Natural occurrence of the entomopathogenic fungi *Beauveria bassiana* as a vertically transmitted endophyte of *Pinus radiata* and its effect on above- and below-ground insect pests

M.-C. Lefort[1,2], A.C. McKinnon[1], T.L. Nelson[1] and T.R. Glare[1]

[1]*Bio-Protection Research Centre, PO Box 85084, Lincoln University, Lincoln 7647, Christchurch, New Zealand*
[2]*Department of Natural Sciences, Unitec Institute of Technology, Auckland, New Zealand*
Corresponding author: Marie-Caroline.Lefort@lincolnuni.ac.nz, mlefort@unitec.ac.nz

Abstract The New Zealand forest industry would greatly benefit from a successful way of controlling insect pests. The entomopathogenic fungus, *Beauveria bassiana*, could hold such potential. It has previously been shown to be capable of endophytic colonisation of the Monterey pine *Pinus radiata*. We investigated *B. bassiana* transmission in *P. radiata* and whether this fungus, while acting as an endophyte, was beneficial to this tree species by testing its effect on above- and below-ground insect feeders. *Beauveria bassiana* was detected in *P. radiata* seedlings which had not previously been exposed to the fungus, indicating a vertical mode of transmission. The presence of the fungus negatively affected the fitness of below-ground insects feeding on the plant by reducing their survival by over 10% and their weight by about 5%. This vertically-transmitted beneficial endophyte of pine could be used cost-effectively to control insect pests in commercially-grown *P. radiata* plantations.

Keywords Biological control, *Helicoverpa armigera*, *Costelytra zealandica, Pinus radiata, Beauveria bassiana*

INTRODUCTION

Pinus radiata D. Don (Pinales: Pinaceae) is a fast-growing softwood intensively used in the forest industry around the world (Mead 2013). This species is the most extensively planted commercially-produced tree in New Zealand (New Zealand Ministry of Primary Industry 2014). The cost-effective management of forests requires both biological and socio-economic factors (Mead 2013) to be considered in order to maximise the return on investment. *Pinus radiata* is highly susceptible to a range of damaging insect species, comprising both above- and below-ground feeders. The common terrestrial insect pathogenic fungi *Beauveria* spp. Vuillemin (Ascomycota: Hypocreales), occurs on multiple hosts in most regions of the world (Bissett & Widden 1988; Rehner et al. 2011), and could be used in a cost-effective way to control insect pests in commercially-grown trees. *Beauveria bassiana* (Bals.) Vuill., has been reported to be endophytic in a variety of plant species (Vidal

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& Jaber 2015), including in *P. radiata* (Reay et al. 2010; Brownbridge et al. 2012). There is some evidence that while acting as a plant endophyte, *Beauveria* can reduce pest damage (Gurulingappa et al. 2010; Akello and Sikora 2012; Castillo Lopez et al. 2014) by inhibiting insect development and reproduction and may also be antagonistic to plant pathogens (Ownley et al. 2008; Ownley et al. 2010).

Although much of the literature reports endophytic establishment of *B. bassiana* following artificial inoculation, natural occurrence in *P. radiata* seed, needles and roots was reported for 15% of trees tested (Reay et al. 2010), supporting the possibility that the fungus could be vertically transmitted. (Quesada Moraga et al. 2014). The role of endophytic *B. bassiana* as an active and protective agent in *P. radiata* has not been demonstrated (Brownbridge et al. 2012). The present study aimed to evaluate the prevalence of endophytic *B. bassiana* in the Monterey Pine *P. radiata* sourced from forestry seed lines, and to test whether the occurrence of the fungi reduces the fitness of above- and below-ground insect pests. The invasive corn earworm *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae) and larvae of the New Zealand beetle *Costelytra zealandica* (White) (Coleoptera: Scarabaeidae), two phytophagous pests reported to cause significant damage to *P. radiata* pines (Chapman 1984; Herman & Davidson 2000), were chosen as models of above- and below-ground insect feeders respectively for this study.

