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ORIGINAL ARTICLE

Soil application of *Beauveria bassiana* GHA against apple sawfly, *Hoplocampa testudinea* (Hymenoptera: Tenthredinidae): Field mortality and fungal persistence

Weronika Świergiel[1], Nicolai V. Meyling[2], Mario Porcel[1] and Birgitta Rämert[1]

[1]*Department of Plant Protection Biology, Unit of Integrated Plant Protection, Swedish University of Agricultural Sciences (SLU), Alnarp, Sweden and* [2]*Department of Plant and Environmental Sciences, University of Copenhagen, Copenhagen, Denmark*

Abstract Low impact alternatives to synthetic insecticides for the control of apple sawfly (*Hoplocampa testudinea* Klug) are scarce encumbering pest management in organic apple orchards. We investigated the soil persistence and field efficacy of the entomopathogenic fungus *Beauveria bassiana* (Balsamo) Vuillemin (BotaniGard) against apple sawfly under common organic orchard practices. We also assessed the efficacy of *B. bassiana* GHA and *Metarhizium brunneum* Petch (indigenous strain) against sawfly in the laboratory. Larvae treated with either fungus in the laboratory died faster than control larvae and displayed 49.4%–68.4% mycosis. In the field, *B. bassiana* density remained high in the week after application, during larval descent to the soil. Fungal density decreased to 25% at 49 d after application and to 0.4% after 55 weeks. Molecular markers revealed that the majority of fungal isolates recovered comprised the applied *B. bassiana* strain GHA. Larvae pupating in soil cages in the orchard for 49 d displayed 17% mycosis. The high efficacy under laboratory conditions was not seen in the field. *B. bassiana* application resulted in densities above the upper natural background level during the growing season, but reversion to background levels occurred within a year. It remains to be investigated whether this has a detrimental effect on nontarget organisms. Additional work is needed to bridge the knowledge gap between laboratory and field efficacy in orchards.

Key words biological control; crop protection; entomopathogenic fungi; integrated pest management; *Metarhizium*; organic orchard

Introduction

The apple sawfly *Hoplocampa testudinea* Klug (Hymenoptera: Tenthredinidae) is an important univoltine pest of apple in Europe and North America (Pyenson, 1943; Vincent & Mailloux, 1988; Cross *et al.*, 1999). The sawfly hibernates in the soil as prepupae and emerges in the flowering period of apple to oviposit in the flower calyx (Miles, 1932; Graf *et al.*, 2001; Ciglar & Barić, 2002). The damage caused by the hatched sawfly larvae comprises superficial tunneling under the fruitlet skin and direct radial entry into the fruitlet and feeding on the kernel (Miles, 1932; Dicker & Briggs, 1953). In the Netherlands, sawfly populations double every year if no control measures are undertaken (Zijp & Blommers, 2002). Apple sawflies are known to be controlled by natural enemies, that is, parasitiods, nematodes and entomopathogenic fungi (Babendreier, 1996; Jaworska, 1992). Apple sawfly control in Swedish conventional orchards relies on the use of nonselective synthetic insecticides at the time of egg hatch (Manduric, 2014; Sjöberg *et al.*, 2014), while control in European organic orchards commonly has

Correspondence: Weronika Świergiel, Department of Plant Protection Biology, Swedish University of Agricultural Sciences (SLU), P.O. Box 102, SE-230 53, Alnarp, Sweden. Tel: +46 18 67 10 00; email: weronika.swiergiel@slu.se

relied on the use of a commercial or homemade extract from the shrub *Quassia amara* L. (Simaroubaceae) (Ascard & Juhlin, 2011). Another control opportunity, with soil application of entomopathogenic fungi (EPF) or nematodes, appears at the time of larval descent to the soil for hibernation. Jaworska (1979, 1981) demonstrated high apple sawfly mortality in laboratory and semifield trials with the fungal species *Metarhizium anisopliae-sensulato (s.l.)*, *Beauveria bassiana s.l.*, *Isaria farinosa* (Holmsk.), *Isaria fumosorosea* (Wize.), *Aspergillus flavus* Linkand *Lecanicillium lecanii* (Zimm.) (all Ascomycota: Hypocreales).

Biological control products based on EPF are not authorized within the European Union if they "persist in the environment in concentrations considerably higher than the natural background levels, taking into account repeated applications over the years, unless a robust assessment indicates that the risks from accumulated plateau concentrations are acceptable" (European Commission, 2005). The lack of studies on the persistence and movement of biological control agents applied in the field has been identified as a bottleneck to their development and regulation in Europe (Mudgal *et al*., 2013).

