Efficacy of entomopathogenic fungi against large pine weevil, *Hylobius abietis* and their additive effects when combined with entomopathogenic nematodes.

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Disclosure statement

No potential conflict of interest was reported by the authors.

Key Message

- Entomopathogenic fungi (EPF) and nematodes (EPNs) were applied to conifer stumps alone and in combination for control of the large pine weevil, *Hylobius abietis*.
- Combinations of EPF and EPN had additive but not synergistic effects against *H. abietis*.
- EPN alone or in combinations with EPF reduced significantly *H. abietis* emergence.
- Application method did not affect the efficacy of EPF against *H. abietis*.
- EPF persist up to two years post-application. *Beauveria caledonica* is a new promising EPF species.

Abstract

The large pine weevil *Hylobius abietis* L. is an important pest of reforestation in northern Europe. In field trials we assessed the efficacy of entomopathogenic fungi (EPF) alone and in combination with entomopathogenic nematodes (EPN) against the immature stages, which develop in the stumps of felled conifer trees. We used commercial strains of *Metarhizium brunneum* (Petch) (Met52) and *Beauveria bassiana* (Bals.-Criv.) Vuill. (1912) and a strain of *Beauveria caledonica* isolated from the pest's habitat. The EPN used were *Steinernema carpocapsae* (Weiser) and *Heterorhabditis downesi* (Stock, Griffin, and Burnell). Efficacy was assessed both by infection of weevils in the pine stumps and by numbers of adult weevils emerging in traps erected above the stumps. EPF infected up to 23% of pine weevils, at depths of up to 25 cm below ground. Three methods of application of EPF were compared, but application method did not affect infection rates. When applied at half doses, EPF and EPN had additive effects: *M. brunneum* and *S. carpocapsae* caused a reduction in pine weevil emergence of 58% relative to control, *M. brunneum* and *H. downesi* 93%, and *B. caledonica* and *H. downesi* 86%. However, EPN alone offered good suppression of *H. abietis* populations and suppression by the mixture did not surpass the suppression afforded by EPN on their own. Our trials showed that *B. caledonica* is a promising species, rivalling the success of the other two EPF species. Furthermore, applied EPF and especially *M. brunneum* can persist for up to two years in the forest setting. It also appears that different fungi can have differential action against weevils above-below ground and therefore combinations of EPF may be beneficial. Based on our trials, further development of strains and application methods are required before EPF can be recommended for suppression of pine weevil.

Keywords

Forest pest, Entomopathogenic nematodes, Entomopathogenic fungi, Biocontrol; large pine weevil

Author Contribution Statement

LM, CG and KK conceived/designed research. LM conducted experiments. AK, CDW and POT assisted with trials. LM and AK analysed data. LM wrote manuscript. All authors read and approved manuscript.

Abbreviations

Entomopathogenic fungi (EPF), entomopathogenic nematodes (EPN)

Introduction

The large pine weevil *Hylobius abietis* (L.) (Coleoptera: Curculionidae) is a widespread pest of plantation forestry in 15 European countries, where it is a threat to 3.4 million hectares of forest (Långström and Day 2007). The immature weevils develop in the stumps of recently felled conifer trees and upon emergence the adult weevils feed on newly planted trees and can kill up to 100% of unprotected stock (Leather *et al.* 1999). A single adult can damage or kill several trees, therefore even small numbers of adults can have an economic impact on sites that have been replanted (Wainhouse *et al*. 2004). Without chemical control measures it is estimated that the resulting damage would reach €140 million for Europe (Långström and Day 2007). However, use of chemical insecticides is to be minimised in accordance with principles of sustainable forest management as well as the Sustainable Use of Pesticides directive (2009/128/EC). Therefore, there is a need for alternatives for incorporation in an integrated pest management approach to this pest.

Entomopathogenic nematodes (EPN) are a promising tool in the management of the pine weevil. They are applied to stumps in an inundative fashion when late instar larvae or pupae of the first generation of weevils are present, between one and two years after trees are felled (Torr et al. 2005; Brixey et al. 2006; Dillon et al. 2006, 2007, 2008; Williams et al. 2013, Kapranas et al., 2017a). Entomopathogenic fungi (EPF) are widely used against soil-inhabiting beetles including black vine weevil, *Otiorhynchus sulcatus* (Fabricius) (Ansari et al., 2006; Keller and Schweizer, 2007; Shah et al., 2007; Eckard et al., 2014) and may persist well in soil environments (Scheepmaker and Butt, 2010). Although EPF would initially seem to be less suitable than the actively host-seeking EPN for use against a pest inhabiting such a cryptic habitat, the limited information

available to date suggests that they may have a place in pine weevil suppression. Firstly, all developmental stages of *H. abietis* were killed by *Metarhizium robertsii* (Metschn.) Sorokīn, *Metarhizium brunneum (Petch)*, and *Beauveria bassiana* (Weiser.) in laboratory assays (Ansari and Butt, 2012). Secondly, *M. brunneum* and *B. bassiana* were also shown to infect pine weevils in stumps in field trials and had an additive effect when applied together with EPN (Williams *et al*., 2013; Evans et al., 2015). Thirdly, *H. abietis* are naturally infected with EPF in the field, including *Beauveria caledonica* (Glare *et al*., 2008, Williams *et al*., 2013). *Beauveria caledonica* appears to be a common natural pathogen of bark and wood-boring beetles of conifers in their natural habitat (Glare et al., 2008, Reay et al. 2008; Williams et al., 2013; Wegensteiner et al., 2015; Draganoval et al., 2017), making it an attractive candidate for further study against such pests, but efficacy of applied *B. caledonica* against *H. abietis* has not been assessed.

There is growing evidence that combinations of EPN and EPF may act synergistically against insects, including several coleopteran pests (Anbesse *et al*. 2008, Ansari *et al*. 2004, 2006, 2008, 2010; Wu et al. 2014; Wakil et al. 2017). Synergy can be defined as the interaction of two or more organisms, substances or agents to produce a combined effect greater than the sum of their individual effects. However, depending on the combination of agents and the test conditions, the result may be merely additive (when the sum of two treatments combined is equal to the sum of the effect of the two treatments used separately), or even antagonistic (the combination of two or more agents results in an overall effect that is less than the sum of their individual effects). The underlying mechanisms of synergistic interactions are unclear, but it is suggested that one agent may stress or alter the behaviour (e.g., feeding or movement) of the host, making it more susceptible to another agent. For example, insects infected by the *Metarhizium brunneum* may be less mobile, allowing EPN more time to penetrate the host (Ansari et al. 2004). Moreover, combined applications may make hosts more susceptible through suppressing their immune system, prolonging developmental stages or by the two treatments acting on different components of the host population (Lacey *et al.* 2015; Mc Namara et al. 2017). If a combination of biocontrol agents results in synergy, then the potential cost of using these agents may be reduced by allowing lower application rates. Moreover, if the agents target different components of the pest population, then the overall effect of the combination may be greater than could be achieved by using one or the other agent on its own, irrespective of application rate.