MATERIALS AND METHODS

Investigation of *Beauveria bassiana* transmission in *Pinus radiata*

Detection of Beauveria bassiana in emergent seedlings

This study used *P. radiata* seedlings grown from commercial seeds (PF Olsen seed, GF Plus; Amberley & Kaingaroa and Seddon, NZ). To determine if *Beauveria* was present in the seed, thirty seeds were surface sterilised (1 min 70% EtOH, 7 min 0.04% NaClO, washed 2 x 1 min sterile distilled H_2O (sdH_2O)), and grown on 1% water agar media in sterile conditions for approximately 10 days at 22°C. Seedlings were cut into 2 cm sections and placed on *Beauveria* semi-selective media (BSM) plates and incubated for 21 days at 20°C in the dark (Brownbridge et al. 2012). A further twenty seeds were germinated and subsequently whole seedlings were surface sterilised following the same procedure, then cut transversally with one half of the radicle stored at 4°C for microscopic analysis. The rest of the radicle was processed to obtain DNA for *Beauveria* endophyte detection by PCR.

Identification of Beauveria cultured from seedlings

Samples from fungal cultures obtained from surface sterilised plant sections were grown 24-48 hours in PDB (Difco) at ~22°C at 160 rpm. Hyphae were ground using liquid nitrogen, and DNA was extracted using a Plant Genomic DNA Extraction System (Viogene, Taiwan) following the manufacturer's protocol and re-suspended in 200 µl of water. DNA from pure fungi was amplified for a fragment of TEF1-α gene using the primers EF349 (5'- TGGCCACCAGCACTCACTAC) and EF1685R (5'-ATGTCACGGACGG-CGAAA) (Reay et al. 2010). PCR products were sequenced to confirm identity and checked for identification using BLASTn (Altschul et al. 1990), then aligned with sequences obtained from previously published phylogenies (Rehner et al. 2011) to confirm species identification.

PCR detection of Beauveria in planta

Primers designed to amplify a 431 bp fragment of EF-1α were optimised specifically for direct detection of endophytic *Beauveria* sp. from all *P. radiata* DNA samples in a one-step PCR experiment (primer sequences as follows EF52F 5'-3': TGACAAGCTCAAGGCCGAG and EF481R 5'-3': GGAGGGCTCAAGCATGTTGT). Amplifications were carried out in a thermal cycler using 40 cycles of 45 sec at 95°C, 45 sec at 60°C and 90 sec at 72°C. Pure *P. radiata* DNA previously obtained (McKinnon, 2011) was also included in each run as a plant DNA positive control to

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ensure specificity of the reaction. PCR products were sequenced and checked for identification using BLASTn and aligned with TEF-1α sequences obtained from the Genbank (NCBI) database and local (unpublished) sequence data to conduct a phylogenetic analysis. Nucleotide alignments were performed in Genious Pro ver. 5.6.5. (Biomatters Ltd, NZ) using the Clustal W. algorithm with default parameters and phylogenetic analysis was conducted in MEGA version 6 (Tamura et al. 2013). The evolutionary distances were computed in MEGA using the Maximum Composite Likelihood method (Tamura et al. 2011) and were in the units of the number of base substitutions per site. The final analysis involved 15 nucleotide sequences. All positions containing gaps and missing data were excluded. There were a total of 260 positions in the final dataset.

All pine DNA samples were simultaneously amplified with the universal plant primers psbA3_f (Sang et al. 1997) and trnHf_05 (Tate & Simpson 2003) to confirm that DNA was present and the PCR effective.

The sensitivity of the PCR detection assay was tested against a serial dilution of *B. bassiana* DNA spiked into pure plant DNA and sdH_2O. The DNA concentration was ascertained using a Qubit® Fluorometer and the number of genome/TEF1-α gene copies calculated per microliter of sample. Using this dilution series as gDNA template, the EF52F and EF481R primers were able to amplify as little as 3 copies of the *Beauveria* TEF-1α gene (equivalent to 3 CFU/µl water), with bands visualised on 1.2% agarose gel in TAE.