The limited number of scientific field studies of sawfly control produced to date mostly focus on pesticide application and timing at egg hatch (e.g., Chaboussou, 1961; Graf *et al*., 2002; Kienzle *et al*., 2006; Psota *et al*., 2010). To the best of our knowledge, no previous study has investigated the potential for apple sawfly control or EPF persistence using a formulated product at the highest recommended field dose and common organic orchard practices. It is important to identify the short-term persistence of the fungal biocontrol agent throughout the first week after soil application during the critical peak larval descent to the soil (Sjöberg *et al*., 2014). Furthermore, an assessment of indigenous EPF levels, the persistence of the applied EPF by the end of the field season and the long-term persistence a year after application would be valuable information for decision makers in the regulation of biocontrol products.

The aim of this study was to address these knowledge gaps by assessing: (i) the effect of a commercial strain of *Beauveria bassiana* (Balsamo) Vuillemin and an isolate of the most common indigenous EPF species in orchard soil against apple sawfly larvae under laboratory conditions; (ii) relative apple sawfly mortality and mycosis after soil application of a commercial *B. bassiana* product; and iii) short- and long-term persistence of the commercial *B. bassiana* product.

Materials and methods

Apple sawfly collection

Apples infested with sawfly larvae were picked from Danish and Swedish organic apple orchards in 2012 and 2013 and placed in boxes with a grid base and an additional box placed underneath to collect the descending larvae on the following day. During harvest in the field, the infested apples were kept in the shade and sprayed with water frequently to maintain high humidity and low temperature. Only large and vital 5th instar larvae were chosen for the trials. The selected larvae were kept in a climate chamber at 99% RH, 12 : 12 L : D and 12.5 °C, and were offered apple pieces until the experiments were performed.

Effect of entomopathogenic fungi on apple sawfly in the laboratory

A laboratory experiment (LE, Table 1) was set up to assess the effect of an isolate of the *B. bassiana* strain GHA biocontrol product (BotaniGard wettable powder [WP], Lindesro, Sweden) and an indigenous isolate of *Metarhizium brunneum* Petch against apple sawfly larvae. A commercial strain of *B. bassiana* was chosen because this species has been shown to be virulent to apple sawfly and is available as a WP, which facilitates commercial-scale application in a tree crop.

The experiments were performed on 21 and 24 May 2012. Conidia suspensions were prepared from *M. brunneum* isolate KVL 14–90 and from *B. bassiana* GHA, which was reisolated from a field experiment in 2011 and confirmed by molecular identification as described below (FE1, Table 1), isolate KVL 14–87. The isolates are stored at -80 °C in the culture collection at the University of Copenhagen, Department of Plant and Environmental Sciences. The isolates were cultured on Sabouraud Dextrose Agar (SDA; Merck KGaA, Germany) for 25 d at 20 °C. Conidia were harvested by flooding the culture plate with 0.05% sterile Triton-X 100 (VWR, Sweden) and releasing with a spatula. Suspensions were transferred to 50-mL centrifuge tubes (114 mm × 28 mm, Sarstedt, Sweden) and washed twice with sterile 0.05% Triton-X 100 solution after centrifugation for 3 min at 3000 r/m (Eppendorf Centrifuge 5702). Stock solution concentrations were established on 20 May using a hemocytometer (Fuchs-Rosenthal 0.0625 mm^2, depth 0.200 mm, VWR, Sweden) under light microscopy (LeitzWetzlarDialux 20) at 400× magnification. These stock solutions were stored at 5 °C and used on both experimental days. Germination

Table 1 List of experiments performed, including a short description, site and date.

Experiment	Description	Date	Site
Laboratory experiment (LE)	Effect of *Beauveria bassiana* GHA product and indigenous *Metarhizium brunneum* against the apple sawfly.	June 2013	Laboratory
Field experiment 1 (FE1)	Assessment of entomopathogenic fungi in soil. Indigenous fungi occurrence and surface soil applied *B. bassiana* persistence at 0, 43 and 55 weeks after application.	May 2011 to July 2012	Kivik Musteri commercial orchard
Field experiment 2a (FE2a)	Mortality and mycosis of the apple sawfly after field application of *B. bassiana* GHA with soil incorporation and mechanical weeding.	June to August 2013	Dammstorp commercial orchard
Field experiment 2b (FE2b)	Assessment of entomopathogenic fungi in soil. Indigenous fungi occurrence and soil incorporated *B. bassiana* persistence 1, 8 and 49 d after application.	April to August 2013	Dammstorp commercial orchard

tests were performed by plating 0.1 mL of 10^{-3} solutions on 2 replicate SDA plates. After 24 h incubation in the dark at 20 ± 1 °C, conidia with germination tubes longer than the width of the conidia were considered germinated (Inglis, 2012). Germination tests showed on average (± SD) 99% ± 0.8% and 98% ± 0.4% germination for *B. bassiana* and 99% ± 0.8% and 99% ± 1.5% germination for *M. brunneum* on 21 and 24 May, respectively.

