Enhancing the efficacy of fungal pathogens using a synergistic chemical, Imidacloprid

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Final Report

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VG07039 (31 May 2009)

Sassan Asgari and Mike Furlong
University of Queensland

• **Project:** VG07039

• **Investigators:** Sassan Asgari and Mike Furlong

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Media Summary

Sub-lethal concentrations of imidacloprid can significantly enhance the efficacy of fungal pathogens in a wide range of pest insects, thus increasing their potency as biological control agents. In this study the interactions between the entomopathogenic fungus *Beauveria bassiana* and imidacloprid when co-applied to diamondback moth (DBM; *Plutella xylostella*), cabbage cluster caterpillar (CCC; *Crocidolomia pavonana*) and green vegetable bug (GVB; *Nezaria viridula*) were investigated. The insecticide did not increase *B. bassiana* infection in target pest insects when co-applied with the pathogen. To understand the dynamics of the *B. bassiana*-imidacloprid interaction in GVB, DBM ad CCC a series of experiments was conducted which examined the effects of sub-lethal doses of imidacloprid on the feeding behaviour of target insects and compared the effects of the insecticide and starvation stress on their immune responses and their susceptibility to the pathogen. Imidacloprid had a limited impact on GVB and CCC feeding behaviour and neither starvation stress nor application of imidacloprid affected the susceptibility of either insect to *B. bassiana.* Although both starvation and imidacloprid caused insects to lose weight, neither treatment compromised the immune response of either insect within 24 h. In DBM imidacloprid compromised larval feeding but, unlike starvation stress, it did not increase DBM susceptibility to *B. bassiana*. Starvation stress significantly compromised the immune response of DBM within 12 h and, although imidacloprid had a similar effect on the immune response within 24 h, this delayed effect was too late to enhance *B. bassiana* host invasion which occurs approximately 12 h after contact with the host cuticle. Starvation and insecticide induced stresses can compromise the immune response of DBM and could provide a new approach to improving pest management tactics for this important pest. In order to exploit this for pest control, insects must either be stressed prior to application of the fungal biological control agent or methods must be developed to rapidly induce sufficient stress so that the immune response is compromised before the pathogen begins invasion of the host.

Technical summary

In laboratory species-specific dose-response bioassays, the three target insects, diamondback moth (DBM; *Plutella xylostella*), cabbage cluster caterpillar (CCC; *Crocidolomia pavonana*) and green vegetable bug (GVB; *Nezaria viridula*) were susceptible to imidacloprid when it was applied to the foliage upon which they fed. The LC_{50} (95%CI) against GVB (second instar nymphs), DBM (third instar larvae) and CCC (second instar larvae) was estimated to be 0.019 (0.007-0.036) ppm, 0.175 (0.096-0.259) ppm and 0.164 (0.120-0.214) ppm respectively; reflecting the greater susceptibility of GVB (Hemiptera) to the insecticide when compared to DBM and CCC (both Lepidoptera). The entomopathogenic fungus *Beauveria bassiana* was pathogenic to all three target pests when applied directly to the cuticle and when applied to the foliage upon which they fed. For each insect the LC_{50} (conidia ml^{-1}) for topical applications of the pathogen (4.0×10^7 [2.4×10^7–6.9×10^7], 5.8×10^4 [1.4×10^4–1.2×10^5] and 4.6×10^6 [1.4×10^6–2.8×10^7] for GVB, DBM and CCC respectively) was significantly lower than the LC_{50} for foliar applications (2.8×10^8 [9.3×10^7-2.4×10^9], 1.6×10^5 [7.9×10^4 –2.4×10^5] and $>3 \times 10^7$ for GVB, DBM and CCC respectively). Topical applications were more effective than foliar applications, and foliar applications of *B. bassiana* were particularly ineffective against CCC larvae. Of the three insect species investigated, DBM larvae were most susceptible to the pathogen in both topical and foliar assays whereas CCC

larvae were the least susceptible. In topical assays the LC_{50} for third instar DBM larvae was 80-fold lower than the LC_{50} for second instar CCC; these larvae are of very similar size and the difference reflects the greater intrinsic susceptibility of DBM to *B. bassiana*.

The interaction between a sub-lethal (LC_{20}) dose of imidacloprid and *B. bassiana* was investigated in dose-response bioassays in which the insecticide was fed to test insects simultaneously with foliar or topical applications of the pathogen. Insect mortality was then compared with mortality induced by appropriate applications of the pathogen alone. Treatment with imidacloprid (LC_{20}) did not increase mortality induced by topical or foliar applications of *B. bassiana* in any of the insects tested. In other insects it has been hypothesised that sub-lethal doses of imidacloprid enhance *B. bassiana* infection by affecting insect behaviours which impact the acquisition and/or the retention of conidia or by inducing physiological stresses. In GVB, imidacloprid (LC_{20}) caused significant weight loss in 24 h that was comparable to 24 h starvation but had no effect on the number of feeding sites established by nymphs, indicating that insect movement was not affected by the insecticide. In DBM, imidacloprid (LC_{20}) caused significant weight loss in 24 h but this was not as great as the weight loss sustained by individuals which were starved for 24 h. The insecticide also promoted the establishment of significantly more feeding sites, indicating that DBM movement was increased. In CCC, imidacloprid (LC_{20}) had no effect on larval weight loss in 24 h but 24 h starvation did. Similarly the insecticide had no effect on the number of feeding sites established by CCC, indicating that its movement was not affected by imidacloprid. In bioassays which investigated the effect of 24 h starvation stress on test insects, starvation had no effect on the susceptibility of GVB or CCC to *B. bassiana* but it significantly increased the susceptibility of DBM to the pathogen.

The immune response of GVB nymphs and CCC larvae was not affected by 24 h starvation or 24 h exposure to imidacloprid (LC_{20}), indicating that neither treatment compromised the capacity of these insects to combat *B. bassiana* when challenged. In DBM larvae the immune response was compromised by 12 h of starvation but only after 24 h exposure to imidacloprid. *Beauveria bassiana* conidia invade the host haemolymph approximately 12 h after attachment to host cuticle. The study indicates that starved DBM are likely more susceptible to *B. bassiana* as their immune response is compromised by the time that *B. bassiana* begins to invade. Although imidacloprid compromises the immune response after 24 h of exposure, it has no effect on the immune response 12 h after exposure providing a likely explanation for its failure to increase the susceptibility of DBM to the pathogen. The study clearly demonstrates that stress in DBM (but not in GVB or CCC) can compromise the immune response and increase susceptibility to *B. bassiana*; however stress must be induced rapidly and anti-feedant compounds which do not completely preclude larval feeding are unlikely to facilitate *B. bassiana* infection.

1. Introduction

Imidacloprid, a chloronicotinyl insecticide, is widely used, and is highly effective, against a range of insects such as whiteflies, aphids, mirids, thrips and beetles. It has recently been shown to have significant potential for the control of some lepidopteron pests of *Brassica* crops (Walsh and Furlong, 2008). The novel mode of action of imidacloprid makes it an important tool for the management of pests (e.g. green peach aphids) which have developed resistance to older, conventional insecticide chemistries. However, its widespread use exposes target insect pests to significant selection pressures and the development of resistance to this important pest management tool is a significant threat to its sustainable use in the Australian horticultural industry.

The effective life of chemical insecticides can be prolonged by the incorporation of biological and/or microbial control strategies into insecticide resistance management programmes. Such approaches ameliorate the development of resistance to chemical insecticides by killing target insects irrespective of their resistant or susceptible status; they have the added advantage that they can reduce the overall use of insecticides. Entomopathogenic fungi can be effective control agents of a wide range of insect pests and are potential candidate organisms for incorporation into resistance management strategies. The pathogens can be applied as aqueous or oil formulations of infective spores which infect susceptible insects via the cuticle but leave crop plants and most non-target insects unharmed. *Beauveria bassiana* and *Metarhizium anisopliae* are two species of entomopathogenic fungi known to be effective control agents of a wide range of pests in vegetable crops. The efficacy of these pathogens is often increased when target insects are also exposed to sub-lethal doses of imidacloprid. This synergistic interaction has the potential to increase the effectiveness of fungal pathogens in the field, reduce the rate of imidacloprid required for effective control and help offset the development of resistance to imidacloprid.

Synergistic interactions between imidacloprid and entomopathogenic fungi have previously been reported for a range of insect pests including termites (Boucias et al. 1996, Ramakrishnan et al. 1999), the citrus root weevil (Quintela and McCoy 1997, 1998), Bihar hairy caterpillar, *Spilarctia oblique*, (Puwar and Sachan, 2006), subterranean burrower bug *Cyrtomenus berg* (Jarmillo et al, 2005) and Colorado Potato Beetle (Furlong and Groden, 2001). However, an antagonistic interaction between *B. bassiana* and imidacloprid was reported when the agents were used together against whitefly (James & Elzen, 2001); it was hypothesized that *B. bassiana* caused a behavioural response that reduced insect feeding and uptake of imidacloprid. In other systems, where unequivocal synergistic interactions have been reported, changes in the behaviour of target insects in response to intoxication with imidacloprid have been implicated in the mechanism of the interactions (Boucias et al. 1996; Quintela and McCoy 1997, 1998). In the case of the synergistic interaction between *B. bassiana* and imidacloprid when co-applied to larvae of the Colorado Potato Beetle the role of starvation induced stress factors, possibly including compromising of the insect immune response, has been shown to be significant (Furlong and Groden, 2001 and 2003).

In this project, we investigated the interaction between imidacloprid and *B. bassiana* in two major lepidopteron pests, diamondback moth (DBM; *Plutella xylostella*) and cabbage cluster caterpillar (CCC; *Crocidolomia pavonana*), and one hemipteran pest, green vegetable bug (GVB; *Nezaria viridula*). Based on the outcome of the interaction in each case, we explored the physiological or behavioural mechanisms behind the interaction.

2. Materials & methods

A series of experiments was conducted to investigate the toxicity of imidacloprid and the pathogenicity of *B. bassiana* to the three vegetable pests targeted. The combined action of sub-lethal doses of imidacloprid and a range of doses of *B. bassiana* were then investigated to identify neutral, synergistic or antagonistic interactions and understand the underlying mechanisms of the interactions. All three species of insects selected for investigation in the study are pests of *Brassica* vegetable crops, while GVB is also a pest of other vegetable crops, including green beans. Diamondback moth assays were conducted on Chinese cabbage (*Brassica campestris* var. pekinensis cv Wombok) while CCC assays were conducted on common cabbage (*Brassica oleracea* var. capitata cv Warrior); GVB assays were conducted on the pods of green beans (*Phaseolus vulgaris)* or on cotton dental wicks saturated with a solution of artificial diet.

2.1. Toxicity of imidacloprid to target insects

Dose response experiments were conducted to determine the susceptibility of target insects to imidacloprid. Assays utilised Confidor 200SC, a commercially available formulation of imidacloprid which is widely utilised in horticultural crops in Australia.

2.1.1 Toxicity of foliar applications of imidacloprid applied to cabbage and Chinese cabbage leaves to CCC and DBM larvae

A leaf dip assay was developed to test the response of DBM and CCC to a range of imidacloprid concentrations. Test concentrations consisted of aqueous solutions of imidacloprid and 0.03% Tween-80 (to act as a leaf wetting agent); control solutions consisted of 0.03 % aqueous Tween-80 solution alone. Leaf discs (4.5 cm) were individually cut from the central portion of single cabbage or Chinese cabbage leaves as appropriate and then dipped in an imidacloprid test solution or control solution for 10 s. Treated leaf discs were placed, abaxial surface uppermost, on a corrugated sheet of aluminium foil and allowed to dry for approximately 2 h. Treated leaf discs were then transferred to Petri dishes (5 cm) lined with moistened filter paper. Ten leaf discs were prepared for each imidacloprid test solution and the control. Depending on the assay, five early third instar DBM larvae or five early second instar CCC larvae were introduced to each leaf disc. All Petri dishes were placed into a sealed plastic box (to maintain humidity and leaf quality) and the box was then placed in a 23°C incubator for 24 hours. After feeding on treated leaves for 24 h, larvae were transferred to clean Petri dishes (5 cm) containing fresh untreated Chinese cabbage or cabbage leaf discs as appropriate and moistened filter paper. Larval mortality in control and all imidacloprid treatments was recorded daily for one week.