Visualisation of endophytic fungi with transmission electron microscopy

To visualise the fungus in the seedlings, stored pine samples were prepared for preliminary light microscopic analysis as described above. The direct PCR detection method determined that only one of the twenty seedlings was positive for *B. bassiana.* Light microscopy on fixed and sectioned material confirmed that 19 other pine samples contained no obvious fungal endophytes. The single positive seedling sample was fixed for TEM by immersion for 2 hours at room temperature (RT) in 2% glutaraldehyde/3% formaldehyde in 0.1M phosphate buffer (pH 7.2), followed by 3 washes in 0.1M phosphate buffer (pH 7.2) and then stored in 1% formaldehyde in 0.1M phosphate buffer pH 7.2 at 4°C. Samples were processed in 1% osmium tetroxide in 0.05M phosphate buffer (pH 7.2) at RT for 1, 2 and 4 h, then washed three times in 0.05M phosphate buffer. Dehydration was through an acetone series at RT, then embedded in an epoxy resin (Procure 812, ProSciTech Pty, Australia) at 60°C for 22 h. Sections were stained with uranyl acetate followed by lead citrate and examined with a Morgagni 268D TEM (FEI Company, Oregon, USA) operating at 80kV and fitted with a 40 µm objective aperture. Images were captured digitally.

Effect of endophytic *Beauveria bassiana* on insects in *Pinus radiata*

Model system

Third instar larvae of the New Zealand grass grub, *Costelytra zealandica,* were collected from a dairy pasture recently converted from a pine forest in the North Island of New Zealand (Tokoroa, 38°15'36.74"S 176°00'35.21"E). First instar caterpillars of *Helicoverpa armigera* were obtained from Plant & Food Research, Auckland. Thirty-five pine seedlings were grown and monitored for the presence of *Beauveria* endophytes over the course of two years. Trees were cultivated in a glasshouse for the entire duration of the study and grown in 12-14 months old potting mix comprised of 80% composted bark, 20% pumice grade 1-7 mm, 5 g/l osmocote extract, 1 g/l horticultural lime and 1 g/l hydraflo wetting agent. The potting mixes were also screened for the presence of *B. bassiana* prior to use by plating on BSM.

Detection of endophyte in two year old P. radiata trees

To determine the presence of *Beauveria* sp. endophytes, small sections of young roots and one needle fascicle were randomly sampled from

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each pine tree, rinsed under cold water and stored individually at 4°C. From these samples, a 5 cm long fragment of one root and one entire needle from the fascicle were surface sterilised (5 min in 2.5% sodium hypochlorite, rinsed in sdH_2O, then 1 min in 70% ethanol and washed x 3 in sdH_2O). Root and needle samples were snap frozen and ground in a sterile mortar. DNA extractions were then performed as described. DNA extracts were used in PCR to ascertain which trees were positive for endophytic *Beauveria*.

Effect of endophytes on below-ground insects

Costelytra zealandica larvae (n=96) were weighed on a 0.01 g portable digital scale and their initial weight recorded. Subsequently, using a block design by initial larval weight (i.e. block 1: 0.14 g, block 2: 0.15 g, block 3: 0.16 g and block 4: 0.16 g) to minimise the amount of unexplained variation in the subsequent statistical analysis, larvae were allocated to two different feeding treatments and kept in individual cells in multi-well cell culture plates at 15°C. Half of the larvae were fed *ad libitum* with chopped roots from pines endophytically infected with *B. bassiana* (i.e. treatment), while the other half were fed with chopped roots from pines with no detectable *Beauveria* sp. (i.e. control). Fresh roots were added to the cells twice a week over five weeks. As indicators of fitness, larval survival and growth (measured as weight gain) were recorded weekly, as well as the final weight of each larva at the end of the experiment.

Statistical analyses on the effect of the endophytic occurrence of *B. bassiana* in *P. radiata* on larval survival were carried out by Chi square testing. Larval growth was analysed by analysis of variance (ANOVA, complete block design) after exclusion of the larvae that died before the end of the experiment. Statistical tests were conducted with R version 2.12.1 (R Development Core Team 2011) and GenStat® version 14.1 (VSN International, Hemel Hempstead).

