target mortality by EPF to non-pest species can be minimised through the implementation of more targeted application methods such as autoinoculation, where the beetles are used to vector a pathogen to the wider pest population, or direct application to fallen or dumped fruit and nuts to decrease emergence rates [16]. There has been limited research assessing EPF for *Carpophilus* beetles, whilst other biopesticides such as bacterial-based insecticides and entomopathogenic nematodes (EPN) have been tested in laboratory assays and field trials [17–19]. The only EPF study to date assessing mortality on *Carpophilus* spp. investigated the potential of autodissemination of *Beauveria bassiana* for the control of the corn pest *C. lugubris* [20]. Higher mortality was seen in the testing of commercial *B. bassiana* isolate AF-4 compared with strains isolated from *Carpophilus* beetles (which did not elicit significant mortality over the two-week incubation period) highlighting the need for a wide range of strains isolated from more than just the specific target insect [20]. Looking to another nitidulid beetle assay, small hive beetle, adult *Aethina tumida,* showed significantly increased mortality rates from treatment with *B. bassiana* compared with *Metarhizium anisopliae* and *Hirsutella illustris* [21].

With previous nitidulid assays generally indicating higher efficacy from *B. bassiana* isolates and the commercial availability of a *B. bassiana* product in Australia, this species was chosen to screen in the current study. Six *B. bassiana* isolates obtained from a range of adult coleopteran hosts and one isolate from *Musca domestica* were selected to screen for efficacy in killing *C. truncatus* and *C. davidsoni*. Bioassays were conducted that assessed time-dependant mortality over 15 days and mycosis frequency on adult beetles and on final instar larvae assessing mortality after 10 days of incubation.

2. Materials and Methods

2.1. Insects

Adult *C. truncatus* beetles were collected from almond orchards in Robinvale, Victoria between November 2015 and March 2016, and maintained in culture on a diet of soybean meal, almond meal and sugar. *Carpophilus davidsoni* beetles were collected from peach orchards in Invergordon, Victoria in February and March 2016. The *C. davidsoni* culture was maintained on a diet consisting of wheat germ, brewer's yeast, agar and water supplemented with Vanderzant vitamin mixture for insects. All cultures were maintained in environment-controlled cabinets (25 °C, 16:8 light: dark, 40% RH). Pre-pupating larvae were separated and held in containers of moist vermiculite or used in assays. Adults up to

one-week post-eclosion were collected from vermiculite containers for use in bioassays. The sex ratio of the beetle cultures was determined by visual identification of male external morphology in ten samples of ten beetles each: a 50% male-female sex ratio was observed in all cultures.

2.2. Fungal Isolates

Seven isolates of *B. bassiana* were screened in this study. The commercial strain Velifer® (Plant Protection Research Institute (PPRI) 5339) is the only product available in Australia and was supplied by BASF (VIC, Australia). Non-commercial isolates were supplied by the Queensland Department of Agriculture and Fisheries (QLD, Australia) and have accession in the Brisbane pathogen herbaria (BRIP). Details of these isolates are given in Table 1. All fungal isolates were grown on oatmeal agar plates for 2–3 weeks at 25 °C 14:10 (L:D). The sporulating plates were dried over silica gel in a desiccator for 3 days, after which the dry spores were scraped into separate sterile vials and stored at 4 °C. Spore suspensions were prepared for each replicate on each day of experimentation with a new vial of dry spores. Spores of each fungal isolate were suspended in sterile 0.05% Tween 80, numerated with a Neubauer Improved Hemocytometer and adjusted to 10^7 conidia/mL. The viability of the spores in each suspension was assessed for each isolate by plating 100 µL of a 10^7 conidia/mL spore suspension at the end of each assay, onto PDA plates and incubating at 25 °C for 18 h. Conidia were considered to have germinated when the germ tube length was greater than the spore width. The first 100 spores seen were counted, this was replicated four times across different fields of view. Only assays with a germination rate over 90% were considered valid.

Table 1. Details of isolates of *Beauveria bassiana* screened against *Carpophilus* spp.