2.1.2 Toxicity of imidacloprid applied to green beans to GVB nymphs

Imidacloprid was applied to the pods of green beans using a modified version of the method described above. Approximately 30 ml of each imidacloprid test solution was poured into the base of a Petri dish (9 cm diameter). Ten 2 cm-long bean pod sections were then stood vertically in the solution so that the lower 0.5 cm was immersed. Three Petri dishes were prepared in this manner for each test solution and the control. Bean pods remained in the solutions for 3 h and were then removed and carefully placed on a corrugated sheet of aluminium foil and allowed to dry for approximately 2 h. Treated portions were then placed individually into SOLO cups; 30 bean

portions were prepared for each test solution and the control. Single second instar GVB nymphs were introduced into each cup and all cups were then sealed with an aerated lid. All cups were transferred to a 23°C incubator and the GVB nymphs were allowed to feed for 24 h before they were individually transferred to fresh cups containing a 2cm-long portion of fresh untreated bean. The insects were examined every day for 8 days and mortality recorded; bean portions were replaced with fresh material as required.

2.1.3 Toxicity of imidacloprid in artificial diet applied to dental wicks to GVB nymphs

An artificial diet for GVB was prepared by mixing L-Cysteine (0.25 gl^{-1}/), Vitamin C (0.5 gl^{-1}) and streptomycin (0.16 mgl^{-1}) in sterile water. The diet was supplemented with imidacloprid to produce a range of concentrations for testing. The highest concentration of imidacloprid tested (5×10^{-2} ppm) represented the recommended foliar application rate for Confidor. Artificial diet to which no imidacloprid was added served as a control. Each test solution (1.6 ml) was pipetted onto single 2cm-long portions of dental wick held in SOLO cups; 30 dental wicks were prepared for each test solution and the control. Single second instar GVB nymphs were introduced into each cup and all cups were then sealed with an aerated lid. All cups were transferred to a 23°C incubator and the GVB nymphs were allowed to feed on the saturated wicks for 24 hours before they were individually transferred to fresh cups containing a 1cm-long portion of a green bean. The insects were examined every day and mortality recorded; bean portions were replaced with fresh material as required.

2.2. Pathogenicity of *B. bassiana* to target insects

Dose response experiments were conducted to determine the susceptibility of target insects to *B. bassiana*. Assays utilised *B. bassiana* conidia from the GHA strain derived from the commercial product Botanigard; this strain of *B. bassiana* is pathogenic to a wide range of insect pests. Two application methods were employed. In foliar assays insects were exposed to *B. bassiana* conidia applied to the substrate (common cabbage, Chinese cabbage or green beans) while in topical assays conidia were applied directly to the insect cuticle.

2.2.1 Pathogenicity of *B. bassiana* to CCC and DBM larvae when applied to cabbage and Chinese cabbage leaves respectively

A modification of the leaf dip assay, developed to test the response of DBM and CCC to imidacloprid, was used to investigate the response of test insects to *B. bassiana*. Formulated *B. bassiana* conidia were mixed with distilled water and 0.03% Tween-80 (to act as a leaf wetting agent); control solutions consisted of 0.03 % aqueous Tween-80 solution alone. Leaf discs (4.5 cm) were individually cut from the central portion of single cabbage or Chinese cabbage leaves as appropriate and then dipped in a *B. bassiana* test suspension or control solution for 10 s. Treated leaf discs were placed, abaxial surface uppermost, on a corrugated sheet of aluminium foil and allowed to dry for approximately 2 h. Treated leaf discs were then transferred to Petri dishes (5 cm) lined with moistened filter paper. Ten leaf discs were prepared for each *B. bassiana* test suspension and the control. Depending on the assay, five early third instar DBM larvae or five early second instar CCC larvae were introduced to each leaf disc. All Petri dishes were placed into a sealed plastic box (to maintain humidity and leaf quality) and the box was then placed in a 23°C incubator

for 24 hours. After feeding on treated leaves for 24 h, larvae were transferred to clean Petri dishes (5 cm) containing fresh untreated Chinese cabbage or cabbage leaf discs as appropriate and moistened filter paper. Larval mortality in control and all *B. bassiana* treatments was recorded daily.

2.2.2 Pathogenicity of *B. bassiana* to GVB nymphs when applied to green beans

A modification of the leaf dip assay developed to test the response of DBM and CCC to foliar applications of *B. bassiana* was used to investigate the response of GVB to the fungus. Formulated *B. bassiana* conidia were mixed with distilled water and 0.03% Tween-80 (to act as a wetting agent); control solutions consisted of 0.03 % aqueous Tween-80 solution alone. Bean portions (2 cm-long) were dipped in a *B. bassiana* test suspension or control solution for 10 s. Treated portions were then placed on a corrugated sheet of aluminium foil and allowed to dry for approximately 2 h before being transferred to SOLO cups. Thirty bean portions were prepared for each *B. bassiana* test suspension and the control and single second instar GVB nymphs were introduced to each. All SOLO cups were placed into a sealed plastic box (to maintain humidity and leaf quality) and the box was then placed in a 23°C incubator for 24 hours. After feeding on treated leaves for 24 h, nymphs were transferred to clean SOLO cups containing fresh untreated bean portions. Mortality in control and all *B. bassiana* treatments was recorded daily.

2.2.3 Pathogenicity of *B. bassiana* to GVB nymphs and CCC and DBM larvae when applied topically

Test suspensions of *B. bassiana* were prepared as previously described (section 2.2.1). Third instar DBM larvae and second instar CCC larvae were held individually in small Petri dishes (5cm diameter) lined with dry filter paper while second instar GVB nymphs were held individually in SOLO cups. All insects were held for 5 minutes on a cooling table set at -5°C immediately before treatment. Using a Gilson micropipette, 0.5 µl of control or *B. bassiana* test solution was then carefully applied to the dorsal surface of immobilized insects. When the droplet had dried, the dry filter paper in the DBM and CCC Petri dishes was moistened and a small portion of Chinese cabbage leaf added; depending on the assay, a 2cm long portion of dental wick impregnated with 1.6 ml of GVB artificial diet or a 2 cm long portion of green bean was placed in each of the SOLO cups containing treated GVB nymphs. Thirty insects of each test species were treated in this manner for each test concentration of *B. bassiana* and for the control. All Petri dishes and SOLO cups were placed into sealed plastic boxes and the box was then placed in a 23°C incubator for 24 h. After feeding for 24 h, DBM and CCC larvae were transferred to clean Petri dishes (5 cm) containing fresh untreated Chinese cabbage leaf discs and moistened filter paper and GVB nymphs were transferred to fresh SOLO cups containing a dental wick impregnated with diet or a fresh green bean portion. All insects were monitored daily and mortality was recorded daily until the assay end point (appropriate end points determined in preliminary assays: DBM end point= 8 days; CCC end point= 9 days; GVB end point= 14 days).

2.3. Combined effects of imidacloprid and *B. bassiana* on GVB, DBM and CCC mortality

Assays performed in 2.1 above identified the response of each test insect to imidacloprid when applied to the surface of plant material upon which the insects subsequently fed and, in the case of

GVB, when incorporated into artificial diet. Appropriate sub-lethal concentrations (LC_{20}) of imidacloprid were then determined for each species of test insect. Bio-assays investigated the effect of co-applying a sub-lethal concentration of imidacloprid with *B. bassiana* on the efficacy of the pathogen to GVB, CCC and DBM.

The interaction between imidacloprid and *B. bassiana* in test insects was studied in foliar assays where insects were exposed to plant material treated with an appropriate LC_{20} of imidacloprid and a range of concentrations of *B. bassiana;* in controls insects were exposed to plant material treated with a range of concentrations of *B. bassiana* but no imidacloprid. The interaction was also studied in topical assays where insects were exposed to plant material treated with an appropriate LC_{20} of imidacloprid immediately after topical application with one of a range of concentrations of *B. bassiana* conidia; in controls *B. bassiana* treated insects were provided with untreated plant material.

2.3.1 Combined effects of imidacloprid and *B. bassiana* on GVB mortality

2.3.1.1 Foliar *B. bassiana* application- imidacloprid interaction assay against GVB

Bean portions were treated with a range of *B. bassiana* concentrations (3×10^6- 3×10^9 conidia ml^{-1}) by dipping the bean portion into the *B. bassiana* test solution for 10s and allowing them to air dry as previously described (section 2.2.2). Six *B. bassiana* concentrations plus a control (0.03%Tween-80) were tested; 30 second instar nymphs were tested in each treatment and the control. In a concurrent assay, second instar nymphs were exposed to bean portions which had previously treated with a range of *B. bassiana* concentrations (3×10^6- 3×10^9 conidia ml^{-1}) which had been formulated in an aqueous solution of the LC_{20} of imidacloprid (0.004 ppm). Six *B. bassiana* concentrations plus a control (LC_{20} of imidacloprid formulated in 0.03%Tween-80) were tested; 30 second instar nymphs were tested in each treatment and the control. Insects and appropriately treated bean portions were held in SOLO cups for 24 h and then all bean portions were replaced with untreated portions of bean. Mortality in all treatments was recorded daily.

2.3.1.1 2.3.1 Topical *B. bassiana* application- imidacloprid interaction assay against GVB

Second instar GVB were topically treated with a range of *B. bassiana* concentrations (1×10^6- 1×10^8 conidia ml^{-1}) as previously described (section 2.3.3). Six *B. bassiana* concentrations plus a control (0.03%Tween 80) were tested and 60 second instar nymphs were treated with each. Thirty insects from each of the control and the six *B. bassiana* treatments were then placed into a SOLO cup containing a portion of green bean which had been dipped in 0.03% Tween-80 solution and the remaining 30 insects from each of the control and *B. bassiana* treatments were placed into a SOLO cup containing a portion of green bean which had been treated with the LC_{20} of imidacloprid. The bean portions were removed after 24 h and replaced with fresh untreated bean. Mortality in all treatments was recorded daily.

2.3.2 Combined effects of imidacloprid and *B. bassiana* on DBM mortality

2.3.2.1 Foliar *B. bassiana* application- imidacloprid interaction assay against DBM

Third instar DBM larvae were treated with one of five concentrations of *B. bassiana* (1×10^4- 1×10^6 conidia ml^{-1}) by exposing them to Chinese cabbage leaf discs which had previously been dipped in the relevant solution for 10 s. Controls were exposed to Chinese cabbage leaf discs which had been dipped in 0.03% Tween-80. In a concurrent bioassay, third instar larvae were exposed to Chinese cabbage leaf discs which had been dipped in one of six concentrations of *B. bassiana* (1×10^4- 1×10^6 conidia ml^{-1}) which had been formulated with the LC_{20} of imidacloprid (0.049ppm; Table 1). Controls were exposed to leaf discs which had been treated with the LC_{20} of imidacloprid alone. Thirty insects were used in the controls and in each treatment. After 24 h each leaf disc was replaced with an untreated leaf disc. Mortality in all treatments was recorded daily.

2.3.1.1 Topical *B. bassiana* application- imidacloprid interaction assay against DBM

Third instar DBM larvae were topically treated with one of six concentrations of *B. bassiana* (3×10^3- 1×10^6 conidia ml^{-1}) or a control solution (Tween-80). Insects were then placed individually into Petri dishes (5 cm diameter) containing either Chinese cabbage leaves treated with 0.03% Tween-80 or Chinese cabbage leaves treated with the previously determined LC_{20} of imidacloprid (0.049 ppm; Table 1). After 24 h each leaf disc was replaced with an untreated leaf disc. Mortality in all treatments was recorded daily.

2.3.3 Combined effects of imidacloprid and *B. bassiana* on CCC mortality

2.3.3.1 Foliar *B. bassiana* application- imidacloprid interaction assay against CCC

Third instar CCC larvae were treated with one of 5 concentrations of *B. bassiana* (3×10^5- 3×10^7 conidia ml^{-1}) by exposing them to leaf discs which had previously been dipped in the relevant solution for 10 s. Controls were exposed to cabbage leaf discs which had been dipped in 0.03% Tween-80. In a concurrent bioassay, third instar larvae were exposed to cabbage leaf discs which had been dipped in one of five concentrations of *B. bassiana* (3×10^5- 3×10^7 conidia ml^{-1}) which had been formulated with the LC_{20} of imidacloprid (0.067 ppm; Table 1). Controls were exposed to leaf discs which had been treated with the LC_{20} of imidacloprid alone. Thirty insects were used in the controls and in each treatment. After 24 h each leaf disc was replaced with an untreated leaf disc. Mortality in all treatments was recorded daily.