Effect of endophytes on above-ground insects

Helicoverpa armigera caterpillars (n=80) were weighed on a 0.0001 g readability digital scale and their initial weight recorded. A block design by initial weight, to minimise the amount of unexplained variation, was then followed to allocate the caterpillars to two different feeding treatments. Individual caterpillars were kept in 35 ml plastic containers containing a small pine cutting of 30 mm long placed in 2 ml eppendorf tubes filled with tap water to ensure that the cuttings remain turgid for the duration of the experiment. Containers were maintained in an incubator at 16:8 L:D cycle, at 23°C and 55% humidity. Half of the caterpillars were given cuttings from pines endophytically infected with *B. bassiana* (i.e. treatment), while the other half were fed with cuttings from control pines containing no endophytes (i.e. control). Pine cuttings were replaced twice during the two week experiment. Mortality and growth (weight gain) were recorded after two weeks. Statistical analyses on the effect of the endophytic occurrence of *B. bassiana* in *P. radiata* on caterpillar survival were carried out by Chi square testing. Growth of the caterpillars was analysed by analysis of variance (ANOVA, complete block design). Statistical tests were conducted with R version 2.12.1 (R Development Core Team 2011) and GenStat® version 14.1 (VSN International, Hemel Hempstead), respectively.

RESULTS

Electron microscopy

A single emerged seedling out of twenty was positive for *Beauveria* and processed for TEM. Ultrathin sectioning showed fungal hyphae (Figure 1) which was only detected in PCR *Beauveria*-positive seedlings. Two to three hyphal strands were found intercellular in the pine samples (Figure 1). No fungus grew from the germinated seedling sections placed on semi-selective BSM, presumably because the amount of fungus was too low to remain viable after sterilisation.

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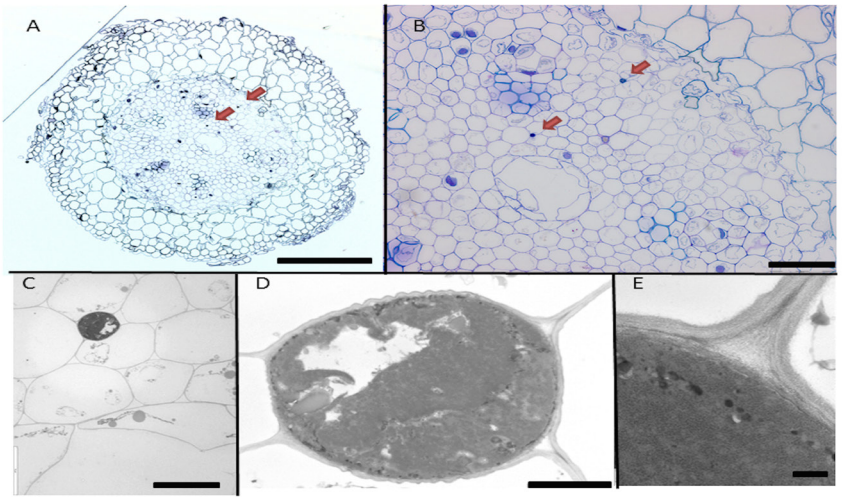

Figure 1 Sections through a *Pinus radiata* radicle showing putative *Beauveria* sp. hyphae. **A.** Thin section stained with toluene blue, with hyphae in intercellular spaces (red arrows) (bar = 200 µm). **B.** Close up on thin section (bar = 50 µm). **C, D and E.** TEMs of hyphae (bar = 10 µm, 0.2 µm and 2 µm) **C.** Hypha pushing between plant cells. **D.** Close up of fungal and plant cell wall interaction. **E**. Magnified view of C.