On each experimental day, suspensions of 1×10^7 and 5×10^7 conidia/mL were prepared. A positive control with 0.05% Triton-X 100 and a negative control with sterile water, were included. Ten larvae per treatment were individually inoculated in 4 replicates during each experimental day. Each larva was gently transferred with a brush to an individual 30-mL medicine cup (Hammarplast, Sweden) and topically inoculated with 2 μL suspension of each treatment, resulting in 2×10^4 or 1×10^5 conidia/larvae. The solutions were vortexed (Vortex-genie 2, Scientific Industries, USA) at maximum speed for 5 s before each inoculation and applied with a pipette to the dorsal side of the first thorax segment. Medicine cups with larvae were incubated in plastic boxes lined with moist paper at 20 °C. After 24 h, the larvae were transferred to new medicine cups with 2 mL sterile sand (0.8–1.2 mm quartz, Rådasand, Sweden) moistened with 400 μL sterile tap water. Incubation continued at 20 °C and the larvae were assessed for mortality daily for 7 d, then every second day up to day 19. Sterile tap water was added to the sand when needed. Dead larvae were surface-sterilized by dipping for 5 s in 10% sodium hypochlorite (Sigma-Aldrich, Sweden), rinsed twice for 5 s in deionized water and subsequently individually incubated at 20 °C with moist filter paper in medicine cups. The emerging fungi were morphologically identified (Humber, 2012).

Field sites and experimental design

Two separate field experiments (Table 1) were performed in 2 commercial organic apple orchards. The 1st experiment (FE1) assessed the long-term persistence of the applied *B. bassiana* product, while the 2nd experiment (FE2) evaluated apple sawfly mortality (FE2a) and fungal persistence during the field season (FE2b). Both orchards were managed with annual organic fertiliser applications, mechanical weeding, applications of the organic fungicide sulfur against apple scab (*Venturia inaequalis* [Cooke] Wint.) and insecticides approved for organic production in Sweden. FE1 was performed at Kivik Musteri in Svineberga, Sweden (55°40′1.67"N, 14°15′28.79"E) in 2011–2012 on a silt loam composed of 9.2% clay, 30.6% silt, 60.2% sand, and 3% soil organic matter. FE2 was performed at Dammstorp in Malmö, Sweden (55°36′1.95"N, 13°6′7.58"E) in 2013 on a silt loam composed of 14% clay, 20% silt, 66% sand, and 4.1% soil organic matter. The Dammstorp field site was chosen for the evaluation of sawfly mortality based on its high natural population of apple sawfly, indicating favorable conditions for the target pest.

In both FE1 and FE2, the experimental design consisted of 2 adjacent rows of apple trees divided into 7 blocks. Two treatments, application of fungi and control, were randomly distributed within each block. Treatments and blocks were separated by buffer zones according to the layout of each orchard (2.5 m in FE1, 4 m in FE2). Each treatment plot consisted of 8 subplots comprising the distance between 2 trees × row width, (1.25 m × 1.0 m in FE1 and 0.8 m × 1.0 m in FE2). The apple cultivar was Holsteiner Cox in FE1 and Rubinola in FE2. At each site, a temperature logger (Tinytag talk 2, Intab, Sweden) was placed in the soil at approximately 5 cm depth to measure

the temperature once per hour from mid-April to the end of June.

BotaniGard application in the field

In FE1 and FE2 (2011 and 2013; Table 1), the commercially available EPF *B. bassiana* strain GHA (BotaniGard WP, year 2011 Lot No.: 22WP101201, expiration date 12/31/2011 and year 2013 Lot No.: 22WP130201 expiration date 02/28/2014) was applied at the start of the peak larval descent to the soil, on 18 June in 2011 and 24 June in 2013. The application rate was 1.22 g (5.37×10^{10} CFU) per m^2 soil. This amount corresponds to the highest recommended dose for soil application of 12.2 kg BotaniGard WP/ha, mixed in 400 L/ha water in 2011 and 600 L/ha in 2013. Water amounts were adapted to the application equipment used. BotaniGard WP was added to the water and shaken by hand in 5-L or 10-L containers before filling up the tank. The solution was continuously shaken during application. In FE1, an electric backpack sprayer (El Solo, Accu-Power 416, SOLO Kleinmotoren GMBH), 2.6 bar with a 4-nozzle boom (standard flat fan nozzles) was used. In FE2, application was performed using a front-mounted experimental tractor sprayer equipped with boom with 2 nozzles (Lechler flat fan 110–04) 50 cm apart. A driving speed of 3 km/h and 2.6 bar pressure with a flow of 1.5 L/min was set to achieve the planned application rate.

Application in both field trials was performed in low sunlight conditions and was followed by light rainfall within hours. In FE1, the fungus was applied to the soil surface, while in FE2 it was incorporated into the soil to 12 cm depth by tractor-driven mechanical weeding. In both field trials mechanical weeding was performed according to common practice. In FE2b, where the impact of weeding on fungal persistence during the field season was of interest, the weeding occasions were 24 June, 9 July, and 2 August 2013.