2.3.3.2 Foliar *B. bassiana* application- imidacloprid interaction assay against CCC

Third instar CCC larvae were topically treated with one of 5 concentrations of *B. bassiana* (3×10^5- 3×10^7 conidia ml^{-1}) or a control solution (Tween-80). Insects were then placed individually into Petri dishes (5 cm diameter) containing either cabbage leaves treated with 0.03% Tween-80 or cabbage leaves treated with the previously determined LC_{20} of imidacloprid (0.067 ppm; Table 1). Thirty insects were used in the controls and in each treatment. After 24 h each leaf disc was replaced with an untreated leaf disc. Mortality in all treatments was recorded daily.

2. 4. Feeding and behavioural studies

In feeding studies, the stresses induced in GVB, DBM and CCC by sub-lethal doses of imidacloprid were measured and compared with the stresses induced by different starvation regimes. These studies were complimented by an investigation of the feeding behaviours of the three test insect species exposed to imidacloprid treated and control food sources.

2.4.1 GVB feeding and behavioural studies

The effect of imidacloprid and sub-optimal food sources on the feeding and behaviour of early second instar GVB nymphs was investigated by exposing them to one of eight feeding regimes:
Control feeding regimes: 2 cm portion of green bean, 2 cm long dental wick saturated with artificial diet.
Imidacloprid feeding regimes: 2 cm of green bean treated with an LC_{20} of imidacloprid, 2 cm long dental wick saturated with artificial diet containing an LC_{20} of imidacloprid, 2 cm long dental wick saturated with artificial diet containing an LC_{50} of imidacloprid.
Starvation regimes: 2 cm long dental wick saturated with diluted (33%) artificial diet, 2cm long dental wick saturated with distilled water, no food or water.
For each regime, early second instar nymphs ($n \geq 20$) were individually weighed and introduced singly into a SOLO cup which contained an appropriately treated bean portion, dental wick or neither a bean portion nor a dental wick. All nymphs were incubated at 23°C for 24 h and then re-weighed.

The effect of imidacloprid on the feeding behaviour of second instar GVB nymphs was investigated by comparing feeding on dental wicks saturated with artificial diet or artificial diet impregnated with the LC_{20} of imidacloprid. Appropriately treated dental wicks were pushed through a small hole made in the lid of a SOLO cup; a single early second instar GVB nymph was then placed into the bottom of the cup and observed carefully for the next 60 minutes. Each treatment was replicated 20 times. The number of times that each nymph probed its food source, the total amount of time spent feeding and the number of feeding sites established was recorded.

2.4.2 DBM feeding and behavioural studies.

The effect of exposure to untreated foliage, foliage treated with an LC_{20} of imidacloprid or starvation for 24 h on the weight gain of early third instar DBM larvae was investigated. Chinese cabbage leaf discs were treated with an LC_{20} of imidacloprid as previously described while control leaf discs were treated with a solution of 0.03% Tween-80. Early third instar larvae were individually weighed and then introduced singly to Petri dishes (5cm diameter) containing a moistened filter paper and a Chinese cabbage leaf disc treated with an LC_{20} of imidacloprid, a moistened filter paper and a Chinese cabbage leaf disc treated with Tween-80 or a moistened filter paper alone. Each treatment was replicated 20 times. All dishes were placed into a sealed plastic container and incubated at 23°C for 24 h. After 24 h insects were reweighed and the weight gain under each feeding regime was calculated.

The effect of imidacloprid on the number of feeding sites established by early third instar DBM in 24 h was investigated by preparing 30 Chinese cabbage leaf discs (4.5 cm diameter) treated with an LC_{20} of imidacloprid and another 30 Chinese cabbage leaf discs (4.5 cm diameter) treated with 0.03% Tween-80.

Each leaf disc was individually placed into a Petri dish (5 cm diameter) and a single early third instar DBM larva was introduced to each. All dishes were placed into a sealed plastic container and incubated at 23°C for 24 h at which time the number of feeding sites established by each larva on each leaf disc was determined.

2.4.3 CCC feeding and behavioural studies

The effect of exposure to untreated foliage, foliage treated with an LC_{20} of imidacloprid or starvation for 24 h on the weight gain of early second instar CCC larvae was investigated. Common cabbage leaf discs were treated with an LC_{20} of imidacloprid as previously described while control leaf discs were treated with a solution of 0.03% Tween-80. Early second instar larvae were individually weighed and then introduced singly to Petri dishes (5cm diameter) containing either a moistened filter paper and a common cabbage leaf disc treated with an LC_{20} of imidacloprid, a moistened filter paper and a common cabbage leaf disc treated with Tween-80 or a moistened filter paper alone. Each treatment was replicated 20 times. All dishes were placed into a sealed plastic container and incubated at 23°C for 24 h. After 24 h insects were reweighed and the weight gain under each feeding regime was calculated.

The effect of imidacloprid on the number of feeding sites established by early second instar CCC in 24 h was investigated by preparing 30 common cabbage leaf discs (4.5 cm diameter) treated with an LC_{20} of imidacloprid and another 30 common cabbage leaf discs (4.5 cm diameter) treated with 0.03% Tween-80.
Each leaf disc was individually placed into a Petri dish (5 cm diameter) and a single early second instar DBM larva was introduced to each. All dishes were placed into a sealed plastic container and incubated at 23°C for 24 h at which time the number of feeding sites established by each larva on each leaf disc was determined.

2.5. Effect of starvation stress and sub-lethal doses of imidacloprid on the susceptibility of test insects to *B. bassiana*

2.5.1. Effect of starvation stress and sub-lethal doses of imidacloprid on the susceptibility of GVB to *B. bassiana.*

The effect of starvation and imidacloprid induced stress on the susceptibility of GVB to *B. bassiana* was investigated by exposing early second instar GVB nymphs which had been topically treated with an LC_{20} solution of *B. bassiana* conidia (as previously described) and control GVB nymphs (treated with 0.03% Tween-80 solution as previously described) to a range of feeding regimes. Treated insects were transferred individually to SOLO cups containing a 2 cm-long bean portion treated with an LC_{20} of imidacloprid (as previously described), a control 2cm-long bean portion or no food source. Thirty *B. bassiana* treated insects and 30 control insects were exposed to each feeding regime. Insects were incubated at 23°C for 24 h and then transferred to clean SOLO cups, supplied with an untreated 2 cm long portion of green bean and returned to the incubator. Mortality and evidence of sporulation were recorded daily.

2.5.2 Effect of starvation stress and sub-lethal doses of imidacloprid on the susceptibility of DBM to *B. bassiana*

The effect of starvation induced stress on the susceptibility of DBM to *B. bassiana* was investigated by exposing early third instar larvae which had been topically treated with a control solution (0.03% Tween-80) or with an LC_{20}, an LC_{50} or an LC_{90} suspension of *B. bassiana* conidia (as previously described) to one of two feeding regimes. Treated insects were transferred individually to Petri dishes (5 cm diameter) which contained both a moistened filter paper and a Chinese cabbage leaf discs (4.5 cm diameter) or Petri dishes (5 cm diameter) which contained a moistened filter paper but no food source. Insects were incubated at 23°C for 24 h and then transferred to individual clean Petri dishes (5 cm diameter) containing a moistened filter paper and an untreated Chinese cabbage leaf disc (4.5 cm diameter). Fifteen *B. bassiana* treated insects and 15 control insects were exposed to each feeding regime and the experiment was repeated 4 times. Mortality and evidence of sporulation were recorded daily.

In a second experiment, the effect of starvation stress on the susceptibility of DBM to *B. bassiana* was investigated in a dose response study as previously described. Following topical application of a range of concentrations of *B. bassiana* to early third instar (n= 60 per test concentration and control), thirty insects exposed to each treatment were transferred individually to Petri dishes (5 cm diameter) containing only a moistened filter paper while the remaining insects were transferred to Petri dishes (5 cm diameter) containing a moistened filter paper and an untreated Chinese cabbage leaf (4.5 cm diameter). All Petri dishes were placed into sealed plastic boxes transferred to a 23°C incubator for 24 h. After feeding for 24 h, DBM larvae were transferred to clean Petri dishes (5 cm diameter) containing fresh untreated Chinese cabbage leaf discs and moistened filter paper. Insects were monitored daily and mortality recorded.

In a third experiment 3.5 ml of tap-water agar (2%) was pipetted into 24 Petri dishes (3 cm diameter) and allowed to set. A single untreated Chinese cabbage leaf disc (2 cm diameter) was placed, adaxial surface upper most, onto the surface of the agar in each dish and all dishes were then transferred to a sealed plastic container and incubated at 23°C for 24 h to allow the moisture level in each leaf disc to equilibrate. Each leaf disc was then blotted with tissue paper to remove any surface condensation, weighed and returned its Petri dish. A further 24 Petri dishes (3 cm diameter) were lined with moistened filter paper. Seventy two early third instar DBM larvae were individually weighed and transferred to individual labelled Petri dishes (5 cm diameter) containing a dry filter paper. Twenty four of these larvae were then topically treated with an LC_{20} concentration of *B. bassiana* as previously described, while the remaining 24 larvae were topically treated with 0.03% Tween-80. Following treatment, *B. bassiana*-treated and control larvae were transferred individually to the previously prepared Petri dishes which contained either a weighed Chinese cabbage leaf disc on tap-water agar (2%) or a moistened filter paper. Each treatment- feeding regime combination was replicated 12 times. All dishes were then placed in a sealed plastic box and transferred to an incubator for 24 h. Each larva and each leaf disc was then individually weighed.

2.5.3. Effect of starvation stress on the susceptibility of CCC to *B. bassiana.*

The effect of starvation induced stress on the susceptibility of CCC to *B. bassiana* was investigated by exposing early second instar larvae which had been topically treated with a control solution (0.03%Tween-80) or with an LC_{50} of *B. bassiana* conidia (as previously described) to one of two

feeding regimes. Treated insects were transferred individually to Petri dishes (5 cm diameter) which contained both a moistened filter paper and a common cabbage leaf disc (4.5 cm diameter) or Petri dishes (5 cm diameter) which contained a moistened filter paper but no food source. Insects were incubated at 23°C for 24 h and then transferred to individual clean Petri dishes (5 cm diameter) containing a moistened filter paper and an untreated common cabbage leaf disc (4.5 cm diameter). Fifteen *B. bassiana* treated insects and 15 control insects were exposed to each feeding regime and the experiment was repeated 4 times. Mortality and evidence of sporulation were recorded daily.

2.6. Effect of starvation stress and sub-lethal doses of imidacloprid on the immune response of test insects

Pro-phenyl oxidase (PPO) production is a key immune response of insects when challenged by fungal pathogens. To determine the effects of starvation and sub-lethal doses of imidacloprid on the immune response of target insects when challenged by *B. bassiana*, the production of melanin (which is the end product of activation of PPO) was measured in control, starvation stressed and imidacloprid treated insects.

Control insects were fed individually on appropriate untreated foliage for 24 h at 23°C; GVB fed on 2 cm-long bean portions, DBM fed on 4.5 cm Chinese cabbage leaf discs and CCC fed on 4.5 cm common cabbage leaf discs. Imidacloprid treated insects fed upon appropriate foliage treated with the appropriate LC_{20} of imidacloprid as previously described. Starved insects were held individually without access to a food source. As in previous experiments the GVB study utilised early second instar nymphs, the DBM study utilised early third instar larvae and the CCC study utilised early second instar larvae. In each experiment 9-15 immature insects were exposed to each feeding regime for 24 h. In a second DBM experiment larvae were only exposed to the feeding regimes for 12 h before the assay.