The aim of the present study was to investigate the ability of EPF, both alone and in combination with EPN, to suppress *H. abietis* populations in the field. Previously, field trials in Wales showed that *M. brunneum* could effectively suppress numbers of weevils emerging from stumps (Evans et al., 2015), while in Ireland,

significant suppression was not achieved by EPF alone, though additive effects with EPN were recorded (Williams et al., 2013). Three EPF strains were utilised in these field studies, commercial strains of *M. brunneum* and *B. bassiana* and a strain of *B. caledonica* isolated from the pine weevil habitat. The efficacy of EPF was investigated alone and in combination with EPN through emergence trapping of adult weevils and destructive sampling of stumps to locate infected immature weevils. The EPN species used were *S. carpocapsae* and *H. downesi*, both of which are promising candidates for pine weevil suppression (Williams et al., 2013; Kapranas et al., 2017a,b). This study extends the work of Williams *et al*. (2013), which was conducted under similar conditions, with the following additions: (1) *B. caledonica* was incorporated into these trials as it infects pine weevils in their cryptic habitat, making it a promising biological control agent for this pest, and additional combinations of EPF and EPN were tested; (2) We assessed if the method of application of EPF can be improved according to recent studies in Wales (Evans et al. 2015); (3) The location of infected weevils (depth and distance in soil) was analysed in order to help explain the relative success of different species and application methods; (4) Persistence of applied EPF in the forest ecosystem was recorded; (5) information on growth patterns of the three EPF species in conifer stumps is presented.

Materials and Methods

Source and culture of entomopathogenic fungi and nematodes

Steinernema carpocapsae (EN03) formulated in vermiculite was provided by e-nema GmbH (Schwentinental, Germany) and was rehydrated, checked for viability and enumerated before use. *Heterorhabditis downesi* (strain K122) is an Irish strain maintained at Maynooth University and was cultured in vivo in late instar *Galleria mellonella* larvae (Mealworm Company, Sheffield, England) as described in Dillon *et al* (2006). A commercial strain of *M. brunneum* (Met52; formerly referred to as *M. anisopliae*) produced by Novozymes (Denmark) was used (National Agrochemical Distributors, Lusk, Dublin).

Dry conidiospores of *B. bassiana* (Bals.) Vuill. (Experimental strain 1694) were supplied by Becker Underwood, West Sussex, UK. *Beauveria caledonica* (2c7b) is a native strain isolated from a soil sample taken close to a pine stump in a felled forest in Hortland, Co. Kildare (Ireland). The soil sample was baited with *G. mellonella* larvae and fungus from the infected cadaver was identified through DNA sequencing of an ITS PCR product (a region of the internal transcribed spacer unit of the ribosomal DNA, ITS4, was amplified by PCR).

To obtain sufficient quantities for the trials both *B. caledonica* and *B. bassiana* were cultured in Sabouraud dextrose liquid medium (Oxoid) in a shaking incubator for 6 days at 25°C and 250 rpm. Basmati rice (500 g) was added to a double-bagged autoclave bag. The opening of the autoclave bag was plugged with a sponge held in place with masking tape and autoclaved at 120°C for 30 minutes. Liquid culture (100 ml) was poured into the bag and mixed with the rice. Bags were placed flat on shelves and turned daily for 10-15 days at 25°C. To remove the spores from the rice, 100 g rice was placed in conical flasks with 100 ml PBST (Phosphate buffered saline with Tween 0.05%) and placed in a shaking incubator at 200 rpm to dislodge spores. The suspension was sieved through a double layer of muslin.

Sites of field studies

Two field studies were carried out over consecutive years in clear-felled lodgepole pine *Pinus contorta* Dougl. var. *latifolia* forests. The 2013 field site was at Glendine, Slieve Bloom, Laois (53°05'45.5"N 7°34'02.2"W, Felled 04-09/2011) and the 2014 site was at Cloondara, Longford (53°44'22.0"N 7°54'03.1"W, Felled 04–05/2013). The soil at both sites was deep peat. At each site, treatments were arranged in a randomized block design, with one stump of each treatment in each block and at least ten blocks. Stumps were marked and colour-coded for different treatments before treatment.

Treatments and Application

In all trials, 500 ml of the suspension of nematode infective juveniles (IJs) and/or fungal conidiospores was applied to the stumps using the standard method of pouring the suspension onto the soil around the stump (Dillon *et al*. 2006) and to the side of the stump. Different application methods were trialed in 2014. Full EPF treatment was $1x10^9$ spores/stump and half treatment was $5x10^8$ spores/stump. Full EPN treatment was $3.5x10^6$ IJs/stump and half treatment was $1.75x10^6$ IJs/stump. Half rates were included to facilitate assessment of interactions between agents in mixed treatments. Control stumps were untreated. *M. brunneum*, *B. caledonica* and *B. bassiana* were diluted to the desired concentration using 0.05% (v/v) Tween 80 as a surfactant. Treatments were made up in 5 L bottles in the laboratory and were regularly agitated while being transported to the field. Treatments were applied on 5.6.2013 (Glendine) and 12.6.2014 (Cloondara).

Assessment of efficacy: destructive sampling and monitoring emergence

Destructive sampling of stumps was carried out using the method of Dillon *et al*. (2006). One quarter of each stump was destructively sampled, four weeks post application. Pine weevils were carefully removed using a forceps and brought back to the laboratory in 24 well plates. Different forceps were used for each treatment. The stage (larva, pupa, callow adult or adult) and infection status (alive, fungus-killed and dead due to other causes) of weevils were recorded in the field, along with the location of the individuals (depth above/below soil level and distance from the bole of the stump). In 2013 the growth patterns of the different species of EPF were also observed in destructively sampled stumps.

Emergence traps, based on Moore (2001) but without the electric shock mechanism, were erected over control and treated stumps on 3.7.13 (in Glendine) and on 24.6.14 (in Cloondara). They were emptied approximately every two weeks throughout the season until weevils ceased emerging in late autumn. Different stumps were used for destructive sampling and emergence trapping (see Tables 1 and 2). All the stumps within a block were used either for destructive sampling or for trapping emerging adult weevils.

Trials

In 2013 the first aim was to compare the efficacy of three EPF (*B. bassiana*, *B. caledonica*, *M. brunneum*) against pine weevil using destructive sampling and emergence trapping for assessment. The second aim was to investigate the use of *M. brunneum* alone and in combination with *S. carpocapsae* for the reduction in numbers of adult weevils emerging. Both EPF and EPN were applied alone as well as in combination. There were seven treatments plus untreated controls (see Table 1).