Apple sawfly mortality after EPF field application

To assess apple sawfly mortality in the field (FE2a in 2013; Table 1), plastic boxes (18 cm wide × 18 cm long × 19 cm deep) were buried in the ground and filled with orchard soil, maintaining the topsoil on top. Both the lid and 2 of the 4 walls were made of fine insect netting (Filbio 0.8 mm × 0.8 mm, Andermatt Biocontrol, Switzerland) and the base was perforated with 1-mm holes to permit humidity exchange with the surrounding soil while preventing sawflies escaping the cage. The average (±SD) percentage germination of the applied product was measured at the beginning (97.8% ± 2.5%) and end (98.5% ± 1.8%) of the field application by plating a 10^{-2} suspension of the spray liquid on SDA as previously described. On the day after *B. bassiana* application (25 June 2013), the fungus was incorporated to 12 cm in the soil by hand, in order to increase the time of larval exposure to the EPF while migrating through the soil. Latex gloves were used and hands were washed in 70% ethanol and rinsed in water between each mixing. Ten larvae picked from Danish and Swedish apple orchards 1–3 d before *B. bassiana* application were placed on the soil surface of each box. Precipitation data were obtained from Sveriges Meteorologiska och Hydrologiska Institut (SMHI, Norrköping, Sweden).

Since only a small proportion of the larvae are infected after spinning a cocoon (Jaworska, 1992), the boxes were removed from the soil after 49 d, at which time all cocoons were formed (pers. obs.). The soil in each box was broken up in tap water in order to recover the cocoons by flotation. The soil was then rinsed through a sieve (mesh size 1 mm × 1 mm) to find any remaining cocoons. The cocoons were placed in individual 30-mL medicine cups with 2 mL sterile sand (0.8–1.2 mm quartz, Rådasand, Sweden) moistened with 400 μL sterile tap water before dissection under a stereomicroscope. Morphological identification was performed for the EPF emerging from the dead larvae (Humber, 2012). Fungi were isolated on SDA.

EPF assessment—soil sampling

The objective of FE1 and FE2b was to assess the occurrence of indigenous EPF and the short- and long-term persistence of the applied *B. bassiana* product. In FE1 (Table 1), soil samples were collected between 2 and 14 May 2011 to evaluate the occurrence of indigenous fungi. Additional soil samples were collected 1–2 d (19–20 June 2011), 43 weeks (10–12 April 2012), and 55 weeks (4–6 July 2012) after application. In FE2b (Table 1), background sampling was performed on 29 April 2013 and additional samples were collected 1 d (25 June), 8 d (1 July), and 49 d (12 August) after application.

On each sampling occasion, soil was sampled from 8 subplots in each treatment and block, resulting in 112 samples. In FE1 in 2011 and 2012, each soil sample was composed of 5 × 5 soil cores, from 0 to 15 cm depth and 2 cm diameter, taken randomly in each subplot within a 10 cm × 10 cm grid covering 80 cm × 120 cm. In 2013, 5 soil cores of 6 cm diameter and from 0 to 5 cm depth were taken randomly within the grid in each subplot and mixed. The 0–15 cm sampling depth in FE1 was chosen to represent the fungal density to which a sawfly would

be exposed while burrowing into the soil for hibernation. Since in FE2 the soil was tilled down to 12 cm depth, and consequently homogenized, a 0–5 cm sample was taken as representative of the entire profile.

In both field trials, soil sampling tools were rinsed in water and left in 70% ethanol for 1 min between each subplot sampling. The soil samples were stored in insulated plastic cool-bags with ice packs during sampling and then transferred to cold storage (4 °C) at the end of each day. Samples were air-dried if too wet for homogenization, homogenized by hand in the bag and weighed (Table 2). Dry weight was established by weighing approximately 5 g of soil before and after drying at 105 °C overnight.

EPF assessment—isolation of fungi from soil by soil baiting

EPF were isolated from FE1 soil samples using insect baits for a qualitative assessment of EPF occurrence. A 155-mL plastic cup (Grathwol A/S) was filled up to 1 cm below the rim with homogenized soil from each sample. The soil was remoistened with deionized water when necessary to achieve equal levels of humidity in all samples. Ten *Tenebrio molitor* L. (Coleoptera: Tenebrionidae) larvae (supplied by Petra-aqua in 2011 and Agama–Martin Kober in 2012) were placed in each cup and all cups were sealed with a ventilated lid and kept upside-down in the dark at 20 °C. During the first week of the assay, the cups were inverted daily in order to keep the larvae moving in the soil. The larvae were checked for mortality once a week. Dead larvae were rinsed with water and placed in individual 30-mL medicine cups with moistened filter paper. Larvae were checked for mycosis weekly for 4 weeks and filter papers were remoistened when necessary. Emerging EPF were isolated on SDA and morphologically identified according to Humber (2012).