	Identification	BRIP Accession/ PPRI Registration	Isolate Host	Date Isolated	% Germination ± SE [1]
B37	*B. bassiana*	61,370	*Musca domestica*	September 2006	97.8 ± 0.26cd
B47	*B. bassiana*	61,378	*Aethina tumida*	October 2009	99.3 ± 0.15e
B48	*B. bassiana*	69,570	*Kushlerorhynchus macadamiae*	January 2016	97.1 ± 0.31bc
B49	*B. bassiana*	69,571	*Paropsisterna tigrinia*	2015	94.2 ± 0.54a
B50	*B. bassiana*	69,572	*Kushlerorhynchus macadamiae*	July 2017	94.5 ± 0.39a
B54	*B. bassiana*	70,673	*Alphitobius diaperinus*	May 2018	96.1 ± 0.28b
Vel	*B. bassiana* Trade name "Velifer®"	PPRI 5339	*Conchyloctenia punctate*	*n*/a	98.4 ± 0.19d

[1] Germination rates are means of replicates ± SEM. Treatment means followed by different letters indicate significant difference ($p < 0.05$) through generalised linear model (GLM) binomial regression analysis.

2.3. Adult Bioassay

Spore suspensions (10^7 conidia/mL) in sterile 0.05% Tween 80 were prepared for each fungal isolate, and a 0.5 mL aliquot was added to a 1.5 mL Eppendorf tube before a single adult beetle was immersed for 10 s. Beetles were gently removed from the suspension with a sterile loop and dried on filter paper for 1 min before transfer to a 30 mL incubation cup (3 cm ø, 4 cm h) with a perforated plastic lid lined with a sterile moistened filter paper disc and supplied with a small amount of species-specific diet. Each cup contained 20 insects of mixed sexes. Control beetles were immersed in sterile 0.05% Tween 80. The treatments were randomly arranged in trays (28 × 24 cm) and incubated continuously in the dark at 24 °C ± 1 and 50–60% RH. 100 µL of dH_20 was added every second day to maintain moisture and diet was given *ad libitum*. Mortality was assessed daily for 15 days. Dead beetles were identified by rigid positioning of their legs and failure to respond to blowing of air. Cadavers from all treatments including the control were surface sterilised in 80% ethanol for 5 s, rinsed in distilled water twice and incubated in sealed 90 mm Petri plates containing 1.5% water agar amended with 0.01% chloramphenicol or moistened sterile filter paper. Verification of mycosis was conducted after 5 days by observation of hyphae under a stereomicroscope. Mycosis was only counted if hyphal growth was characteristic of *B. bassiana* (white filamentous growth and spherical spore clumps, shown in Figure S1). Each replicate containing 20 beetles was repeated 3 times for all fungal isolates. The whole experiment

was replicated on three separate days (180 insects per isolate). The order of each strain on each day and beetle species was randomly selected.

2.4. Larval Bioassay

Suspensions of each fungal isolate were prepared as described above to the lower concentration of 10^6 conidia/mL in sterile 0.05% Tween 80. 2 mL of each isolate suspension was mixed with 2 g of vermiculite in a 30 mL (3 cm ø, 4 cm h) plastic cup with a perforated plastic lid. Ten final instar "wandering stage" larvae were added to each cup, comprising one replicate. Five replicates were completed on each day and repeated on three different days. Control treatments contained 2 mL of sterile 0.05% Tween 80. The treatments were randomly arranged in trays (28 × 24 cm) and incubated at 25 °C, 50–60% RH, in the dark in a controlled environment cabinet. All replicates were assessed for mortality after 10 days. Larval mortality was indicated by a lack of movement when grasped with forceps, colour change (extensive brown melanisation spots and/or cream to pink) or mycelial growth (Figure S2 (Supplementary Materials)). During the incubation period some larvae underwent pupation, death of the pupae was indicated by the same features as the larvae.

2.5. Statistical Analysis

Germination rates were analysed with a generalised linear model (GLM) regression with means separated by a post hoc Tukey HSD test. Mortality counts for adult and larval assay replicates were tested for homogeneity of variance with Bartlett's test across the three trial days, and subsequently combined for analysis with a generalised linear model (GLM) regression analysis using a Binomial distribution with either a "logit" or "cloglog" link depending on the equality of mortality/survival [22]. The treatments were separated with a post hoc Tukey HSD test. Data for presentation were adjusted for control mortality with Abbott's correction [23]. Kaplan–Meier survival analysis was used to illustrate time-response relationship for adult assays. Survival curves were separated by significance with a multiple comparisons log-rank test with a family-wise p-value adjustment method of Benjamini and Hochberg [24]. Statistical analyses were conducted with R statistics Base, Emmeans and Survival packages [25–27].