In 2014 the EPN used was *H. downesi* and EPF used were *B. caledonica* and *M. brunneum.* Both EPF and EPN were applied alone as well as in combination. One aim was to assess if different methods of applying EPF affected their efficacy. The methods tested were: suspension poured in the 'standard' method (applied to sides/base of stump), suspension poured into the gap between bark and stump at the top of stump (top) (Evans *et al*. 2015) and suspension applied to both top and sides (top + sides). The second aim was to assess the effect of single and combined applications of *H. downesi* and either *M. brunneum* or *B. caledonica* on weevil emergence; for this purpose, the treatment was applied to top and sides of the stump, as this combined both the 'standard' method and the method referred to in Evans *et al*. (2015). There were 12 treatments plus untreated controls (see Table 2).

198

199 *Survey for persistence of fungi*

200 In 2015, stumps treated in 2013 (Glendine) were tested for persistence of EPF two years post
201 application. The treatments sampled were stumps treated with *B. caledonica*, *B. bassiana* and *M. brunneum* and
202 controls; ten stumps were sampled per treatment. Eight soil samples (50 ml cores) were taken per stump (at a
203 depth of 0-10 and 10-20 cm and in 4 directions North, South, East, and West), and one composite bark (above
204 ground) sample (volume approx. 50 ml) consisting of at least 4 subsamples. Each soil or bark sample was baited
205 with five *G. mellonella* larvae. This was done by placing five larvae in a plastic tub (6cm x 9.5 cm) with each
206 sample and storing at 20°C, with inversion of the tub every 2-3 days to encourage contact between insect and
207 sample. Larvae were removed once dead but before they were mycosed. All infected larvae were surface-
208 sterilized by flaming after immersion in 70% ethanol before culturing the EPF on Potato Dextrose Agar plates
209 (Oxoid, Ltd, England) covered with a layer of colourless sterile cellophane. Once sufficient hyphal growth had
210 occurred it was peeled off and placed in a 1.5 ml centrifuge tube. Samples were exposed to freeze-thaw and
211 were broken down further using a plastic pestle (Sigma-Aldrich Co. LLC). Qiagen DNEasy Mini Plant Kit and
212 protocol was used to extract DNA. DNA was measured using the Nanodrop 1000 (Mason, Dublin, Ireland). A
213 region of the internal transcribed spacer unit of the ribosomal DNA was amplified using primers (Eurofins)
214 ITS4 (5'TCC TCC GCT TAT TGA TAT GC'3) and ITS5 (5'GGA AGT AAA AGT CGT AAC AAG G'3) in
215 the following reaction mix; 5X MyTaq Reaction buffer (5 µl), Promega GoTaq Polymerase (0.125 µl), forward
216 and reverse primers (1 µl of each), ddH_20 (15.875 µl) and template DNA (2 µl). PCR was carried out for 3 min
217 at 94°C followed by 34 cycles of 1 min at 94°C, 2 min at 55°C, followed by 7 min at 72°C, on an Eppendorf
218 Mastercycler (Eppendorf, Stevenage, UK). Positive and negative (dH_2O) controls were included in each PCR
219 run. PCR products were cleaned up using miPCR purification kit (Metabion) and sequenced by GATC Biotech
220 AG (Germany). Identities of the isolates were confirmed using BLAST.

221

222 *Statistical analysis*

223 Statistical analysis using Minitab V. 16 and GraphPad Prism V.5. were carried out on data from each
224 year separately. Emergence data were transformed to normality using log (x+1) transformation; untransformed
225 data are shown in results. Differences in numbers of adult weevils between treatments were analyzed using
226 ANOVA followed by Dunnett's multiple comparison test comparing each treatment with the control. In 2013,

although the overall ANOVA was not significant, each treatment was compared to the control using a t-test in order to assess what treatments lead to significant weevil suppression.

For emergence data in all field trials the type of interaction (synergistic, additive, or antagonistic) between EPN and EPF was determined using a procedure described by Ansari *et al*. (2008). All combinations of treatments that were trapped for emergence in both years were analysed; The expected additive reduction in emergence ($R_{expected}$) for the EPN–EPF combinations was calculated by $R_{expected} = R_{nematode} + R_{fungus} (1 – R_{nematode})$, where $R_{nematode}$ and R_{fungus} are the observed reduction caused by EPNs and EPF alone (half rates), respectively. Results from a χ^2-test, $\chi^2 = (R_{observed} – R_{expected})^2/R_{expected}$, were compared to the χ^2 table value for 1 degree of freedom. If the calculated χ^2-values exceeded the table value, there would be reason to suspect a non-additive effect that is synergistic/antagonistic, between the two agents. If the differences $R_{observed} – R_{expected} = D$ had a positive value, a significant interaction was then considered synergistic, and if D had a negative value, a significant interaction was considered antagonistic. Analysis involves half rates of all pathogens involved in each combination.

Further statistical analysis was carried out using GENSTAT statistical package (Version 14, VSN International, Hemel Hempstead, UK). Analysis of factors influencing weevil infection rates were performed with generalized linear models, starting from fully saturated models, using where possible empirically estimated scale parameters to account for potential overdispersion, and arriving at the minimum adequate model via backwards model simplification (Crawley 1993). Infection rates among different treatments (either fungi alone in 2013 or fungi and application method in 2014) were compared with logistic analysis and using quasi-binomially distributed errors. Infection rates in relation to depth below soil surface and horizontal distance from the bole of the stump were explored with mixed Generalized Linear Models. Fungal species, method of application (2014), depth and distance were introduced as fixed effects whereas each stump was introduced in the analysis as a random effect. Analysis was run separately for both years.

Results

Field trials in 2013

Overall, there was no significant difference between treatments in the number of emerging adult weevils (ANOVA: $F_{7,79}=1.43$, $p>0.05$). Since the prime aim of our study is to identify agents or combinations capable of reducing weevil emergence relative to the control, and simultaneous comparison of many treatments

may obscure particular comparisons of interest, we compared each treatment to the control separately. Using this approach, we detected a significant difference between control stumps and both full dose *S. carpocapsae* (T=3.143, df=18, p<0.01), and the mixed treatment (T=2.35, df=18, p<0.05). Both treatments caused a reduction in number of weevils emerging relative to the control (Figure 1A). The mixed EPF and EPN treatment had an additive effect with an expected reduction in numbers of weevils emerging of 66% and an observed reduction of 58% (χ^2= 0.011, p=0.92).