Assays to determine the haemolymph phenoloxidase activity were conducted using a modification of the method described by Beck et al. (2000). For each species of insect three larvae or nymphs were washed in 70% ethanol and then crushed in 50 µl of ice-cold phosphate buffer solution (PBS) in a PCR tube; a minimum of three replicates of each treatment was prepared. The PBS-insect mixture was centrifuged at 800g for 15 minutes and then 10 µl of supernatant was mixed with 90 µl of 0.02 M L-DOPA in PBS in a spectrophotometer (Varian Techtron) and absorbancy at 485 nm measured at room temperature for 240 minutes.

2.7. Statistical analysis

Dose response data were analysed by logit analysis using POLO PC (LeOra software 2001) following correction for control mortality. All other statistical tests were performed in StatView Version 5 (SAS Institute, 1998). Where appropriate, data were either subject to analysis of variance (individual means were compared by Fischer's LSD) or compared by a t-test. Prior to analysis of proportional data were subject to arcsin square root transformation. When data were not normally distributed medians were compared by the Kruskall-Wallace test when three or more groups were compared and by the Mann-Whitney U-test when two groups were compared.

3. Results

3.1. Toxicity of imidacloprid to target insects

3.1.1 Toxicity of foliar applications of imidacloprid applied to cabbage and Chinese cabbage leaves to CCC and DBM larvae

When DBM larvae were exposed to a range of concentrations of imidacloprid applied to Chinese cabbage leaves, the logit regression was significant with a slope of 2.43 (± 0.55) (Table 1). The estimated LC_{20} (95%CI) and LC_{50} (95%CI) were 0.049 (0.027- 0.072) and 0.175 (0.096- 0.259), respectively (Table 1). Similarly, when CCC larvae were exposed to a range of concentrations of imidacloprid applied to common cabbage leaves, the logit regression was significant with a slope of 3.83 (± 0.75) (Table 1). The estimated LC_{20} (95%CI) and LC_{50} (95%CI) were 0.071 (0.036- 0.101) and 0.164 (0.120- 0.214), respectively (Table 1).

3.1.2 Toxicity of imidacloprid applied to green beans to GVB nymphs

When GVB nymphs were exposed to a range of concentrations of imidacloprid applied to 2 cm-long portions of green bean, the logit regression was significant with a slope of 2.13 (± 0.54) (Table 1). The estimated LC_{20} (95%CI) and LC_{50} (95%CI) were 0.004 (0.0001- 0.010) and 0.019 (0.007- 0.036), respectively (Table 1).

3.1.3 Toxicity of imidacloprid in artificial diet applied to dental wicks to GVB nymphs

When GVB were exposed to a range of concentrations of imidacloprid formulated in artificial diet and applied dental wicks, the logit regression was significant with a slope of 1.47 (± 0.34) (Table 1). The estimated LC_{20} (95%CI) and LC_{50} (95%CI) were 0.019 (0.008-0.32) and 0.168 (0.090-0.649), respectively (Table 1).

3.2. Pathogenicity of *B. bassiana* to target insects

3.2.1 Pathogenicity of *B. bassiana* to CCC and DBM larvae when applied to cabbage and Chinese cabbage leaves respectively

When DBM was exposed to a range of concentrations of *B. bassiana* applied to Chinese cabbage leaves, the logit regression was significant with a slope of 2.27 (± 0.55) and the estimated LC_{50} (95% CI) was 1.565×10^4 ($7.958 \times 10^4 – 2.382 \times 10^5$) (Table 2). When exposed to a range of concentrations of *B. bassiana* applied to cabbage leaves, CCC larvae did not demonstrate a significant dose response, the highest concentration tested, 3×10^7 conidia ml^{-1} killed 37% of exposed CCC larvae (Table 2).

3.2.2 Pathogenicity of *B. bassiana* to GVB nymphs when applied to green beans

When GVB was exposed to a range of concentrations of *B. bassiana* applied to portions of green bean, the logit regression was significant with a slope of 0.85 (± 0.25) and the estimated LC_{50} (95% CI) was 2.792×10^8 ($9.314 \times 10^7 – 2.357 \times 10^9$) (Table 2).

3.2.3 Pathogenicity of topical applications of *B. bassiana* to GVB nymphs and CCC and DBM larvae

When a range of concentrations of *B. bassiana* was topically applied to DBM larvae the logit regression was significant with a slope of 1.34 (± 0.26) and the estimated LC_{50} (95%CI) was 5.756×10^4 ($1.414 \times 10^4 – 1.149 \times 10^5$) (Table 2). When a range of concentrations of *B. bassiana* was topically applied to CCC larvae the logit regression was significant with a slope of 0.64 (± 0.15) and the estimated LC_{50} (95%CI) was 4.560×10^6 ($1.337 \times 10^6 – 2.758 \times 10^7$) (Table 2). When a range of concentrations of *B. bassiana* was topically applied to GVB nymphs, the logit regression was significant with a slope of 2.17 (± 0.62) and the estimated LC_{50} (95%CI) was 4.001×10^7 ($2.364 \times 10^7 – 6.898 \times 10^7$) (Table 2).

Table 1: Susceptibility of GVB, DBM and CCC to imidacloprid

Insect species	Mode of application[a]	n	Slope (±SE)	LC_{20} ppm (95% CI)	LC_{50} ppm (95% CI)	Heterogeneity (df)
N. viridula (GVB)	Foliar-green bean	210	2.13 (± 0.54)	0.004 (0.0001- 0.010)	0.019 (0.007- 0.036)	1.222 (15)
	Diet/wick	210	1.47 (± 0.34)	0.019 (0.008-0.32)	0.168 (0.090- 0.649)	0.506 (15)
P. xylostella (DBM)	Foliar- Chinese cabbage	210	2.43 (± 0.55)	0.049 (0.027- 0.072)	0.175 (0.096- 0.259)	0.737 (13)
C. pavonana (CCC)	Foliar –common cabbage	180	3.83 (± 0.75)	0.071 (0.036- 0.101)	0.164 (0.120- 0.214)	0.687 (10)

[a] For GVB assays: Foliar green bean, 2 cm portion of green bean soaked in imidacloprid solution for 3h; Diet/ wick 1.6 ml of imidacloprid test solution formulated in artificial diet and absorbed into dental wick. For DBM assay, imidacloprid applied to Chinese cabbage leaf discs by leaf dip method. For CCC assay, imidacloprid applied to common cabbage leaf discs by leaf dip method.

Table 2: Susceptibility of GVB, DBM and CCC to *B. bassiana* in topical and foliar assays

Insect species	Mode of application	n	Slope (±SE)	LC_{50} conidia ml^{-1} (95% CI)	Heterogeneity (df)
N. viridula (GVB)	Topical	210	2.17 (± 0.62)	4.001×10^7 (2.364×10^7 – 6.898×10^7)	0.656 (15)
	Foliar	210	0.85 (± 0.25)	2.792×10^8 (9.314×10^7- 2.357×10^9)	0.501 (15)
P. xylostella (DBM)	Topical	210	1.34 (±0.26)	5.756×10^4 (1.414×10^4 – 1.149×10^5)	1.731 (16)
	Foliar	210	2.27 (±0.55)	1.565×10^5 (7.958×10^4 – 2.382×10^5)	0.219(16)
C. pavonana (CCC)	Topical	180	0.64 (±0.15)	4.560×10^6 (1.337×10^6 – 2.758×10^7)	1.677 (13)
	Foliar [a]	180	-	$< 3 \times 10^7$	-

[a] Full dose response experiment conducted (3×10^5- 3×10^7 conidia ml^{-1}). 37% of test insects killed following exposure to foliage treated with 3×10^7 *B. bassiana* conidia ml^{-1}; only 10% of treated insects sporulated. When exposed to foliage treated with 1×10^7 *B. bassiana* conidia ml^{-1}, 34% of test insects were killed but only 3% sporulated. At lower *B. bassiana* treatments no insects sporulated.

3.3. Combined effects of imidacloprid and *B. bassiana* on GVB, DBM and CCC mortality

3.3.1 Combined effects of imidacloprid and *B. bassiana* on GVB mortality

There was no evidence for a significant synergistic interaction between imidacloprid and *B. bassiana* in GVB in either the foliar or topical assays; 95% confidence intervals for the estimated LC_{50}s for *B. bassiana* overlapped in the presence and absence of imidacloprid in both sets of assays (Table 3).

3.3.2 Combined effects of imidacloprid and *B. bassiana* on DBM mortality

There was no evidence for a significant synergistic interaction between imidacloprid and *B. bassiana* in DBM in either the foliar or topical assays; 95% confidence intervals for the estimated LC_{50}s for *B. bassiana* overlapped in the presence and absence of imidacloprid in both sets of assays (Table 3).

3.3.3 Combined effects of imidacloprid and *B. bassiana* on CCC mortality

There was no evidence for a significant synergistic interaction between imidacloprid and *B. bassiana* in CCC. In the topical assays, the 95% confidence intervals for the estimated LC_{50}s for *B. bassiana* overlapped in the presence and absence of imidacloprid (Table 3), while in the foliar assays there was no evidence of a dose response to *B. bassiana* by CCC either in the presence or absence of imidacloprid (Fig. 1). Despite running the foliar assays on three separate occasions, it was not possible to generate a dose response curve for CCC to *B. bassiana* by this application method.

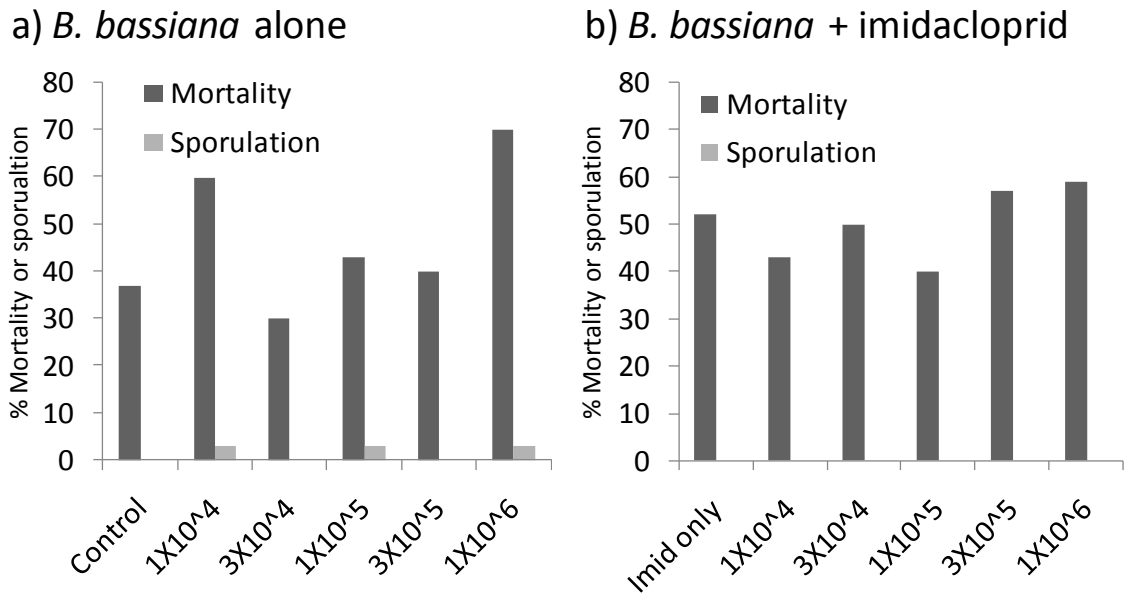

Figure 1: Foliar assays. Response of *C. pavonana* larvae to **a)** foliage treated with a suspension of *B. bassiana* conidia alone and **b)** foliage treated with a suspension of *B. bassiana* co-applied with an LC_{20} of imidacloprid (0.067 ppm).