EPF assessment—isolation of fungi from soil by selective media

EPF in soil samples from FE1 were also isolated and quantified by plating soil suspensions on selective agar media (SM) modified from (Strasser *et al*., 1996). SM was prepared by adding 500 μL dodine solution (2.225 g dodine [VWR], 20-mL distilled water, 27.775 mL 96% ethanol) to 32.5 g SDA/500 mL distilled water autoclaved at 120 °C and 20 bar for 20 min. After cooling to 60 °C, 500 μL chloramphenicol solution (0.1 g/mL 96% ethanol; Duchefa Biochemie, Haarlem, The Netherlands) and 500 μL streptomycin sulfate solution (0.5 g/mL sterilized distilled water; Duchefa Biochemie) were added.

Soil suspensions were plated on SM. Dilutions were determined in pilot studies to achieve a reasonable number of colonies per plate (Table 2). The suspensions were prepared by adding soil to 0.05% Triton-X 100 (Table 2), leaving it for 3 min and then stirring (mediMAG, Labassco, Sweden) at 1100 r/m for 2 min using sterile magnetic stir bars (25 mm × 6 mm, VWR, Sweden). Following 10 s of sedimentation, the solution was plated on triplicate SM plates, resulting in 336 plates per occasion. The plates were sealed with parafilm and incubated in the dark at 20 °C. CFUs were counted on 3–4 occasions (Table 2) and morphologically identified according to Humber (2012). Newly emerging CFUs were only included until day 13 after plating to avoid inclusion of secondary colonies. A selection of CFUs morphologically identified as *B. bassiana* or *Metarhizium* spp. was used for molecular identification in 2011 and 2012.

EPF assessment—molecular detection

In FE1, *B. bassiana* CFUs on SM plates were isolated on SDA by selecting 1–3 CFUs from 1–3 replicate plates of each subsample. In addition, 1–2 isolates of *B. bassiana* from infected bait larvae were obtained from each soil subsample. Clean cultures were incubated on SDA until sporulation. Conidia were inoculated into sterile flasks with sterile liquid growth medium consisting of 2% peptone (Lab M Ltd., UK), 3% sucrose (Merck, Germany), and 0.2% yeast extract (Merck, Germany). The flasks were incubated for 3 d at room temperature on a shaker (170 r/m) and the resulting fungal cells were filtered and lyophilized. DNA was extracted from fungus material using a DNeasy Plant Mini Kit (Qiagen GmbH, Copenhagen, Denmark) according to the manufacturer's instructions. Conidia from each isolate were harvested in sterile 10% skimmed milk (Arla, Denmark), transferred to 1.5-mL cryovials containing 0.8-mL sterile glycerol (Sigma, USA) and kept at -80 °C.

Strain-specific SCAR marker $SCA14_{445}$ for *B. bassiana* GHA was applied according to Castrillo *et al*. (2003) by using primer pairs OPA14 F_{445} (5′-TCTGTGCTGGCCCTTATCG-3′) and R_{445} (5′-TCTGTGCTGGGTACTGACGTG-3′). PCR was performed on a Biometra T1 Thermocycler in 50-μL volumes consisting of 1× Phusion HF buffer (Finnzymes, Espoo, Finland), 200 μmol/L of dNTP, 1 μmol/L of each primer, 0.5 U of proofreading Phusion polymerase (Finnzymes) and 1 μL of genomic DNA. Cycling conditions consisted of 2 min initial denaturation at 98 °C, followed by 30 cycles of 15 s at 98 °C, 45 s at 63 °C, and 45 s at 72 °C, ending with a final extension of 7 min at 72 °C. Next, 5 μL portions of the

Table 2 Field experiments with soil application of *Beauveria bassiana*. Soil sampling occasions, dilutions, plated volumes and Colony Forming Units (CFU) count occasions for the 2011–2012 and 2013 field experiments (FE).

Sampling date	Dilutions	Plated volume	Day of CFU count
Year 2011 (FE1)			
1 Day after app.	5 g soil/98 mL 0.05% Triton-X 100	0.2 mL	4, 7, and 10
Year 2012 (FE1)			
Week 43 and 55 after app.	20 g soil/80 mL 0.05% Triton-X 100	0.1 mL	5, 9, and 13
Year 2013 (FE2b)			
Background sampling, April	5 g soil/95 mL 0.05% Triton-X 100	0.1 mL	4, 8, 13, and 22
Day 1 and 8 after app.	7 g soil/93 mL 0.05% Triton-X 100, 1 mL soil solution/8 mL 0.05% Triton-X 100	0.1 mL	4, 8, 13, and 22
Day 49 after app.	Dilution 1[†]: 7 g soil diluted in 133 mL 0.05% Triton-X 100	0.1 mL	4, 8, 13, and 22
	Dilution 2[‡]: 1 mL of dilution 1 in 5.43 mL 0.05% Triton-X 100	0.1 mL	4, 8, 13, and 22

[†]Dilution 1 corresponds to the 2013 background dilution.