Destructive sampling was carried out on ten blocks four weeks after application and another ten blocks eight weeks after application. The two assessment periods were combined for analysis as they were not significantly different from each other. The infection rate in untreated control stumps was 4.6%; these fungi were not identified. Infection rates among different fungal treatments (excluding the untreated controls) were compared with logistic analysis and using quasi-binomially distributed errors. Infection rates differed significantly among the three fungal species used ($F_{2,55}$ = 3.29, P = 0.045, Figure 2). Infection rates in relation to depth below soil surface and horizontal distance from the bole of the stump were explored with mixed Generalized Linear Models. Infection rates of weevils were significantly different among fungal species ($F_{2,607}$= 7.83, P< 0.001, Figure 2). Infection rates of weevils were negatively influenced by depth ($F_{1,607}$ = 6.16, p= 0.013, Figure 3A) and distance ($F_{1,607}$=21.85, P <0.001. Figure 3B). Growth morphology of the three EPF species was also observed during destructive sampling (Figure 4).

Field trials in 2014

There was a significant difference in the number of emerging adult weevils between treatments ($F_{7,8}$=3.73, p=0.001). Full *H. downesi* and the mixed treatment *M. brunneum* and *H. downesi* were significantly different to the control stumps (p<0.05); both treatments caused a reduction in pine weevil emergence (Figure 1B). Both of the mixed EPF and EPN treatments had an additive effect. For *M. brunneum* and *H. downesi* the expected reduction in number of pine weevils emerging was 86% and the observed reduction was 93% (χ^2= 0.86, p= 0.93). For *B. caledonica* and *H. downesi* the expected reduction in number of pine weevils emerging was 90% and the observed reduction was 86% (χ^2= 0.001, p= 0.97). All treatments that included EPN reduced emergence below the suggested threshold of 20 weevils per stump (Kapranas *et al*., 2017).

Efficacy of different methods of application

Another aim of the study was to compare the effect of different application methods on EPF efficacy against pine weevil. To investigate this, destructive sampling was carried out on five blocks four weeks after application and on another five blocks eight weeks after application. Only 0.34% of weevils in control stumps showed signs of fungal infection. Infection rates among different fungal treatments (controls excluded) were compared with logistic analysis and using quasi-binomially distributed errors. There was no significant difference in weevil infection rates among different treatments (fungal species and application method) ($F_{5,57}$ = 0.41, P = 0.838) (Figure 5). Fungal species and depth were not significant factors in determining infection rates (Fungus: $F_{1,682}$=1.37, P=0.242; depth: $F_{1,682}$=0.14, P=0.712) but their interaction was significant ($F_{1,682}$ = 3.46, p = 0.041, Figure 6). Distance did not significantly influence weevil infection rates by EPF ($F_{1,682}$ = 0.73, p=0.193). All developmental stages of *H. abietis* were found to be infected (Table 3) as previously shown (Ansari and Butt 2012; Williams et al 2013).

Persistence of entomopathogenic fungi post application

Two years following application, *B. bassiana* and *B. caledonica* were recovered from around stumps treated with each of the three fungal species and from control stumps while *M. brunneum* was only recovered at stumps to which it had been applied (Fig. 7). Thus, *M. brunneum* appears to persist in the environment at two years post application.

Discussion

In this study we assess the efficacy of using EPF alone and in combination with EPN for suppression of the large pine weevil *H. abietis.* Using EPF alone for this purpose might be inefficient because it is difficult for the non-motile conidiospores to reach the weevils feeding within the roots of the stumps. Nonetheless, their persistence for long periods in some environments could provide long term control effects (Scheepmaker and Butt, 2010). Williams *et al*. (2013) investigated the efficacy of EPN and EPF (*B. bassiana* and *M. brunneum*) applied to stumps to suppress *H. abietis* emergence and suggested that if choice of EPF strain and application technologies are optimised, EPF may present a viable option for pine weevil management in the future. *Beauveria. caledonica* is a naturally occurring pathogen of pine weevil larvae (Glare et al., 2008; Williams *et al*., 2013; van Vlaenderen, Griffin and Meade, unpublished), which justified its inclusion in our current trials.

313 Moreover, its proposed use against pine weevils is strengthened by the fact that it appears to be restricted to
314 forest coleopterans (Reay *et al.* 2008) and hence its impact on non-target insects could be low.

315

316 *EPF for suppression of large pine weevil and their persistence*

317 EPF used alone had varying success both across the different field trials and the three fungal species
318 trialed. In 2013, *B. caledonica* was the most promising of the three species applied at full dose, with an overall
319 observed reduction in emergence of 38% as well as the highest proportion of infected weevils. This was the first
320 time this species has been trialed against *H. abietis* and the results highlight its potential application as a
321 biocontrol agent. *B. bassiana* was not effective in suppressing pine weevil in the 2013 field studies, similarly to
322 what was reported by Williams *et al*. (2013) and so it was not included in the 2014 trials. In 2014, *B. caledonica*
323 was found to be as effective as *M. brunneum*, infecting up to 14.7 and 17.3 % of the pine weevils within stumps,
324 respectively.

325 We observed that the three EPF had distinct growth morphologies on weevil cadavers in the stump. As
326 is typical for the species, *M. brunneum* sporulated on the cadaver. Spores could be dispersed within the space
327 between bark and stump by water or insects (Roy *et al*., 2010). For *B. bassiana*, hyphal growth radiated out
328 from the cadaver and spread along the underside of the bark, which could distribute spores closer to other pine
329 weevils within the stump, suggesting that it might be better able to recycle and give extended control within
330 stump; however, recycling potential was not examined in this work. *B. caledonica* hyphae grew out from the
331 cadaver, but instead of remaining in the under-bark space, it exited through the bark: this may be useful for
332 infecting insects in the soil, including emerging *H. abietis* adults.

333 The ability of entomopathogenic Hyphomycetes species to persist in an environment is another
334 important attribute of a successful biocontrol agent. For propagules that exhibit good persistence, there will be a
335 higher probability of an insect coming in contact with sufficient propagules to cause disease (Inglis et al. 2001).
336 There is evidence that *M. brunneum* persisted in the forest soil environment at two years post application, which
337 may have implications for utilising EPF in prophylactic biocontrol, though initial tests of this approach were not
338 promising (Williams et al., 2013). It is unknown whether the fungus recovered two years post-application was
339 the original inoculum or resulted from recycling in pine weevils or other hosts. Persistence of EPF for up to two
340 years following application is not unusual, but there are few studies conducted in conifer forest agroecosystems
341 (Scheepmaker and Butt, 2010). Little is known about the natural ecology and population dynamics of EPF in

conifer forests (Reay et al., 2008; Ormand et al., 2010), but advances in this knowledge would be invaluable in designing more effective biocontrol strategies.