Table 3: Interactions between *B. bassiana* and a sub-lethal dose of imidacloprid when simultaneously applied to GVB, DBM and CCC

Insect species	*B. bassiana* application method	Treatment	n	LC_{50} conidia ml^{-1} (95% CI)	Nature of Interaction
N. viridula (GVB)	Topical	*B. bassiana* alone	210	1.8×10^6 (4.5×10^5 - 3.7×10^6)	
		B. bassiana + LC_{20} Imidacloprid[1]	210	1.3×10^6 (6.0×10^4 - 3.7×10^6)	None
	Foliar	*B. bassiana* alone	210	1.8×10^8 (2.8×10^7 - 1.1×10^9)	
		B. bassiana + LC_{20} Imidacloprid[1]	210	4.3×10^8 (1.0×10^8 - 1.2×10^9)	None
P. xylostella (DBM)	Topical	*B. bassiana* alone	210	6.1×10^4 (2.2×10^4 – 1.4×10^5)	
		B. bassiana + LC_{20} Imidacloprid[2]	210	1.8×10^4 (1.4×10^3 – 6.0×10^4)	None
	Foliar	*B. bassiana* alone	210	3.8×10^5 (1.7×10^5 - 1.8×10^6)	
		B. bassiana + LC_{20} Imidacloprid[2]	210	2.5×10^5 (6.3×10^4 - 7.2×10^5)	None
C. pavonana (CCC)	Topical	*B. bassiana* alone	180	3.8×10^6 (1.3×10^6 – 9.9×10^6)	
		B. bassiana + LC_{20} Imidacloprid[3]	180	2.2×10^6 (0.6×10^6 – 5.4×10^6)	None

[1] LC_{20} (95%CI) imidacloprid against GVB on beans= 0.004 ppm (0.0001- 0.010)
[2] LC_{20} (95%CI) imidacloprid against DBM on Chinese cabbage= 0.049 (0.027- 0.072)
[3] LC_{20} (95%CI) imidacloprid against CCC on cabbage= 0.071 (0.036- 0.101)

3.4 Feeding and behavioural studies

3.4.1 GVB feeding and behavioural studies

Second instar GVB nymphs gained significantly more weight when they fed on portions of green bean than when they fed on artificial diet or were starved ($F_{7,1}$= 13.92, P<0.0001; Fig. 2). Starved insects which did not have access to water, insects which fed on artificial diet treated with sub-lethal concentrations of imidacloprid (LC_{20} or LC_{50}) and insects which fed on bean portions treated with an LC20 of imidacloprid all experienced significant weight loss (Fig. 2), indicating that these treatments induced significant feeding stress in GVB nymphs.

When second instar GVB nymphs were exposed to dental wicks saturated with artificial diet or artificial diet impregnated with an LC_{20} of imidacloprid, they probed significantly more often on control wicks than on imidacloprid treated wicks ($F_{1,38}$= 25.5; *P*<0.0001); Fig 3 A) and spent a lower proportion of their time feeding on imidacloprid treated wicks when compared with control wicks ($F_{1,38}$= 103.89; *P*<0.0001; Fig 3C). However, the presence of imidacloprid in the diet in the wicks had no effect on the number of feeding sites established ($F_{1,38}$= 1.57; *P*=0.218; Fig3B).

3.4.2 DBM feeding and behavioural studies

Starvation for 24 h had a significant effect on the weight change of DBM larvae when compared to control larvae that had unlimited access to food and larvae that fed on foliage treated with an LC_{20} of imidacloprid ($F_{2,57}$= 52.52 P<0.0001; Fig. 4). Larvae feeding on imidacloprid treated foliage gained approximately 33% of the weight gained by control larvae, while starved larvae lost weight (Fig. 4), indicating that larvae feeding on imidacloprid treated foliage experienced feeding stress but that these larvae experienced a lower level of feeding stress than 24 h starved larvae.

When DBM larvae fed on foliage treated with an LC_{20} of imidacloprid, they established a significantly greater number of feeding sites than DBM larvae which fed on untreated foliage ($F_{1,51}$=5.30, *P*=0.025; Fig. 5).

3.4.3 CCC feeding and behavioural studies

Starvation for 24 h had a significant effect on the weight change of CCC larvae when compared to control larvae that had unlimited access to food ($F_{2,56}$=57.95, *P*<0.0001; Fig. 6). Feeding on foliage treated with an LC_{20} of imidacloprid did not affect the weight change of CCC larvae when compared with control larvae (LSD, P>0.05; Fig. 6), indicating that this concentration of imidacloprid did not induce starvation stress in CCC larvae.

Treatment of foliage with an LC_{20} of imidacloprid had no effect on the number of feeding sites established by CCC when compared with the number established on untreated foliage ($F_{1,54}$=0.21, *P*=0.649;Fig. 7).

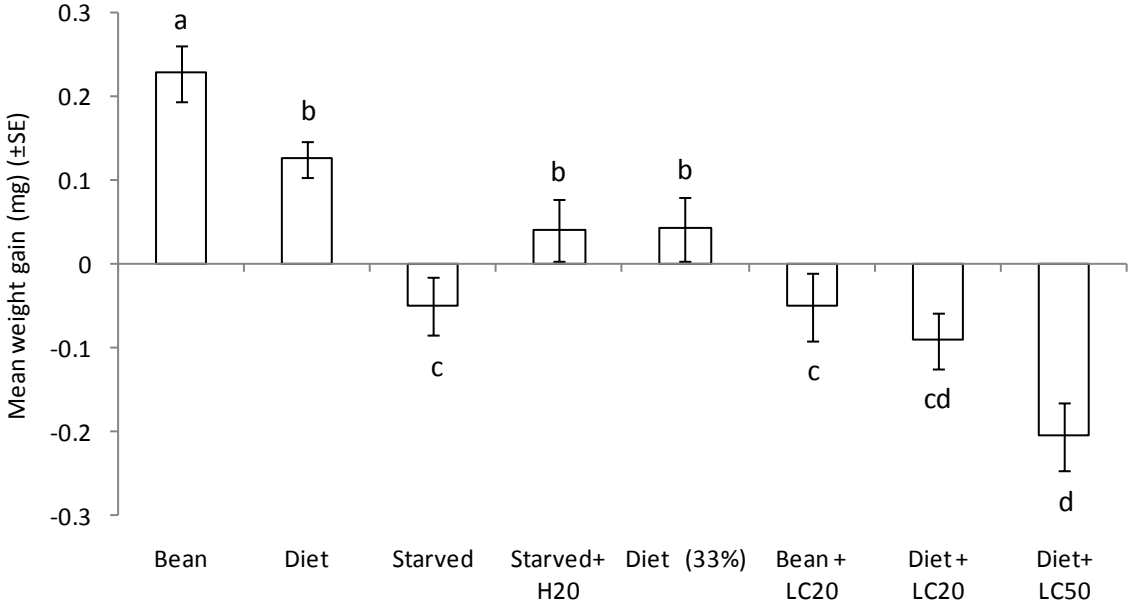

Figure 2: The effects of diet (bean or artificial diet), starvation and sub-lethal (LC_{20} and LC_{50}) concentrations of imidacloprid on 24 h weight gain of second instar GVB nymphs ($F_{7,143}$=13.92, P<0.0001; columns marked by different letters are significantly different(LSD; P<0.05).

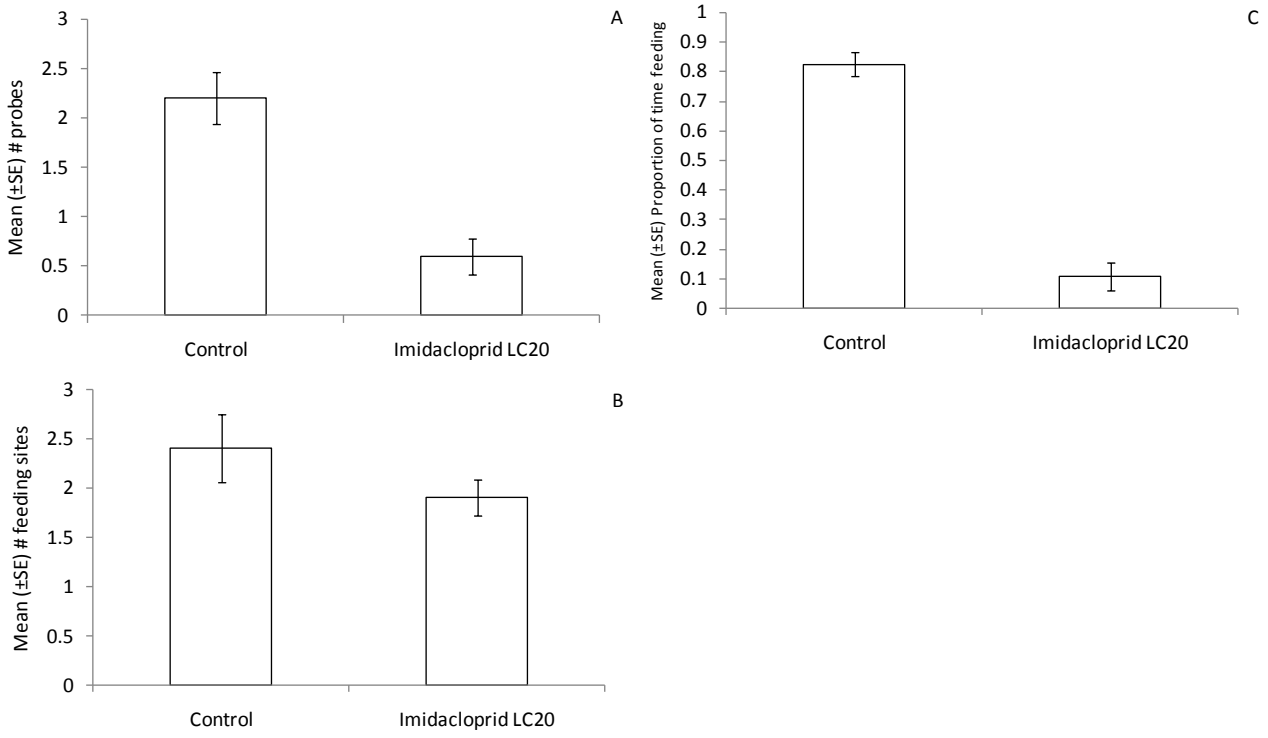

Figure 3: The effect of imidacloprid (LC_{20}) on the feeding behaviour of second instar GVB nymphs during one hour of observation. (A) Probing behaviour: Nymphs probed artificial diet soaked wicks formulated with an LC_{20} of imidacloprid less frequently than control (artificial diet alone) soaked wicks ($F_{1,38}$= 25.5; P<0.0001). (B) Establishment of feeding sites: There was no difference in the number of feeding sites established on imidacloprid treated and control wicks ($F_{1,38}$= 1.57; P=0.218). (C) Proportion of time spent feeding: Nymphs spent significantly less time feeding on

imidacloprid treated wicks than on control wicks ($F_{1,38}$= 103.89; P<0.0001; proportional data arcsine-square root transformed prior to analysis, data back transformed for presentation).

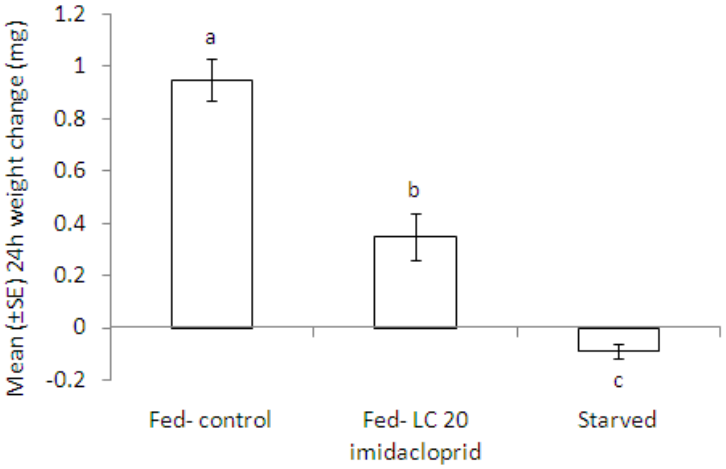

Figure 4: The effects of starvation and exposure to foliage treated with an LC_{20} of imidacloprid on the weight gain of third instar DBM larvae ($F_{2,57}$= 52.52, P<0.0001; columns marked by different letters are significantly different).

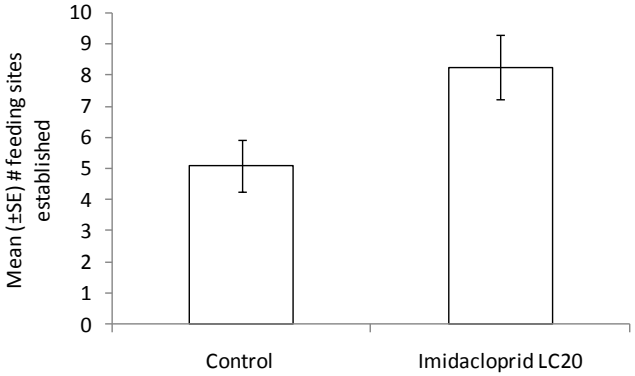

Figure 5: The effect of imidacloprid (LC_{20}) on the feeding behaviour of third instar DBM larvae ($F_{1,51}$=5.30, P=0.025).