3. Results

3.1. Adult Beetle Bioassay

In experiments using adult insects, *C. truncatus* was found to have the lowest mortality from treatment with *B. bassiana*, with Abbott's corrected mean mortality ranging from 4.4%–19%, compared to *C. davidsoni* mortality, which ranged from 20%–52%. The mortality rates of the two beetle species were significantly different ($p < 0.0001$) (Figure 1). The *C. truncatus* assay showed that the most virulent strain was B54, with total mortality significantly higher than B47, B48 and B49, using GLM regression analysis ($p < 0.05$). Kaplan–Meier Survival analysis comprising cumulative mortality and survival time indicated that the isolate B54 was significantly more virulent than all other test strains except Velifer (Figure 2). Control mortality for this species was 3.9%. *Carpophilus davidsoni* adult beetles were also more susceptible to isolate B54, which was deemed significantly different to all other isolates except B50, using Kaplan–Meier Survival analysis ($p < 0.05$) (Figure 2). GLM regression analysis on total mortality counts indicated a significant difference only between B54 and the less virulent strains B48, B49 and Velifer®. Control mortality for this species was 16.9%. The mortality rate across the test isolates follows a similar trend as C. *truncatus*, but with a greater magnitude of mortality. Comparison of LT50 is not possible from this assay given the <50% total mortality for most treatments.

Mycosis across the two beetle species did not follow a consistent trend across all isolates (Table 2). B54 was, however, among the highest mycosis inducing isolates across both beetle species. For *C. truncatus* the mycosis rate was lowest for Velifer® (at 39%) with all other isolates producing external hyphae in at least 77% of cases with the peak at 98% and 100% for B54 and B48, respectively.

The only statistically significant difference in this case was between B54 and Velifer due to the low replicate numbers obtained in this trial ($p < 0.05$). *Carpophilus davidsoni* overall had a lower rate of mycosis compared with *C. truncatus* ($p < 0.05$), peaking with isolates B49 and B54 at 80% and 81%, respectively. The lowest mycosis rates were related to isolates B37 and B47 with 40% and 56%, respectively, B37 was significantly different ($p < 0.05$) to B48, B50, B54 and B49.

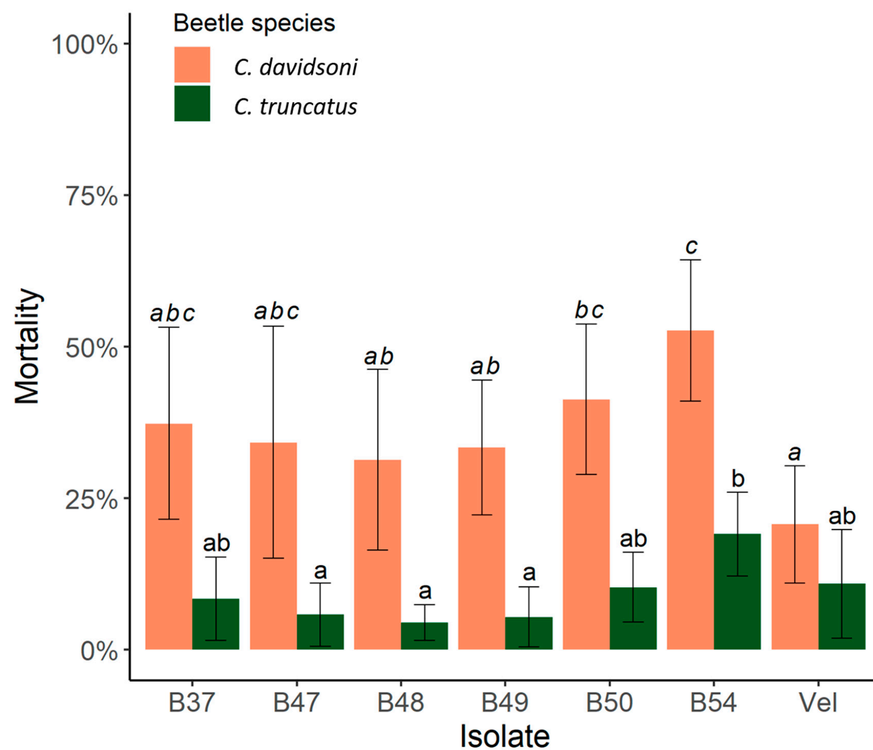

Figure 1. Cumulative Abbotts's corrected mortality of adult *Carpophilus* spp. immersed in a suspension of *B. bassiana* conidia (10^7 conidia/mL) and incubated for 15 days. Different letters denote significant difference ($p < 0.05$) performed on untransformed data with GLM analysis with post-hoc Tukey HSD test within beetle species. Error bars represent 95% confidence intervals.