Efficacy of combining of entomopathogenic fungi and nematodes

EPN offered better suppression of *H. abietis* populations than EPF, with the most effective treatments for reducing weevil emergence being EPN alone or in combination with EPF. These field trials strengthen the use of EPN, which actively seek out hosts, as a viable control method for pine weevils. *Heterorhabditis downesi* alone or combination with EPF reduced weevil emergence by 83-93%, with a reduction of 60-72% for *S. carpocapsae*. The levels of reduction by these two EPN species is in agreement with previous studies (Dillon *et al.* 2006, 2007, Torr *et al.* 2007, Williams, 2013, Kapranas et al. 2017a). The highest observed reduction in emergence across all treatments and years was a combination of *M. brunneum* and *H. downesi* (93%).

All three EPF and EPN combinations tested resulted in additive rather than synergistic effects. One possible explanation for the lack of synergistic action of EPN+EPF is that application rates of *H. downesi* already resulted in relatively high levels of control; even half rate resulted in reduction of weevils by 83%, and therefore synergistic effects might have been better observed by using even lower rates of EPN than the ones we used. However, the effect was also additive in 2013 where the 40% weevil suppression rate by half-dose *S. carpocapsae* left ample room for detection of synergy. Moreover, there was no evidence of synergy of EPN and EPF combinations in the Williams et al. (2013) study. Another approach may be to apply EPF earlier in the season. EPF may weaken the insect or modulate their immune system, ultimately making them more susceptible to subsequent pathogens (McNamara *et al*., 2017; Ansari *et al*., 2006; Ansari *et al*. 2004). Immune suppression by EPF has been demonstrated for pine weevil larvae (McNamara, 2016). Early application would also coincide with prevalence of late instar larvae, the stage which was found to be most likely to be infected both in the present study and in Williams et al. (2013).

Methods of application

Similar field studies to the 2013 trials reported in this work were carried out in Wales from 2009-2012 to assess EPF and EPN at a range of doses against pine weevils (Evans *et al.* 2015). *Metarhizium brunneum* was tested alone and in combination with *S. carpocapsae* and results indicated that all treatments were effective in suppressing emergence of pine weevil adults even at low doses, indicating a potential reduction in cost (Evans *et*

al. 2015). In those studies, *M. brunneum* reduced weevil emergence by about 60%, a much more successful result than observed in Ireland both in our 2013 study and previously by Williams et al. (2013). As the application method in Wales differed to that used in Ireland, an investigation into the effect of application method on treatment efficacy was included in the 2014 field study to see if it might explain the differences in results. In Wales, suspension was applied to the gap between the bark and stump at the top, while in Ireland it was applied to the sides of stumps and surrounding soil. Another study testing different application methods of EPN for pine weevil suppression showed that application on top vs application around the stump can have significant effects on EPN efficacy and that weevil suppression relative to suggested thresholds can be improved by altering the method of EPN application depending on the nematode species (Kapranas et al. 2017b). These two application methods were tested, along with a combination of both methods. However, application method did not have a significant effect on efficacy of EPF against pine weevils. Thus, the difference in efficacy of treatments in this field study in comparison to results from Wales cannot be explained by application method, nor can success of the two EPF species tested in our trials be enhanced by altering application method in this way. In addition, both sets of trials employed the same strain of *M. brunneum*, but other factors such as tree species, soil type and climatic factors may be at play.

Fungal parasitism of weevils in relation to depth and distance

In 2013, infection rates of *H. abietis* were negatively influenced by depth and distance for all EPF, thus it was harder for EPF to reach weevils further from their site of application, for instance weevils located deep in the roots of stumps. EPF-infected *H. abietis* were found up to 16 cm above and 25 cm below soil level, again highlighting the advantage of being able to infect weevils further from where the EPF is applied. Williams *et al.* (2013) reported that applied EPF reached *H. abietis* at depths of up to 18 cm. In 2014, the significant interaction of EPF species and depth shows that different EPF species behave differently at different depths. *Beauvaria caledonica* infected a higher proportion of weevils above soil level (at depths >1cm) while *M. brunneum* infected a higher proportion of weevils below soil level (at depths >-1cm). This may indicate potential for dual EPF application to control pine weevils, if the EPF are more effective at different depths perhaps together they could target a larger number of weevils, conferring greater control. EPF combinations can have promising results, for example *M. flavoviride* and *B. bassiana* have been used in combination to overcome the constraints of temperature in controlling thermoregulating grasshoppers (Inglis *et al.* 1997).

Conclusions

The results for EPF in two years' trials reported here together with the three similar eradicant trials reported by Williams et al. (2013) together provide a comprehensive assessment of three EPF, both alone and with EPN. Based on the five years of trials conducted in Ireland to date, we can conclude that strains and methods tested to date do not on their own result in adequate infection or suppression of pine weevils, nor could they be recommended for use in combination with EPN. On the positive side, EPF infected pine weevils at depths of 25 cm below soil level and distances of 25 cm from the bole. Here, we applied EPF in a conventional manner- aqueous suspension of spores applied to the soil/tree surface, relying on the application water, rainfall and possible phoretic hosts to carry them to the weevils developing under the tree bark, including deep below soil level. Considering the difficulties of access to weevils, the level of success achieved in these trials is encouraging and should stimulate a search for superior strains adapted to this environment, and improvement of application methods. For example, Goble et al. (2016) applied microsclerotia of *M. brunneum* to tree bark in a hydromulch to target a wood-boring pest, the Asian longhorned beetles *Anoplophora glabripennis* (Motschulsky). The native EPF *B. caledonica* emerged as a potential candidate for biological control of large pine weevil with efficacy rivalling or surpassing the commercial strains tested. Its use certainly warrants further investigation, but a search for superior strains is also warranted. Our trials and those of Williams et al. (2013) used available examples of three EPF species. As there is considerable intra-specific variation in EPF (Amiri-Beshel *et al*., 2000; Quesada-Moraga & Vey 2003; Ansari & Butt, 2012), there is scope for selecting a strain of fungus that would be better adapted to the pest and habitat than the three tested here. The natural occurrence of diverse species of EPF infecting pine weevils (Williams et al., 2013, van Vlaenderen, Griffin and Meade unpublished; Popowska-Nowak et al., 2016; Wegensteiner et al., 2015) presents a source of potential isolates that may be suited to this purpose, such as the *B. caledonica* strain used in these field trials. Further research could be directed on alternative methods of application EPN and EPF to the inundation method as the forest industry will require effective non-pesticide methods for weevil control in the near future due to legislation (SUD) and registration challenges.

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Table 1. Treatments in 2013 field trial, giving number of stumps used for erection of emergence traps or for destructive sampling to record locations and stages of infected insects

Species	Full dose	Half dose
B. bassiana	10 traps + 20 destructive	
B. caledonica	10 traps + 20 destructive	
M. brunneum	10 traps + 20 destructive	10 traps
S. carpocapsae	10 traps	10 traps
M brunneum + S. carpocapsae		10 traps
Untreated	10 traps + 20 destructive	

Table 2. Treatments in 2014 field trial, giving number of stumps used for erection of emergence traps or for destructive sampling (dest.) to record locations and stages of infected insects. Agents were applied either in full or half dose. Full dose rates of EPF were applied in each of three manners (to sides and top of stump or to either sides or top only).