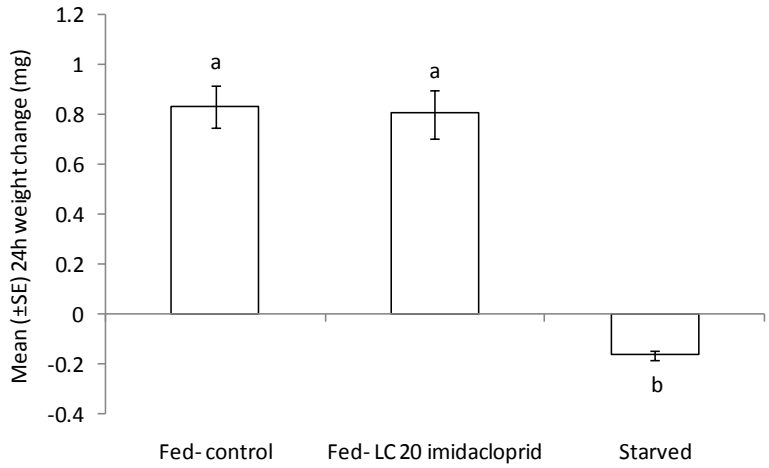

Figure 6: The effects of starvation and exposure to foliage treated with an LC_{20} of imidacloprid on the weight gain of second instar CCC larvae ($F_{2,56}$=57.95, P<0.0001; columns marked by different letters are significantly different).

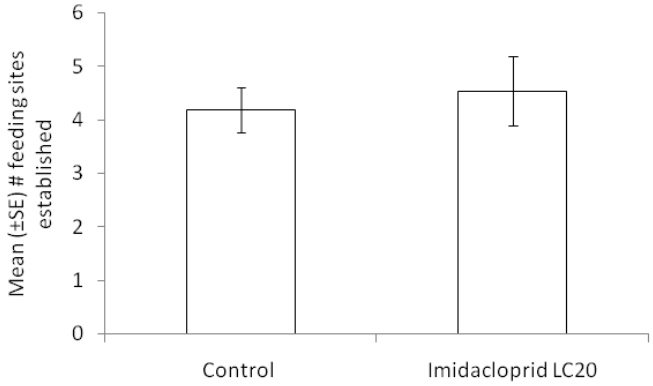

Figure 7: The effect of imidacloprid (LC_{20}) on the feeding behaviour of second instar CCC larvae ($F_{1,54}$=0.21, *P*=0.649).

3.5. Effect of starvation stress and sub-lethal doses of imidacloprid on the susceptibility of test insects to *B. bassiana*

3.5.1. Effect of starvation stress and sub-lethal doses of imidacloprid on the susceptibility of GVB to *B. bassiana*

In studies to investigate the effect of starvation and imidacloprid induced stress on the susceptibility of GVB to *B. bassiana,* treatment significantly affected GVB mortality ($F_{5,12}$= 6.51, *P*=0.004; Fig. 8a) but there was no significant difference between the mortality rates of any treatment exposed to *B. bassiana* (LSD; *P*>0.05; Fig 8a). Similarly, there was no significant difference between the sporulation rates of any of the treatments exposed to *B. bassiana* ($F_{5,12}$= 6.23, *P*=0.004; Fig 8a). There was no significant difference between the median time to death in any of the treatments exposed to *B. bassiana* (Kruskall-Wallis H(df,2) = 4.43, *P*= 0.11; Fig. 8b) nor was there any significant difference between the time to sporulation in these treatments (Kruskall-Wallis H(df,2)=0.87, *P*= 0.65; Fig. 8b).

3.5.2. Effect of starvation stress and sub-lethal doses of imidacloprid on the susceptibility of DBM to *B. bassiana*

DBM mortality was significantly increased by starvation stress ($F_{1,24}$= 7.78; *P*=0.010) and by *B. bassiana* application rate ($F_{3,24}$= 86.80; *P*<0.0001) (Fig. 9a). In insects exposed to LC_{20} and LC_{50} concentrations of *B bassiana* mortality was significantly greater in starved larvae than in larvae which had access to food following inoculation (LSD, P<0.05; Fig. 9a) but there was no difference between the mortality rates in starved and fed larvae exposed to an LC_{90} of *B. bassiana* (LSD, P>0.05; Fig. 9a). Similarly, the proportion of *B. bassiana* treated larvae which sporulated was also significantly increased by starvation stress ($F_{1,24}$= 10.47; *P*=0.004) and by *B. bassiana* application rate ($F_{3,24}$= 149.40, *P*<0.0001) (Fig. 9a). The median time to death of *B. bassiana* treated larvae was significantly reduced by starvation (Kruskall-Wallis H= 116.8, p<0.0001; Fig. 9c).

Starvation of DBM larvae for 24 h following topical application of *B. bassiana* significantly reduced the LC_{50} when compared with larvae which were supplied with an unlimited food supply following inoculation (Table 4); indicating that starved larvae were more than 15-fold more susceptible to infection than fed larvae (Table 4).

In feeding studies, topical application of an LC_{20} of *B. bassiana* to larvae did not affected the quantity of foliage consumed in the following 24 h when compared to controls (t=0.219, *P*= 0.829; Fig. 10a). However, treatment did significantly affected the weight gain of larvae ($F_{3,43}$= 20.47; *P*<0.0001; Fig. 10b); fed untreated control larvae gained significantly more weight than *B. bassiana*-treated fed larvae (LSD, *P*<0.05) which in turn gained more weight than both starved control and starved *B. bassiana*-treated larvae (LSD, *P*<0.05) (Fig 10b). The efficiency with which *B. bassiana* treated larvae converted ingested food into body mass was significantly reduced when compared with the rate at which control larvae converted ingested food into body mass with (t=3.342, *P*= 0.003; Fig. 10c).

3.5.3. Effect of starvation stress and on the susceptibility of CCC to *B. bassiana*

CCC mortality was significantly increased by starvation stress ($F_{1,12}$= 12.46; *P*=0.004) and by *B. bassiana* ($F_{1,12}$= 8.86; *P*<0.012) (Fig. 11a). However, starvation did not increase the mortality of *B. bassiana* treated insects when compared with fed *B. bassiana* treated insects (Fig 11a), although starvation did increase the proportion of insects which subsequently sporulated ($F_{1,12}$= 47.61, *P*<0.0001; Fig 11b). The median time to death of *B. bassiana* treated larvae was significantly reduced by starvation (Mann Whitney U- test, *P*=0.006; Fig 11c).

3.6. The effect of sub-lethal doses of imidacloprid and starvation stress on the immune response of test insects

The effect of starvation and imidacloprid treatment on the humoral (non-cellular) immune responses of GVB, DBM and CCC was measured by comparing haemolymph phenoloxidase (PO) activity in treated insects with PO activity in fed control insects. In GVB, there was no significant difference between the PO activity of insects exposed to any of the three treatments ($F_{2,6}$= 2.08, *P*= 0.21; Table 5). In DBM, when larvae were exposed to the three treatments for 24 h PO activity was significantly affected ($F_{2,6}$= 7.29, *P*= 0.025; Table 5); there was no significant difference between PO activity in imidacloprid treated and starved larvae (*P*>0.05) but there were significant differences in PO activity between fed control and imidacloprid treated larvae (*P*<0.05) and between fed control larvae and starved larvae (*P*<0.05). When DBM larvae were exposed to treatments for only 12 h and then subjected to the assay, there was a significant difference between treatments ($F_{2,5}$=7.51, *P*=0.031; Table 5); there was no significant difference between the control fed and imidacloprid treatments (*P*>0.05) but there were significant differences between the control fed and starved treatments (*P*<0.05) and the imidacloprid and starved treatments (*P*<0.05). In CCC, PO activity was not affected by any of the treatments ($F_{2,6}$= 1.028, *P*= 0.413; Table 5).

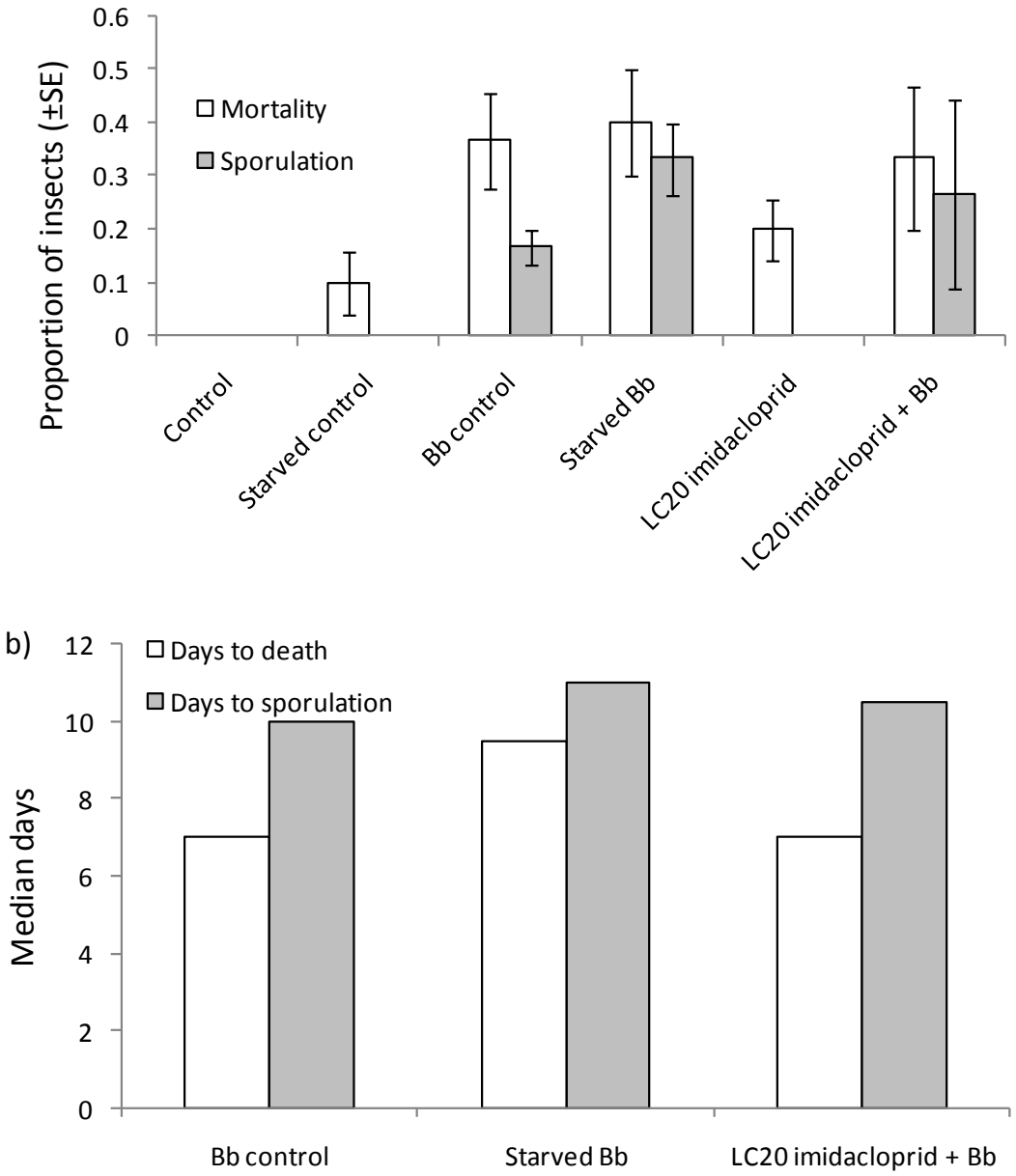

Figure 8. a) Mortality of GVB nymphs following treatment with *B. bassiana* and then exposure to starvation or an LC_{20} of imidacloprid; neither starvation nor feeding on imidacloprid treated bean portions significantly affected mortality ($P>0.05$). Similarly, sporulation rates of GVB nymphs following treatment with *B. bassiana* were not affected by subsequent feeding regime ($P>0.05$). **b)** Neither median time to death (Kruskall-Wallis H(df,2) = 4.43, $P= 0.11$) nor median time to sporulation (Kruskall-Wallis H(df,2)=0.87, $P= 0.65$) of GVB nymphs treated with *B. bassiana* was significantly affected by post inoculation feeding regime.