[‡]Dilution 2 corresponds to the 2013 day 1 and 8 after fungi application dilution.

resulting amplicons were mixed with 2 μL EZ-Vision® One (Amresco, OH, USA) and visualized on 2% agarose gel, with diagnostic 445 bp bands interpreted as the isolate being *B. bassiana* GHA. Lack of bands on the gel for isolates was interpreted as the isolate being indigenous fungi. To confirm that lack of bands was not due to defective DNA quality, and thus inability to produce PCR product, a PCR for each of the isolates was performed for the internal transcribed spacer (ITS), using methods described by Meyling *et al*. (2012). All isolates testing negative for the 445 bp band showed amplicons for the ITS region. The SCAR marker was initially tested against a range of indigenous *Beauveria* isolates and an isolate obtained from the commercial product BotaniGard and the latter showed the diagnostic band, while the former isolates all tested negative. The GHA-positive isolate obtained from BotaniGard was always included as a positive control in all PCR assays.

In both FE1 and FE2b, *Metarhizium* spp. occurred as CFUs on SM and as infections in bait larvae. Given the existence of cryptic species in this genus (Bischoff *et al*., 2009), molecular identification of selected isolates was performed to establish which species occurred. Initially, isolates were selected to represent different subsamples and isolations from selective media and bait larvae. Isolates on SDA were cultured in liquid medium, filtered, lyophilized, and DNA-extracted as described above. PCR was performed on the 5′- end of the gene Elongation Factor 1-alpha (EF1-α) with the primers EF2F and EfjR as described by Meyling *et al*. (2012), using the same methods. Amplicons were sequenced and analyzed as described by Meyling *et al*. (2012) and the resulting DNA sequences were aligned with reference sequences deposited in GenBank, NCBI.

Statistical analyses

Effects of each fungal isolate and concentration relative to the control on larval survival were analyzed by calculation of hazard ratios (HR; relative average daily risk of death) by fitting the survival data using a mixed Cox Proportional Hazards (PH) regression model (Cox, 1972) including experimental repetition as a random factor. Cox PH is expressed as the HR, where a value of one indicates that the 2 treatments cause the same risk of death at any given time. Multiple pairwise comparisons between treatments were performed using Tukey contrasts to correct for significance levels by generating pairwise HR values. Frequencies of mycosis were compared by standard χ^2 tests.

Generalized linear mixed models (GLMMs) were used to analyze the effect of fungus application on the proportion of recovered and dead sawfly cocoons and the occurrence of EPF in the soil. The models included the variable treatment (fungal application vs. control) as explanatory variable and experimental block as a random effect and were generated using a binomial distribution. By using the Agresti–Coull method, 95% confidence intervals (CI) for binomial proportions were calculated.

The density of CFU counts of *B. bassiana* and indigenous fungi were analyzed by GLMMs with a Negative Binomial distribution due to the overdispersion detected when initially modeled using a Poisson

Table 3 Hazard ratios (±SE) and observed relative mycosis of sawfly larvae for each fungal treatment in infection bioassays. The "low" and "high" concentrations used corresponded to 1×10^7 and 5×10^7 conidia/mL, respectively. Test statistics (z) and significance differences relative to control treatments are from multiple pairwise comparisons by Tukey contrasts.

Treatment (n)	Hazard ratio (SE)	z Value	P value, Tukey contrasts	Relative mycosis (%)
KVL14–87 *B. bassiana* GHA low (79)	1.3207 (0.1404)	1.981	0.2737	49.4
KVL14–87 *B. bassiana* GHA high (69)	1.6080 (0.1408)	3.372	0.0067	62.3
KVL14–90 *M. brunneum* low (79)	1.6886 (0.1424)	3.680	0.0021	68.4
KVL14–90 *M. brunneum* high (78)	2.2273 (0.1441)	5.559	<0.0001	65.4

distribution (Lindén & Mäntyniemi, 2011). In all models, experimental block was included as random effect and the amount of soil per sample as an offset variable in order to adjust for the different soil volumes in each CFU count (Zuur*et al*., 2009). *Beauveria bassiana* CFU analyses included treatment as explanatory variable and the different sampling dates were analyzed separately. Indigenous fungi CFU included time as the explanatory variable.

Wald tests were carried out for all GLMMs to test the significance of the explanatory variable. All statistical analyses were performed using R software 3.1.0 (R Core Team, 2014) with packages glmmADMB, multcomp, binom, and coxme.

Results

Effect of EPF on apple sawfly

There was a significant effect of treatment on larval survival ($\chi^2 = 39.1$, df = 5, $P < 0.001$). Although most larvae in the control died by the end of the experiment (14 d), fungus-treated larvae died significantly faster than untreated larvae (HR > 1) in 3 of the 4 fungal treatments (Table 3). No mycosis on cadavers was observed in the positive and negative controls, while 49.4%–68.4% of the fungus-treated dead larvae showed mycosis. These proportions did not differ significantly between the fungal treatments ($\chi^2 = 7.0$, df = 3, $P < 0.073$).