Species	Full dose			Half dose
	Sides and top	Sides	Top	Sides and top
B. caledonica	10 traps + 10 dest.	10 dest.	10 dest.	10 traps
M. brunneum	10 traps + 10 dest.	10 dest.	10 dest.	10 traps
H. downesi	10 traps			10 traps
B. caledonica + H. downesi				10 traps
M. brunneum + H. downesi				10 traps
Control	10 traps + 10 dest.			

573 Table 3. Proportion of *Hylobius abietis* infected by fungus (total n, alive and dead, in parentheses) at each stage
574 of the lifecycle present at destructive sampling of stumps treated with each of three species of entomopathogenic
575 fungus
576
577

Trial	*H. abietis* stage	*B. caledonica* treated stumps	*M. brunneum* treated stumps	*B. bassiana* treated stumps
2013	Larvae	0.36 (128)	0.24 (81)	0.13 (146)
	Pupae	0.11 (72)	0.14 (52)	0.02 (49)
	Callow adult	0 (3)	0 (3)	0 (18)
	Adults	0 (25)	0 (10)	0.1 (21)
2014	Larvae	0.22 (94)	0.44 (57)	-
	Pupae	0.11 (102)	0.17 (132)	-
	Callow adult	0.16 (19)	0.29 (7)	-
	Adults	0.11(84)	0.06 (86)	-

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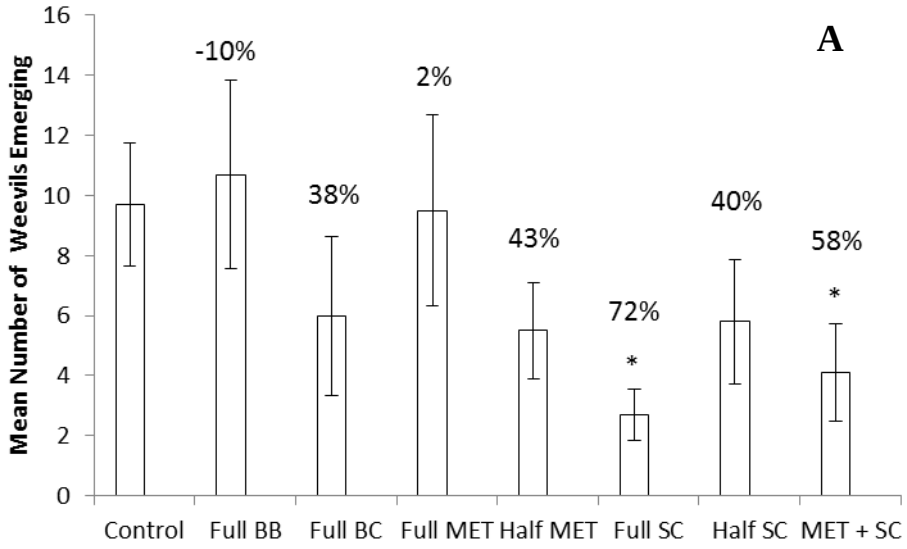


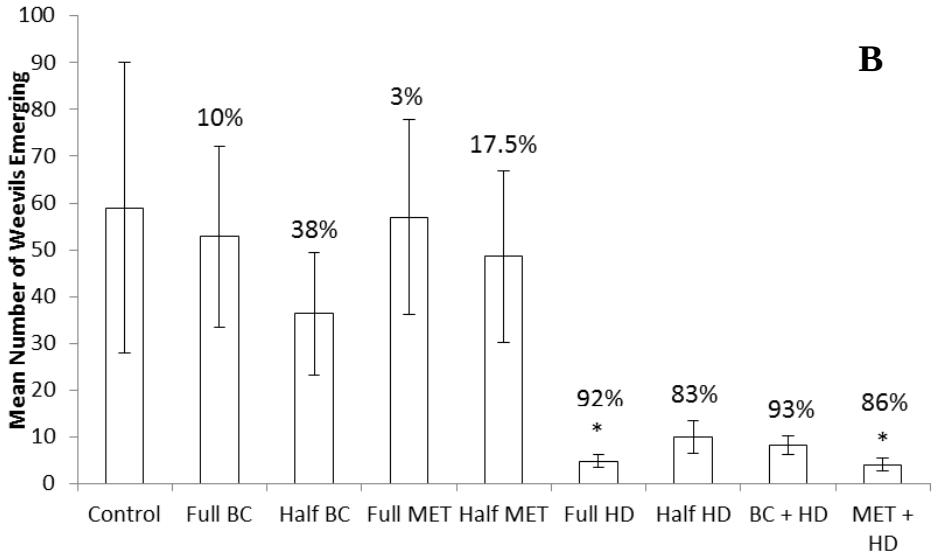
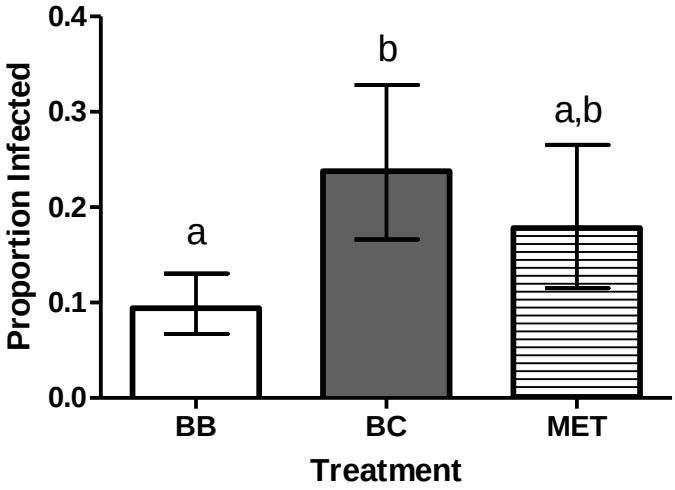


Figure 1. Mean (± SE) number of *Hylobius abietis* emerging in **a.** 2013 and **b.** 2014 field studies. Control: untreated stumps, BB: *Beauveria bassiana* BC: *B. caledonica*, MET: *Metarhizium brunneum*, SC: *Steinernema carpocapsae*, HD: *Heterorhabditis downesi*, Mixed: half rate of each agent and half. Treatments differing from control are indicated by *p<0.05 or **p<0.01, two tailed t-test (2013) or Dunnett's multiple comparison test 2014. Data labels refer to % reduction in emergence relative to control.