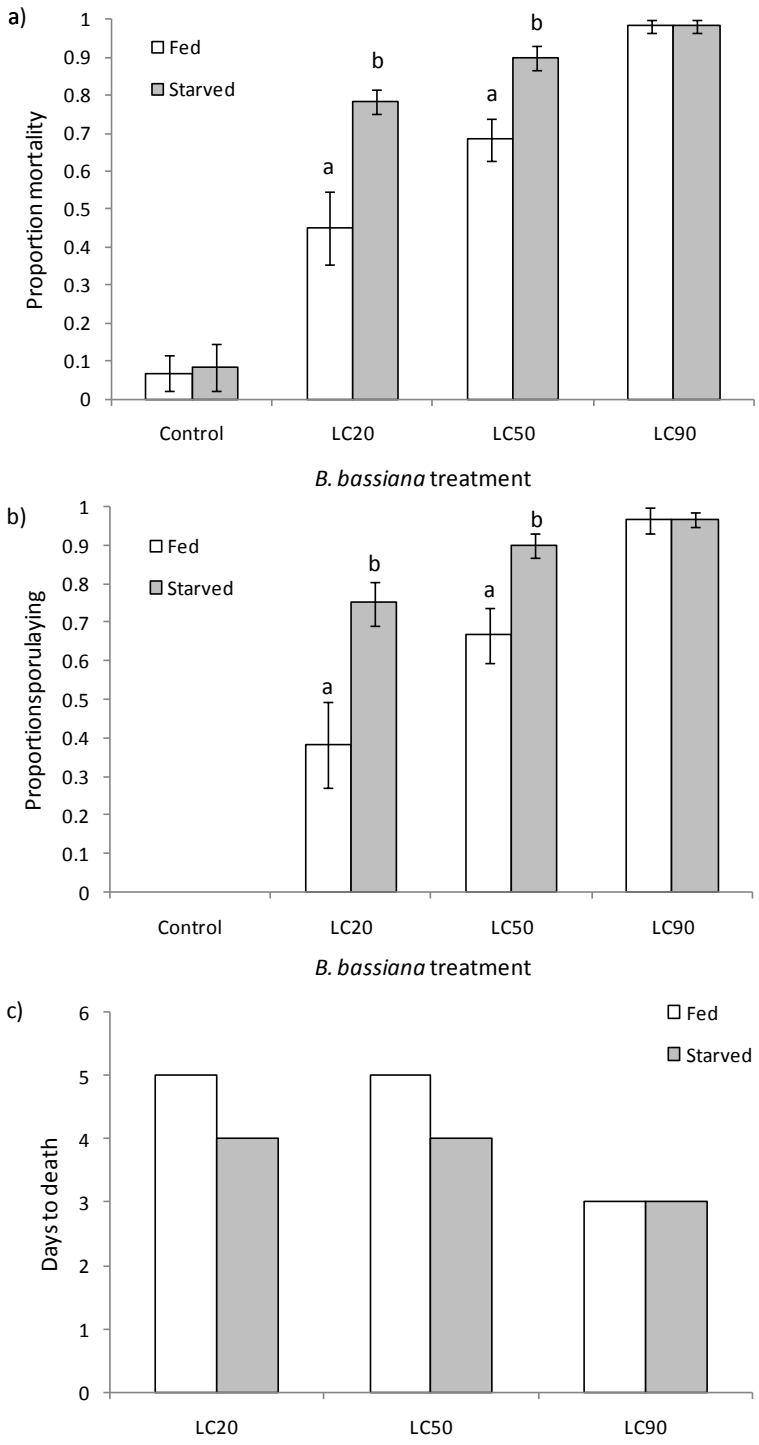

Figure 9. a) Mortality of DBM larvae following treatment with *B. bassiana* and then exposure to starvation or an optimal feeding regime. For each *B. bassiana* treatment columns marked with the same letter are significantly different (LSD; $P<0.05$). **b)** Sporulation of DBM larvae following treatment with *B. bassiana* and then exposure to a starvation optimal feeding regime. For each *B. bassiana* treatment columns marked with the same letter are significantly different (LSD; $P<0.05$). **c)** Median time to death of fed and starved third instar DBM larvae following topical treatment with LC_{20}, LC_{50} or LC_{90} of *B. bassiana*. For LC_{20} and LC_{50} treated larvae, the median time to death was significantly shorter for starved larvae than for fed larvae ($P<0.05$).

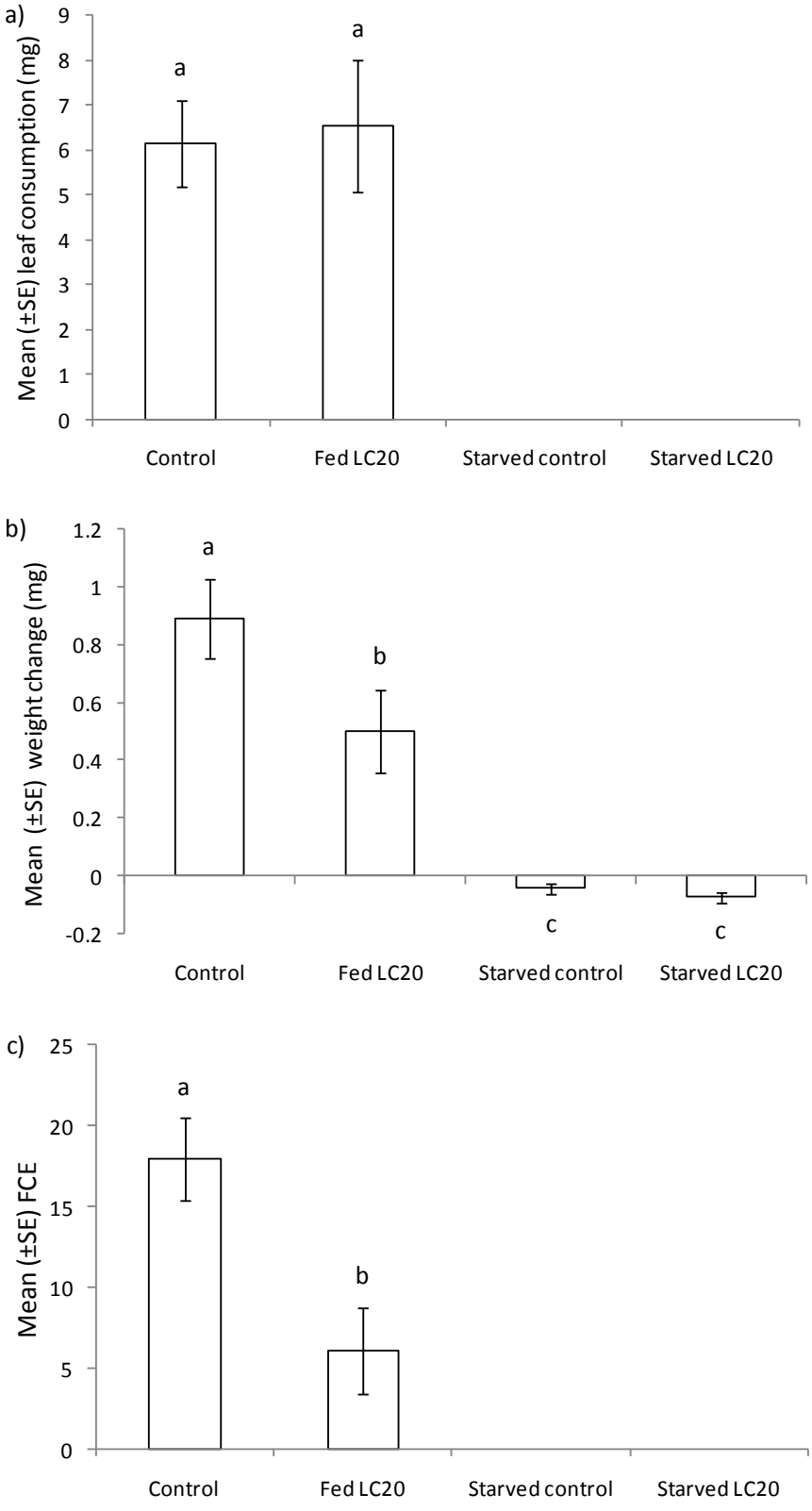

Figure 10. a) 24 h foliage consumption by control and *B. bassiana* (LC_{20}) treated DBM larvae ($P>0.05$). **b)** Weight gain by control and *B. bassiana* (LC_{20}) treated DBM larvae when supplied with food or starved for 24 h; columns marked by a different letter are significantly different ($P<0.05$). **c)** Food conversion efficiency (FCE) of control and *B. bassiana* (LC_{20}) treated DBM larvae in 24 after inoculation; marked by a different letter are significantly different ($P<0.05$).

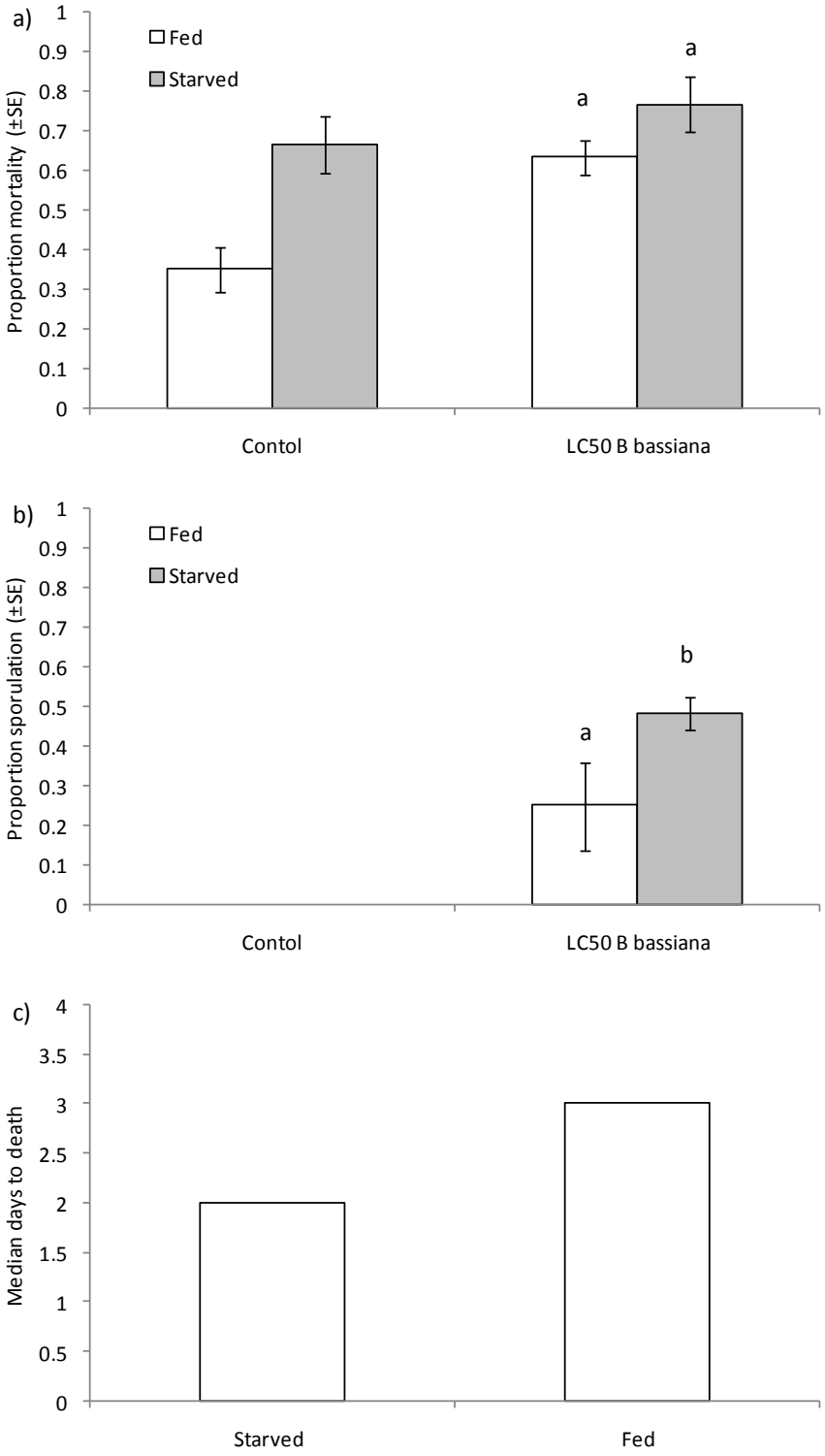

Figure 11. a) Mortality of CCC larvae following treatment with *B. bassiana* and then exposure to starvation or an optimal feeding regime. For *B. bassiana* treatment columns marked by the same letter not are not significantly different (LSD; $P>0.05$). **b)** Sporulation of DBM larvae following treatment with *B. bassiana* and then exposure to a starvation optimal feeding regime. For *B. bassiana* treatment columns marked by a different letter are significantly different (LSD; $P<0.05$). **c)** Median time to death of fed and starved third instar DBM larvae following topical treatment with an LC_{20}, LC_{50} or LC_{90} of *B. bassiana*. For LC_{20} and LC_{50} treated larvae, the median time to death was significantly shorter for starved larvae than for fed larvae ($P<0.05$).