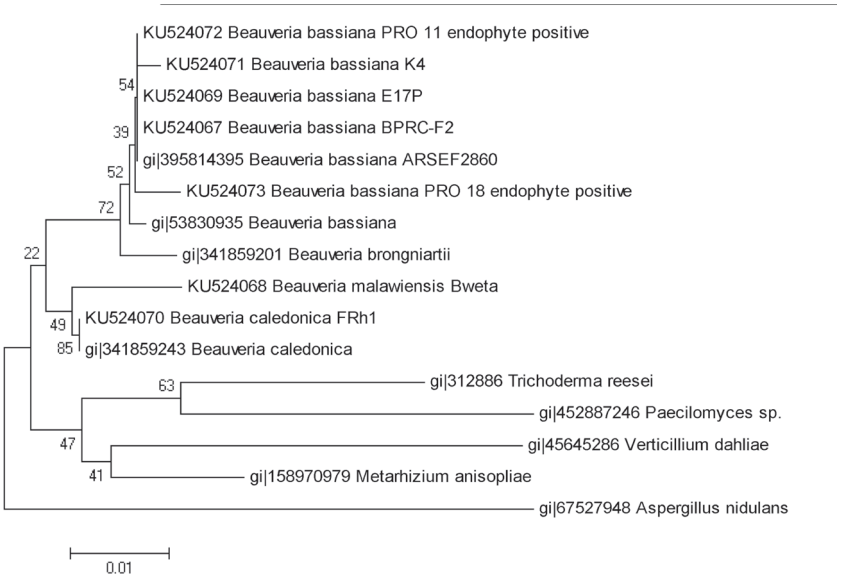

Figure 2 Phylogeny of TEF1-α partial sequences of various *Beauveria* species (constructed with the Neighbor-Joining method). The endophyte positive *P. radiata* samples are shown here aligning with *B. bassiana* (PRO 11 and PRO 18). A *Beauveria* isolate obtained from the same seed-line (BPRC-F2, identical to BPRC-F1) is also shown. Isolates used for comparison are either GenBank obtained or the Lincoln University fungal collection. 2860 =ARSEF 2860 from Genbank ascension ASM28067v1.

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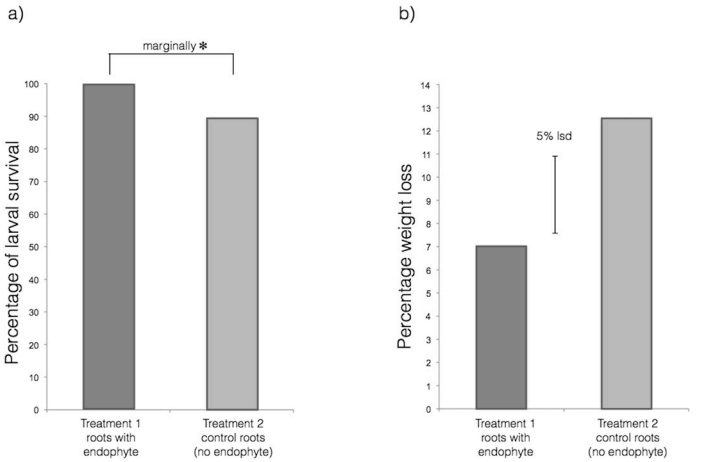

Figure 3 a) *Costelytra zealandica* larval survival when fed with pine roots free of endophyte and with pine roots endophytically infected with *Beauveria bassiana*. **b)** Larval weight variation when fed with pine roots free of endophyte and with pine roots endophytically infected with *Beauveria bassiana*.

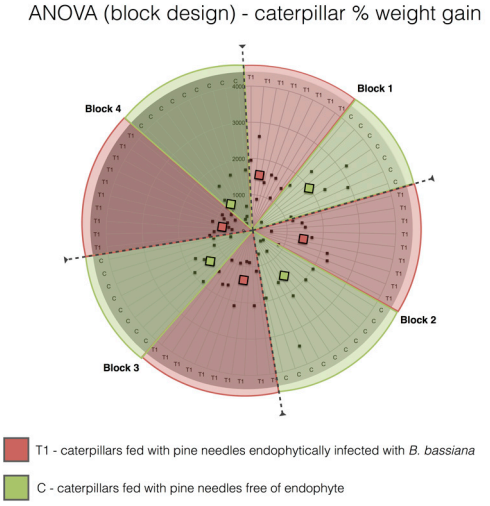

Figure 4 Individual *Helicoverpa armigera* caterpillar percentage weight gain (black dots) and average percentage weight gain per treatment and per block (squares), with the caterpillars fed with pine needles free of endophyte indicated by (C) and the caterpillars fed with pine needles endophytically infected with *Beauveria bassiana* indicated by (T1).