Apple sawfly mortality in the field

There were no significant differences between the percentage of recovered cocoons in treated (76.1%, CI = 72.4%–79.5%) and control plots (74.3%, CI = 70.5%–77.7%) in FE2a ($\chi^2 = 0.7$, df = 1, $P = 0.469$). Amongst the recovered cocoons, there were no significant differences between the percentage of dead larvae in the treatment (41.6%, CI = 37.0%–46.4%) and control (35.8%, CI = 31.4%–40.5%) ($\chi^2 = 1.1$, df = 1, $P = 0.085$). *Beauveria* mycosis was found in 17.0% (CI = 13.7%–20.9%) of the recovered larvae and in 40.8% (CI = 33.8%–48.2%) of the dead larvae in treated plots, while no larvae in control plots showed mycosis. The precipitation on the day of fungus application was 11 mm. Ten days later the accumulated precipitation was 26 mm, but thereafter there was no rainfall for 24 d. The accumulated precipitation was 53.0% (53.2 mm) in July and 36.4% (98.1 mm) in August, of the average precipitation for those months in the period 2008–2013.

Beauveria bassiana persistence and occurrence of other EPF

In FE1, soil baiting showed overall background levels of 3.6% occurrence for *B. bassiana* and 42.0% for *Metarhizium* spp. In total, 44.6% of the subplots showed EPF occurrence. At sampling 1–2 d after application, both the average (± SE) density of *B. bassiana* presented in Figure 1 ($1.49 \times 10^4 \pm 1.00 \times 10^3$ CFU/g dry soil) ($\chi^2 = 345.5$, df = 1, $P < 0.001$) and the 87.5% occurrence presented in Figure 2 ($\chi^2 = 30.00$, df = 1, $P < 0.001$) were significantly higher in treated plots compared with the control (3 ± 1 CFU/g dry soil and no occurrence). The occurrence of *Metarhizium* was lower in treated plots than in controls ($\chi^2 = 13.6$, df = 1, $P < 0.001$; Fig. 2). In samples from treated plots collected in weeks 43 and 55 after application, the observed average density of *B. bassiana* decreased to 0.2% (29.5 ± 6.62 CFU/g dry

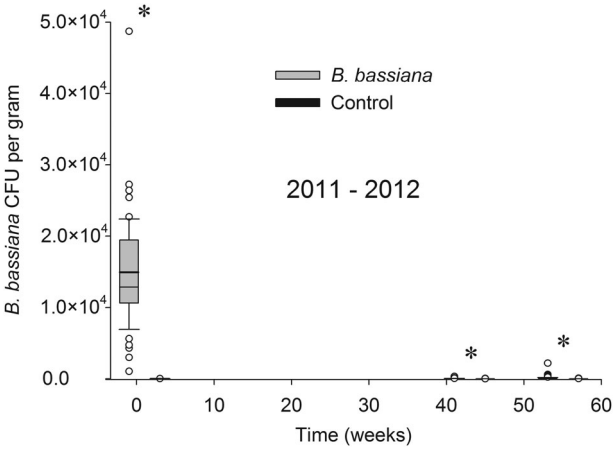

Fig. 1 Boxplot of *Beauveria bassiana* persistence in field experiment 1 (FE1, 2011–2012) at application (0) and 43 and 55 weeks after application of *B. bassiana* strain GHA. Bold lines represent average values and standard lines median values of number of colony forming units (CFUs) per gram of dry soil. *Represents statistically significant difference (GLMM; Wald test, $P < 0.01$).

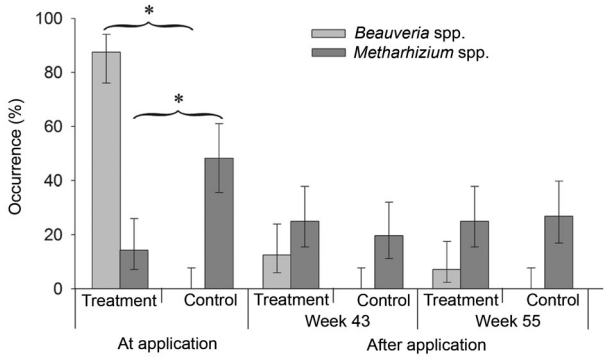

Fig. 2 Entomopathogenic effect of *Beauveria* spp. and *Metarhizium* spp. detected by soil baiting with *Tenebrio molitor* larvae, presented as percentage occurrence at time of application and 43 and 55 weeks after application in field experiment 1 (FE1, 2011–2012). Error bars show Agresti–Coull 95% confidence intervals. *Represents statistically significant difference (GLMM; Wald test, $P < 0.01$).

soil) and 0.4% (53.90 ± 23.82 CFU/g dry soil), respectively, of the density at application. The median density decreased to 0.1% and 0% during the same period. Although low, these densities were significantly higher than in the control ($\chi^2 = 31.4$, df = 1, $P < 0.001$ and $\chi^2 = 28.1$, df = 1, $P < 0.001$, respectively; Fig. 1). However, this did not result in an increased entomopathogenic effect against bait larvae, since there were no significant differences in the occurrence of infected larvae between treated and control plots in weeks 43 and 55 ($\chi^2 = 13.7$, df = 1, $P < 0.056$ and $\chi^2 = 1.6$, df = 1, $P < 0.203$, respectively; Fig. 2).