Table 4: The susceptibility of fed and 24 h starvation stressed third instar DBM larvae to *B. bassiana* in topical application bio-assays.

Treatment	n	LC_{50} (95% CI) conidia ml^{-1}	Ratio: LC_{50}(Fed)/LC_{50}(starved)
Fed	180	6.1×10^4 ($2.2 \times 10^4 - 1.4 \times 10^5$)	
Starved	180	3.9×10^3 (2.4×10^3- 5.8×10^3)	15.6

Table 5: Haemolymph phenoloxidase activity (measured as mean absorbance at 485 nm after 240 mins at room temperature) in GVB, DBM and CCC following starvation, feeding on imidacloprid treated (LC_{20}) plant material or feeding on untreated plant material. For each assay, mean absorbance values followed by a different letter are significantly different ($P<0.05$).

Insect	Treatment	Duration	Mean Absorbance (485 nm) after 240 minutes
GVB	Fed control	24h	0.631 (±0.050)a
	Starvation	24h	0.613 (±0.016)a
	LC_{20} imidacloprid	24h	0.726 (±0.051)a
DBM	Fed control	24h	0.474 (±0.022)a
	Starvation	24h	0.348 (±0.019)b
	LC_{20} imidacloprid	24h	0.292 (±0.052)b
	Fed control	12h	0.537 (±0.031)a
	Starvation	12h	0.420 (±0.039)b
	LC_{20} imidacloprid	12h	0.590 (±0.022)ac
CCC	Fed control	24h	0.338(±0.019)a
	Starvation	24h	0.387 (±0.012)a
	LC_{20} imidacloprid	24h	0.355 (±0.036)a

4. Discussion

In laboratory species-specific dose-response bioassays, the three target insects, DBM, CCC and GVB were susceptible to imidacloprid when it was applied to the foliage upon which they fed. The LC_{50} (95% CI) against GVB (second instar nymphs), DBM (third instar larvae) and CCC (second instar larvae) was estimated to be 0.019 (0.007-0.036) ppm, 0.175 (0.096-0.259) ppm and 0.164 (0.120-0.214) ppm, respectively; reflecting the greater susceptibility of GVB (Hemiptera) to the insecticide when compared to DBM and CCC (both Lepidoptera) (Table 1). *Beauveria bassiana* was pathogenic to all three target pests when applied directly to the cuticle and when applied to the foliage upon which they fed. For each insect the LC_{50} for topical applications of the pathogen was significantly lower than the LC_{50} for foliar applications, indicating that topical applications were more effective than foliar applications (Table 2). Foliar applications of *B. bassiana* were particularly ineffective against CCC larvae (Table 2). Of the three insect species investigated, DBM larvae were most susceptible to the pathogen in both topical and foliar assays whereas CCC larvae were the least susceptible. In topical assays, the LC_{50} for third instar DBM larvae was 80-fold lower than the LC_{50} for second instar CCC (Table 2). These larvae are of very similar size and the difference probably reflects the greater intrinsic susceptibility of DBM to *B. bassiana*, however, the assays were conducted using different food plants for each species and the effect that this may have had on the relative susceptibility of the two insects to *B. bassiana* should be considered.

The interaction between a sub-lethal (LC_{20}) dose of imidacloprid and *B. bassiana* was investigated in dose-response bioassays in which the insecticide was fed to test insects simultaneously with foliar or topical applications of the pathogen. Insect mortality was then compared with mortality induced by appropriate applications of the pathogen alone. Treatment with imidacloprid (LC_{20}) did not increase mortality induced by topical or foliar applications of *B. bassiana* in any of the insects tested (Table 3). In other insects it has been hypothesised that sub-lethal doses of imidacloprid enhance *B. bassiana* infection by affecting insect behaviours which impact the acquisition and/or the retention of conidia (Boucias et al 1996; Quintela and McCoy 1997, 1998) or by inducing physiological stresses (Furlong and Groden 2001). In GVB, imidacloprid had no effect on the number of feeding sites established by nymphs, indicating that insect movement was not affected by the insecticide (Figure 3). Further, although imidacloprid treatment induced significant starvation stress in GVB, which was at least comparable to that induced by 24 h food deprivation (Figure 2), it appears that this level of stress was not severe enough to increase susceptibility to the pathogen. Thus imidacloprid treatment of GVB did not elicit either of the responses known to increase susceptibility to *B. bassiana* in other insects in GVB, providing an explanation for the lack of a significant interaction between the insecticidal agents in the bioassays. Similarly imidacloprid treatment did not affect the movement of CCC (Figure 7) and there was no evidence that it induced any feeding stress in exposed CCC larvae (Figure 6). Accordingly, there was also no evidence that imidacloprid treatment induced either the behavioural or stress levels in CCC required to increase susceptibility to *B. bassiana* infection. In DBM, the insecticide promoted the establishment of significantly more feeding sites, indicating that DBM movement was increased (Figure 5) and induced feeding stress, although not to the same extent as starvation (Figure 4). Thus, although imidacloprid did induce the type of behavioural and stress responses necessary to increase susceptibility to *B. bassiana*, the lack of a significant interaction between the two insecticidal agents indicated that it did not induce either to a sufficient magnitude in DBM to increase either the acquisition of conidia or stress insects sufficiently to cause increased mortality due to *B. bassiana.*

The immune response of GVB nymphs and CCC larvae was not affected by 24 h starvation or 24 h exposure to imidacloprid (LC_{20}), indicating that neither treatment compromised the capacity of these insects to combat *B. bassiana* when challenged (Table 5). In DBM larvae the immune response was compromised by 12 h of starvation but only after 24 h exposure to imidacloprid (Table 5). *Beauveria bassiana* conidia invade the host haemolymph approximately 12 h after attachment to host cuticle (Fernandez *et al*. 2001). These results suggest that starved DBM were more susceptible to *B. bassiana* as their immune response was compromised by the time that *B. bassiana* began to invade their tissues. Although imidacloprid compromised the immune response after 24 h of exposure (Table 5), critically it had no effect on the immune response 12 h after exposure (Table 5), providing a likely explanation for its failure to increase the susceptibility of DBM to the pathogen. The suggestion that starvation stress compromises the immune response of DBM larvae is supported by further experiments which showed that following inoculation with a low dose of *B. bassiana* conidia, larvae gained less weight than un-inoculated controls (Figure 10b), despite consuming as much food (Figure 10a), resulting in a significant reduction in the efficiency with which ingested food was converted to body mass (Figure 10c). This indicates that energy resources were diverted away from growth and towards some other metabolic process. Elicitation of the insect humoral (non-cellular) immune response is energetically costly and it has been shown previously that insects deprived of metabolic energy resources are more susceptible to *B. bassiana* infection (Furlong and Groden 2003). Thus, metabolic stress in DBM (but not in GVB or CCC) can compromise the immune response and increase susceptibility to *B. bassiana*. However, this stress must be induced rapidly so that its effects are manifested prior to or during the critical period when the pathogen begins invasion of the host tissues. It is possible that application of imidacloprid 24 h prior to the application of *B. bassiana* to DBM larvae could increase the susceptibility of larvae to the pathogen as the insecticide has been shown to significantly affect the humoral immune response of the insect by this time (Table 5), however, the interaction is likely to be complicated by the resumption of feeding by imidacloprid intoxicated DBM larvae.

The study clearly demonstrated that there is no interaction between imidacloprid and *B. bassiana* in either GVB or CCC and that this is likely to be due to sub-lethal doses of the insecticide causing no behavioural changes or metabolic stress in either species of insect. While no significant interaction between imidacloprid and *B. bassiana* in DBM was demonstrated, the study showed that this was likely to be because the level of metabolic stress induced by the sub-lethal dose tested was not sufficient to stress DBM adequately at a critical time in the infection process. An extremely important and fundamental finding of this study was that stress in insects can suppress the humoral immune response and that this in turn can lead directly to increased susceptibility to an invading pathogen. To our knowledge this is the first time that such a phenomenon has been demonstrated for a horticultural insect pest. The study thus has wider implications for the combined use of chemical and microbial insecticidal agents in plant protection where the desired aim is to use one agent to synergise the other by stressing the target insect (see Furlong and Groden, 2003). Although target insects may indeed suffer physiological changes which increase their susceptibility to invading pathogens, it is crucial that these changes are induced to coincide with host invasion by the pathogen. As evidenced by the response of DBM to imidacloprid in this study, the consumption of only a small amount of food by target insects may prove sufficient to maintain metabolic stress levels below the threshold required to compromise the immune response and so increase susceptibility to a pathogen. Consequently, any anti-feedant compounds used to increase susceptibility to *B. bassiana* are likely to be ineffective unless they almost completely preclude

larval feeding or unless they are applied sometime in advance of the pathogen. Applications of even very effective anti-feedant compounds are unlikely to achieve this under field conditions due to the difficulties associated with achieving complete plant coverage and dilution of the anti-feedant due to plant growth. However, methods by which the immune response of insects can be compromised to increase their susceptibility to invading pathogens should be explored in order to increase efficacy and reduce the time to kill of the many entomopathogens which are available to growers but currently under utilised in plant protection due to their perceived unreliability and the relatively long time that they take to kill hosts when compared with conventional insecticides.

5. Technology transfer

Two papers are in preparation for submission to an international peer reviewed journal (Journal of Invertebrate Pathology). Articles will also be prepared for publication in Ausveg and the *Brassica* IPM newsletter.

Project outcomes will also be made available to *Brassica* growers in southeast Queensland by presentation of findings at a *Brassica* field day to be organised by Queensland Primary Industries and Fisheries.

6. Recommendations - scientific and industry

Scientific recommendations. The demonstrated link between suppression of the humoral immune response in DBM and its subsequent increased susceptibility to *B. bassiana* is of fundamental scientific importance. Further, the studies showed that it was possible to manipulate the immune response by different feeding regimes and thus increase *B. bassiana* mortality in experimental insects in the laboratory. The challenge now is to develop ways in which this finding can be exploited by the combination of *B. bassiana* (or other related pathogens) and other strategies which compromise the immune response of target insects.

Industry recommendations. The research indicates that it is unlikely that imidacloprid will be used in combination with *B. bassiana* for the control of GVB, CCC or DBM. However, as the results to the study show that different insects have very different responses to imidacloprid (and other metabolic stressors) it is very likely that there are some insect pests of horticultural crops in Australia which could be better controlled by a combination of imidacloprid and *B. bassiana* (and/or other related fungal pathogens). The interaction is likely to be best exploited following a thorough investigation of imidacloprid toxicology and pathogen dynamics in the given system. Despite the lack of a significant synergistic interaction between imidacloprid and *B. bassiana* in these studies, *B. bassiana* could still prove a useful tool to manage imidacloprid resistance in pests against which the insecticide is widely used as no negative interaction was uncovered.

7. Acknowledgments

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