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591 Figure 2. Proportion of *Hylobius abietis* that were infected by fungus at time of destructive sampling compared
592 with logistic analysis and using quasi binomially distributed errors (2013 field study). Infection rates differed
593 significantly among different fungal species ($F_{2,55}$ = 3.29, P = 0.045). Stumps were treated with *Beauveria*
594 *bassiana* (BB), *B. caledonica* (BC), or *Metarhizium brunneum* (MET). Treatments sharing the same letter are
595 not significantly different.

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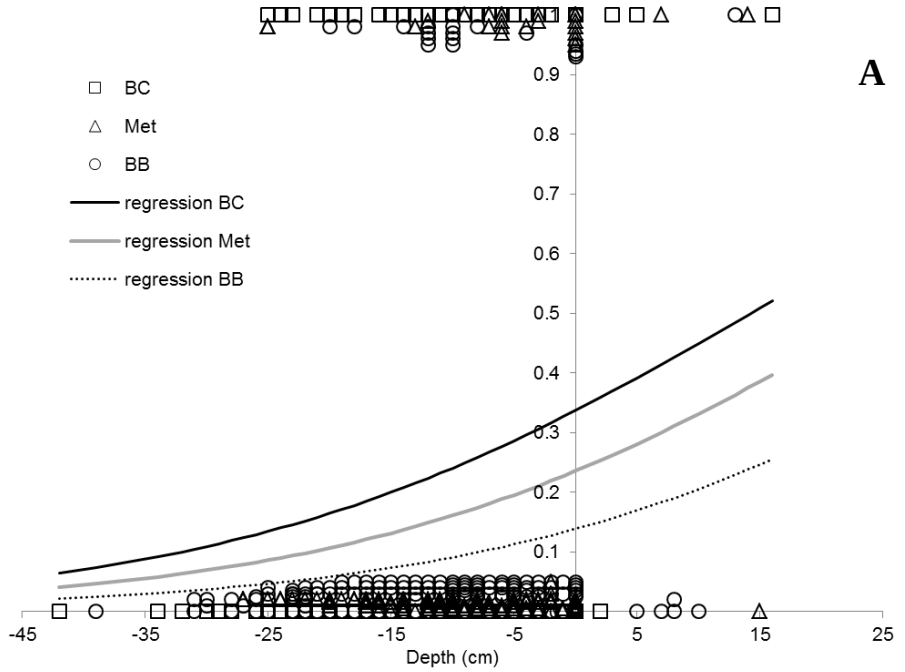

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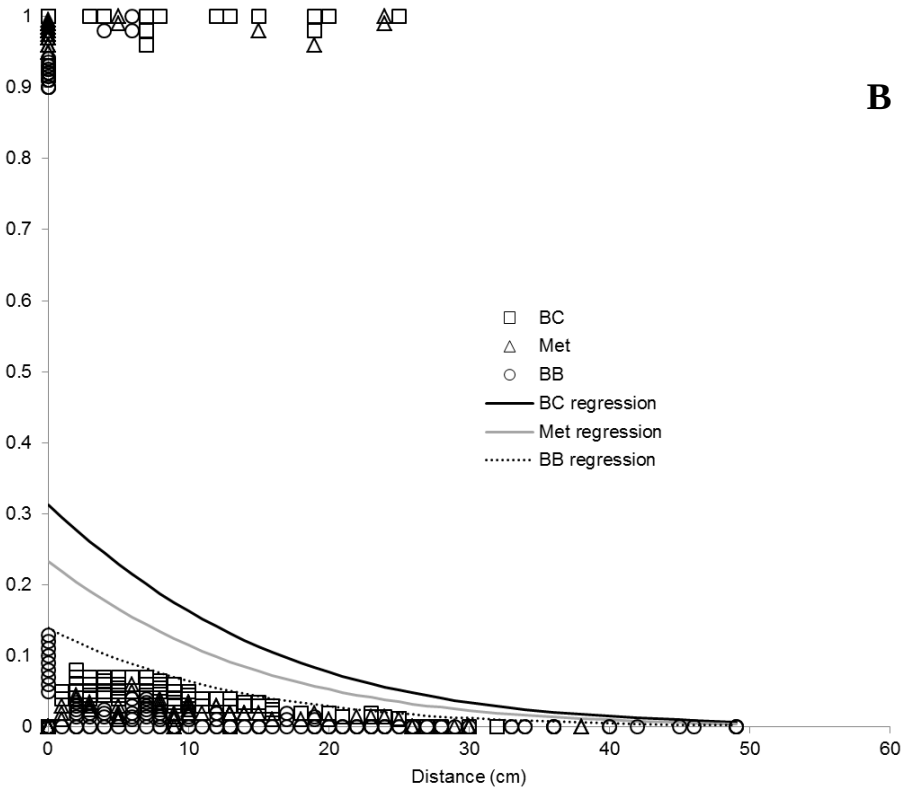
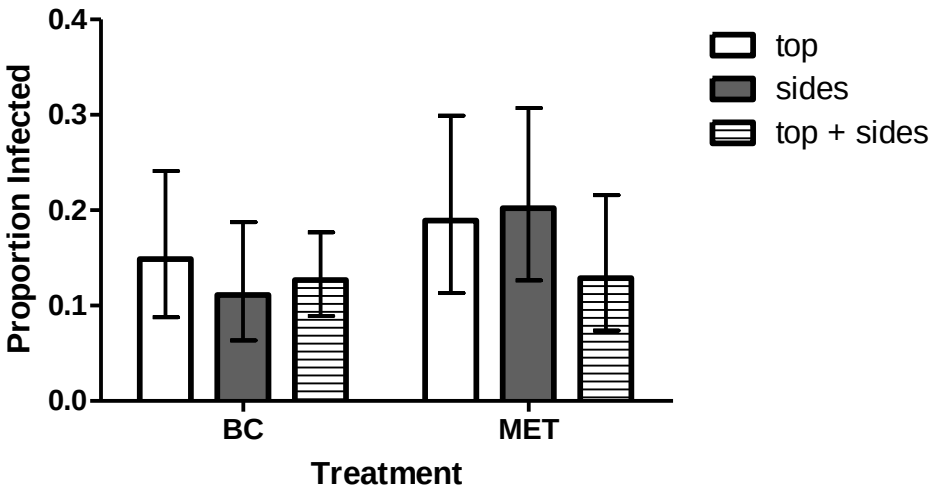


601 Figure 3. Proportion of *Hylobius abietis* infected with EPF, relative to **a.** depth (cm) above (+)/below (-) soil
602 level and **b.** distance (cm) from stump, at time of destructive sampling in 2013 field studies. BB: *Beauveria*
603 *bassiana* BC: *B. caledonica*, MET: *Metarhizium brunneum.* Points show actual data and lines show the fitted
604 logistic regression models. Points are slightly displaced from x axis for clarity.