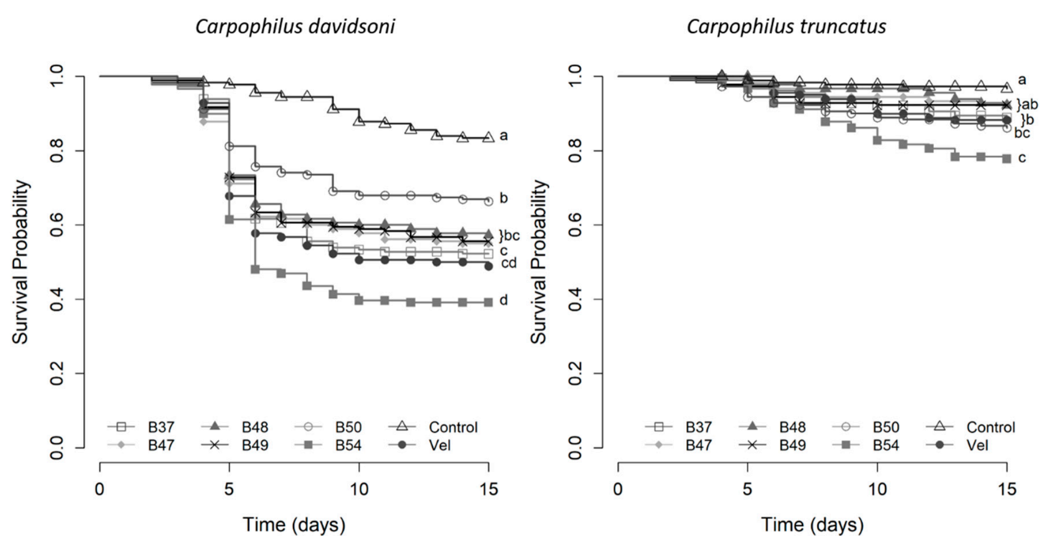

Figure 2. Kaplan–Meier survival curves of adult *C. davidsoni* (**left**) and *C. truncatus* (**right**) indicating time–mortality response. Survival curves followed by different letters denote significant difference ($p < 0.05$) with a multiple comparisons log-rank test.

Table 2. Mean amount of mycosis in surface sterilised adult cadavers of *Carpophilus* spp. incubated for five days after lethal infection with different isolates of *B. bassiana*.

Carpophilus Spp.	B37	B47	B48	B49	B50	B54	Vel	Control
C. davidsoni	40% ± 8.7a	56% ± 8.5ab	73% ± 7.8bc	80% ± 8.5c	69% ± 6bc	81% ± 6c	68% ± 10.1abc	0 ± 0
C. truncatus	75% ± 12.5ab	90% ± 8ab	100% ± 0ab	82% ± 8ab	80% ± 9.2ab	98% ± 2.1b	39% ± 15.5a	0 ± 0

Mycosis rates are means of replicates ± SEM. Treatment means followed by different letters indicate significant difference ($p < 0.05$) through GLM binomial regression analysis.

3.2. Larval Bioassay

For *C. truncatus* the mortality rate, at 73%, was highest in larvae under treatment with the isolate B54 (Figure 3). This isolate was significantly different ($p < 0.0001$) to all other isolates tested on this beetle species, which had mortality rates ranging between 26%–38%. The mortality rate for *C. davidsoni* ranged from 82%–85% for B48, B49, B50 and Velifer® to 68%, 62% and 53% for B54, B37 and B47, respectively. The four higher mortality strains were all significantly different ($p < 0.05$) from the lower two strains. Control mortality for both beetle species was zero. Across the two test beetle species there was no substantial trend of isolate induced mortality.