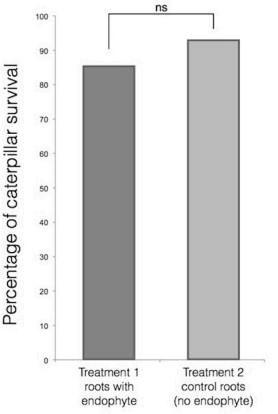

Figure 5 *Helicoverpa armigera* caterpillar survival of caterpillars fed with pine needles free of endophyte and caterpillars fed with needles endophytically infected with *Beauveria bassiana*.

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Endophyte detection in *P. radiata*

From the 30 plants sown and cultured for endophyte isolation, two *Beauveria bassiana* (*sensu stricto* clade A, Rehner & Buckley, 2005) colonies were obtained from the hypocotyl of a single emerged seedlings (isolates designated as BPRC-F1 and BPRC-F2). For the 35 two-year-old pine trees tested, the presence of *B. bassiana* was detected by PCR (confirmed by genotyping) in the roots of two trees (trees PRO 11 and PRO 18, Figure 2). The roots and needles of these two trees were subsequently used as treatment in the two experiments of this study, while the other trees were used as control. The soil in which the trees were grown contained no detectable *Beauveria* species. For the Maximum Composite Likelihood analysis, the optimal tree with the sum of branch length was 0.2288 (Figure 2). The phylogenetic tree branch lengths indicate evolutionary distances and bootstrapping (Felenstein 1985).

Effect of endophytes on below-ground insects

Below-ground insect larvae fed with roots free of endophyte survived marginally better than those fed with roots endophytically infected with *B. bassiana* (at 10% level of significance) (χ^2 = 3.3758, d.f. = 1, p = 0.06616) (Figure 3a). Although the average weight of the larvae tends to decrease over time under both treatments regardless of their initial weight, it was quite noticeable that the weight of the larvae decreased even more (by almost 5%) when fed with roots endophytically infected with *B. bassiana* (ANOVA, $F_{1,86}$ = 10.86, $p < 0.001$) (Figure 3b).

Effect of endophytes on above-ground insects

Caterpillars fed on pine needles from treatment pines (i.e. endophytically infected with *B. bassiana*) showed neither any impairment in their growth (ANOVA, $F_{1,66}$ = 1.03, p = 0.315) regardless of their initial weight (Figure 4) nor survival (χ^2 = 0.5008, d.f. = 1, p = 0.4792) (Figure 5) compared to the caterpillars fed on pine needles from control pines.

DISCUSSION

Our study supports that vertical transmission of *Beauveria* as an endophyte may occur in *P. radiata*. While there was no PCR detection of *Beauveria* from ungerminated seed directly, *Beauveria* was detected in newly emerged seedlings that had no prior exposure to the fungus. Direct observation with TEM of a positive seedling showed the fungus was present in very small quantities, only 2-3 hyphal strands per transverse section. We failed to detect *Beauveria* in the majority of the young seedlings grown from surface-sterilised seed and from the seed itself using the PCR method. However, based on the observations made from the TEM images it is possible that *Beauveria* can be present in very small quantities in seed. The seed casing may also contain some inhibitors to the PCR reaction (although seed DNA did amplify with pine specific primers).

Our study also suggests that plant host endophytic infection by *Beauveria* is relatively rare in nature as *B. bassiana* was isolated from only two pine seedlings, perhaps indicating vertical transmission. However, a similar genotype of *Beauveria* was detected two years later from mature plants sown from the same seed batch. This is consistent with a previous study that reported *Beauveria* was established in pine seedlings from both seeds and roots independently, but only one plant out of thirty was positive for endophytic *B. bassiana* after 9 months (Brownbridge et al. 2012).