Figure 4. *Hylobius abietis* larvae A. alive and B-D in stumps treated with EPF. B *Beauveria caledonica*: Mycelium encases the cadaver and extends from it as a single rhizomorph, which grew through the bark (bark removed for clarity). The extended portion seen at the bottom of the picture had emerged through the bark into the soil. C. *Beauveria bassiana:* hyphal growth covers cadaver and radiates from it, remaining under the bark and D. *Metarhizium brunneum* with minimal extension of hyphae from cadaver.



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616 Figure 5. Proportion of *Hylobius abietis* that were fungal infected at time of destructive sampling compared with
617 logistic analysis and using quasi binomially distributed errors (2014 field study). Stumps were treated with
618 either *B. caledonica* (BC) or *M. brunneum* (MET). There were no significant differences in weevil infection
619 rates among different treatments (fungal species and application method) ($F_{5,57} = 0.41$, $P = 0.838$).

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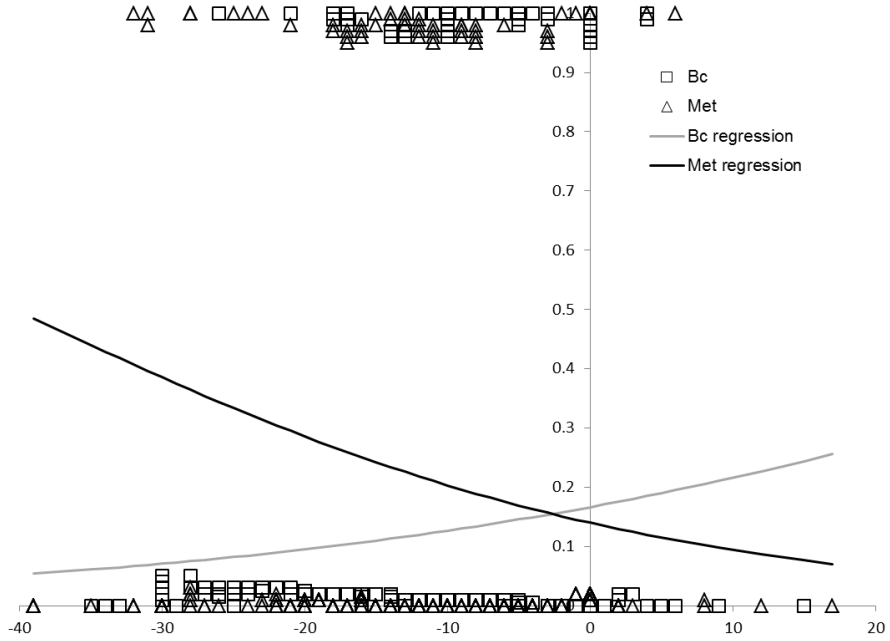

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623 Figure 6. Proportion of *Hylobius abietis* infected with EPF, relative to depth (cm) above (+)/below (-) soil level
624 at time of destructive sampling in 2014 field studies. BC: *Beauveria caledonica*, MET: *Metarhizium brunneum.*
625 Points show actual data and lines show the fitted logistic regression models. Points are slightly displaced from x
626 axis for clarity.

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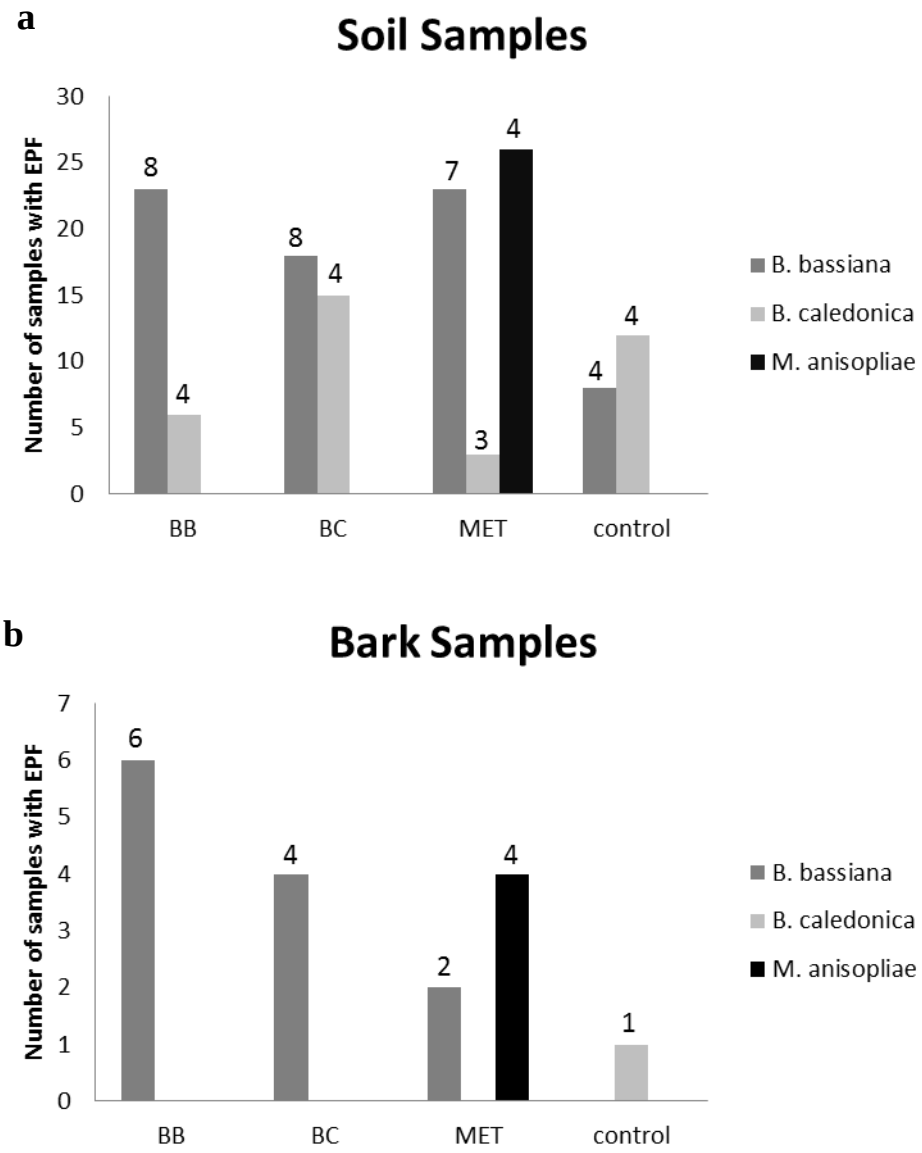

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633 Figure 7. Recovery of three entomopathogenic fungi from (a) soil samples (N=80) and (b) bark samples (N=10)
634 two years after application of the same three fungi to stumps (10 per treatment). Numbers above the bars are
635 numbers of stumps from which the fungus was recovered.

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