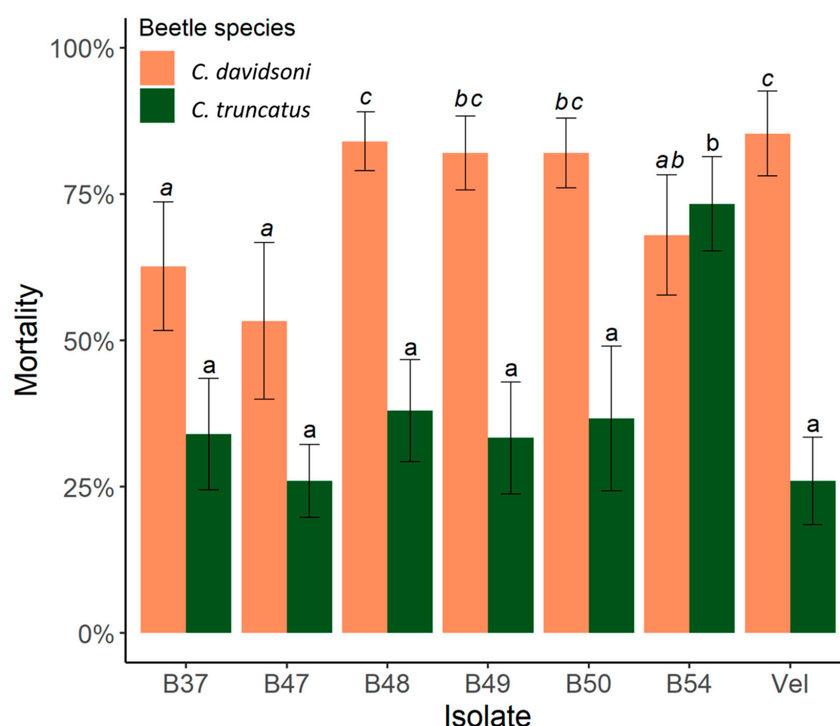

Figure 3. Average mortality rate of larval *Carpophilus* spp. treated with 10^6 conidia/g vermiculite. Different letters denote significant difference ($p < 0.05$) through GLM analysis with post-hoc Tukey HSD test within beetle species. Error bars represent 95% confidence intervals.

4. Discussion

Our study provides encouraging data for the potential development of entomopathogenic fungal (EPF) "biopesticides" to control pest *Carpophilus* beetles in Australia. Whilst all isolates led to significant infection for the larval stage of both insect species, three isolates had no significant infection rate on adult *C. truncatus* beetles. The isolate, B54, was consistently one of the highest performing isolates against both species and life stages.

Adult stages of the two *Carpophilus* species showed considerable differences in the magnitude of mortality following exposure to the *B. bassiana* isolates. The most susceptible of the two beetle species, *C. davidsoni*, had maximum mortality of 52% when inoculated with B54: by comparison, adult *C. truncatus* had a maximum of 19% mortality with the same isolate.

Larval mortality was considerably higher than adult mortality across both *Carpophilus* species in this study. For most isolates *C. davidsoni* was the more susceptible of the two species: the highest mortality of *C. davidsoni* larvae was over 80% when infected with isolates B48, B49, B50 and Velifer®. For *C. truncatus* the mortality rate peaked at 73% with isolate B54, which was significantly higher than all other isolates (which had mortality ranging between 26%–38%). Standard inoculation procedures were employed for both the adult and larval assays to ensure that consistent spore loads were delivered to each insect and results were thus comparable to other EPF bioassays. The immersion treatment protocol used on adult *Carpophilus* beetles in this study gave rise to an average mortality rate that was considerably lower compared to similar EPF assays conducted on other adult Coleoptera: for example nine isolates of *Metarhizium anisopliae* on sweet potato weevil using the same inoculation method, carrier, time and dose-rate, had 100% mortality after 10 days in six of the nine isolates [28]. Similarly, mortality rates between 97.5%–100% for emerald ash borer adults treated with three *B. bassiana* and two *M. anisopliae* isolates under the same application regime, incubated for six days [29]. When comparing larval assay results from another published study, similar larval mortality (up to 85% after 14 days of exposure) was seen with *M. anisopliae* and *B. bassiana* isolates tested on second instar *Delia radicum* larvae with an analogous inoculation procedure: a result that prompted calls for further field trials [30].

The low mortality rates observed from adult *Carpophilus* spp. in these trials when compared with similar assays suggests that direct targeting of the adult life stage may have limited effectiveness in the field. Further study clarifying dose rates with accurate spore loads could determine whether increased dosages have greater effectiveness; and if so, whether these are feasible in terms of field application. By contrast, the high rates of mortality observed in larval assays demonstrate potential for using *B. bassiana* as a biopesticide against *Carpophilus* beetle larvae. Final instar larvae (which were used in this study) might be the most suitable life stage to target with EPF, as during this stage they leave the fruit or nut and burrow into the soil to pupate. Inundative soil application rates of 10^5 to 10^6 conidia per gram of soil are efficacious and economically viable with broadcast applications [31]: the present study applied 10^6 conidia per gram of vermiculite resulting in high mortality rates, indicating that *B. bassiana* may be an effective biopesticide with this application method. The use of a sterile media does increase the efficacy of entomopathogens compared with soil, however nutritive granules can help reduce the fungistatic effect of soil microbiomes [31]. Additionally, abiotic soil factors, such as soil moisture and temperature, present in the field will need to be assessed for suitability for fungal growth [31]. Soil structural qualities that are permissive of the homogenisation of conidia in broad/non-targeted liquid or granular applications will be necessary to support the success of this application strategy [31,32].