Of the many reports of *B. bassiana* as a plant endophyte, few studies have demonstrated the effect of this fungal association on phytophagous insect pests (e.g. Bing & Lewis 1991; Cherry et al. 2004; Akello et al. 2008a,b; Quesada-Moraga et al. 2009; Gurulingappa et al. 2010; Akutse et al. 2013). Using *C. zealandica* and *H. armigera* as models of below- and above-ground insect feeders respectively, we showed that in *P. radiata* *B. bassiana* significantly reduced the fitness of the below-ground insect feeder, while no effect was observed on the above-ground feeder.

Previously, Griesbach (2000) found that a *Fusarium* sp. as a plant endophyte resulted in smaller

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larvae of the Banana Weevil *Cosmopolites sordidus* (Germar) (Coleoptera: Curculionidae) than those feeding on non-inoculated plants. Gurulingappa et al. (2010) found similar effects with the cotton aphid on cotton endophytically infected by *B. bassiana*. This previous research aligns with the weight loss observed in *C. zealandica* larvae fed with pine infected with *B. bassiana* in the present study.

In a majority of studies, no fungal growth was observed on the dead body of the insects, leading authors to hypothesize that the mode of action of *B. bassiana* might be due to the feeding deterrence induced by fungal metabolites or mycotoxins (Vega, 2008; Mantzoukas et al. 2015) rather than by direct mycosis infection. There is evidence that *Beauveria* strains produced different mycotoxins, but this has not been reported *in planta*. This hypothesis is in agreement with our results and would also explain the significant decrease in weight observed in the larvae fed with *B. bassiana*-infected pines.

It is important to note that the overall loss of weight itself, as observed in the larvae across both feeding treatments, could be due to different factors. Indeed, *C. zealandica* usually undergo a non-feeding pre-pupation stage (East & Kain 1981; Wright 1989). This experiment was performed with larvae already weighing between 0.14 and 0.16g. These larvae might have reached their optimal weight to enter pre-pupation during the course of the experiment, hence ceased feeding at an early stage of the trial, which would have resulted in a similar loss of weight under both feeding treatments. Furthermore, the nutritional value of the host plant on which *C. zealandica* larvae fed can affect the fitness of the larvae (Lefort et al. 2015; Lefort et al. 2014). It seems that while *C. zealandica* is able to use *P. radiata* as a host, this plant might not offer all the nutritional qualities required for optimal growth of the insect. Lefort et al. (2014) also suggested the possible occurrence of several races of this species that have become adapted to specific hosts. This might be the reason for pasture-collected insects used in our experiments not gaining weight while feeding on pines.

In our study feeding deterrence appears to be the best explanation of the loss of weight of *C. zealandica*. Our results also suggest that this effect against insect feeders remains limited to the infected tissue, as we did not detect any deterrent effect on the caterpillars fed with pine needles when only the roots were tested positive for the fungi.

In a study by Akello et al. (2008b), endophytic *B. bassiana* was isolated from different parts of banana plants, but to a greater extent from the roots and rhizomes of the plant. In the present study, the sampling of the pine needle and the molecular diagnostic for the occurrence of *B. bassiana* were performed several weeks after the sampling and molecular diagnostic were carried out on the roots. Similarly, the experiments using *H. armigera* caterpillars as the above ground-feeder were performed several weeks after the one on *C. zealandica* larvae. This raised further questions as to whether the mode of action of *B. bassiana* occurs through SAR with a limited lifespan or is truly a localised response, depending on the persistence of the endophyte in the plant.

The presence of *B. bassiana* as a vertically transmitted endophyte capable of reducing insect damage could have implication for pest control in these long-lived, commercially-grown trees. The issues around longevity of colonisation would need to be overcome, but it is possible that a vertically transmitted endophyte could provide a persistent and cost-effective solution for protecting commercial *P. radiata* forests from insects.

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