Low adult beetle mortality to EPF strains that are highly effective against larvae could be advantageous in an autoinoculation strategy that uses adult beetles to vector the pathogen to early instar larvae inside nuts, where they are protected from chemical pesticides [4,33]. Earlier instar larvae are generally more susceptible to EPF than later instars [34,35]. Transfer of the pathogen between inoculated adults and larvae can occur through direct contact between the insects and through contamination of niches and residual nut habitats through sporulation on mycotic cadavers [33,36]. The high rates of mycosis observed with isolate B54 support the use of an autoinoculation strategy as it presents a greater opportunity for the pathogen to spread among pest groups. Moreover, horizontal transmission of the pathogen may be more prevalent as a result of the aggregating behaviour of *C. davidsoni* and *C. truncatus* [5]. For both these species there are highly effective chemical attractant lures (which combine aggregation pheromone and host volatile blends) that could underpin such a program [5]. The aggregation behaviour of these species, cryptic habitat and readily available chemical lures makes them an ideal target for the autodissemination of a fungal pathogen.

Interestingly, the relatedness of the host species for the isolate was not predictive of virulence in this experiment. Strain B47 (*Aethina tumida*) from the same family as *Carpophilus* (Nitidulidae) was one of the least effective treatments for both species and both life stages whereas isolate B37, isolated from *Musca domestica* (common house fly), had higher mortality for both species. The most effective isolate

across all these groups, B54, was isolated from a lesser mealworm, *Alphitobius diaperinus* (Coleoptera: Tenebrionidae). In another study on host specificity of *B. bassiana* isolates, host order did not correlate with higher pathogenicity to test insects in laboratory bioassays [37]. Genotypic variation of individual insect populations may lead to differences in insect susceptibility to EPF to a greater extent than isolate host specificity or relatedness [37]. The result of the present study suggests that there is limited family level host specificity as the isolate collected from the most related (i.e., nitidulid) host was the least effective in this assay. The testing of strains isolated from a wider range of more and less related hosts is, however, needed to further explore this relationship. Successful infection of EPF to a specific insect host is determined by the particular genetic assemblage of virulence factors comprising a pathotype that can be adapted to singular or broad host ranges, and their ability to out-compete the host defence mechanisms in an evolutionary arms race [38,39]. Results from the present study support general pathotype virulence factors being more important in determining host susceptibility rather than taxonomic relatedness of isolate host and target insect [39–41]. The pattern of virulence across adult beetles in the present study does suggest that the isolates have some specificity within the *Carpophilus* genus, and that the decreased magnitude of pathogenicity in *C. truncatus* could be attributed to either a species-specific resistance factor or a population specific genotype susceptibility or tolerance in either population brought about by the pathogenic pressure of their environments [42,43]. The present study only had access to single laboratory populations of each beetle species and therefore was unable to test for the effect of variation in population genotype to resolve this.

While the isolate, B54, showed the most promise as a biocontrol agent with effective mortality against adult and larval *Carpophilus* beetles in laboratory conditions, further studies are required to ascertain the suitability of this isolate under field conditions, where many abiotic and biotic factors can have significant impacts on disease progression and fungal growth [44]. Formulation optimisation either with an oil emulsion, nutritive granules, or amended powder, depending on application strategy, may increase control rates and allow more targeted applications such as an autoinoculation and dispersal system [45]. The selection of a virulent isolate is a crucial first step to developing biopesticide control strategies: the practicalities of broad-scale pest control within these agricultural systems do, however, need to be investigated to determine whether this agent is cost-effective as an additional tool to be incorporated into an IPM strategy.

Supplementary Materials: The following are available online at http://www.mdpi.com/2073-4395/10/8/1207/s1, Diet recipe, original data collection and analysis is attached to this submission.

Author Contributions: Conceptualisation, all authors; methodology, W.B. and D.L.; conduct all experiments and data analyses, W.B.; write the manuscript, W.B.; perform editing, W.B., D.L. and J.P.C. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare there is no conflict of interest.

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