In total, morphologically identified indigenous EPF were found in 92.0% of all subsamples in FE2b. The background soil samples collected in April 2013 and plated on SM showed occurrence of 34.8% *Beauveria* spp., 84.8% *Metarhizium* spp. and 11.6% *Isaria* spp. Quantitative background densities of indigenous EPF (CFU/g soil) are presented in Table 4.

At the time of *B. bassiana* application, its average (±SE) levels increased significantly to $1.45 \times 10^5 \pm 7.92 \times 10^3$ CFU/g dry soil compared with $9.55 \times 10^2 \pm 4.81 \times 10^2$ CFU/g dry soil in the control ($\chi^2 = 28.4$, df = 1, $P < 0.001$; Fig. 3) and 100.0% frequency of occurrence in treated soil samples compared with 46.4% in the control (Fig. 4). However, the occurrence of *Metarhizium* spp. decreased to 35.7% compared with 69.6% in the control ($\chi^2 = 3.5$, df = 1, $P < 0.001$; Fig. 4).

On day 8 and 49 after application, soil baiting showed that *B. bassiana* was still present in all subsamples. *Metarhizium* spp. occurrence levels were comparable to those in control plots ($\chi^2 = 0.2$, df = 1, $P = 0.847$ and $\chi^2 = 0.8$, df = 1, $P = 0.408$, for day 8 and 49, respectively). However, the quantity of *B. bassiana* decreased gradually to $1.01 \times 10^5 \pm 4.29 \times 10^3$ CFU/g dry soil by day 8 after application (30.3% average and 28.6% median decrease) and $3.6 \times 10^4 \pm 2.5 \times 10^3$ CFU/g dry soil (74.9% average and 75.2% median decrease) while the median density decreased with 75.2% by day 49 (Fig. 3). On the latter sampling date, the density was approximately 31 fold higher than the average *B. bassiana* level in the control ($\chi^2 = 16.7$, df = 1, $P < 0.001$). The average density of all EPF in the treated plots was then $3.7 \times 10^4 \pm 2.5 \times 10^3$ CFU/g dry soil. Although low, this was still significantly higher than the average in the control plots ($2.8 \times 10^3 \pm 1.3 \times 10^3$ EPF CFU/g dry soil) ($\chi^2 = 14.0$, df = 1, $P < 0.001$).

Quantification and molecular identification of EPF

Metarhizium spp. were the most frequent morphologically identified fungi in the control plots in both field experiments (Table 4). FE1 showed negligible densities of *Beauveria* spp., while in FE2b the density increased over the season to levels comparable with *Metarhizium* spp. in control plots.

In total, 274 *Beauveria* isolates representing 152 soil subsamples obtained from CFUs on selective media or soil baiting with *T. molitor* were PCR-assayed with GHA diagnostic SCAR primers. From soil samples collected immediately after application in June 2011, all 40 isolates

Table 4 Average (±SE) density of indigenous fungi presented as colony forming units (CFUs) per gram dry soil in background soil samples and control plots at the time of *B. bassiana* soil application and 43 and 55 weeks after application in field experiment 1 (FE1, 2011–2012) and field experiment 2b (FE2b, 2013).

Fungi	Background	At application	Week 43	Week 55	χ^2	df	*P* value
FE1							
Metarhizium spp.	Na	Na	10 ± 3 a	292 ± 197 b	12.1	1	<0.001
Beauveria spp.	Na	3 ± 1	2 ± 1	2 ± 1	0.92	2	0.631
Total	Na	Na	12 ± 3 a	293 ± 197 b	11.86	1	<0.001
FE2b			Day 8	Day 49	χ^2	df	*P* value
Metarhizium							
(dilution 1[†])	763 ± 290	Na	Na	488 ± 276	3.18	1	0.075
(dilution 2[‡])	Na	804 ± 144	637 ± 166	1564 ± 1003	0.7	2	0.699
Beauveria spp.							
(dilution 1)	93 ± 21a	Na	Na	683 ± 384b	37.1	1	<0.001
(dilution 2)	Na	955 ± 481	488 ± 115	1176 ± 800	2.1	2	0.355
Isaria spp.							
(dilution 1)	25 ± 8	Na	Na	30 ± 23	0.1	1	0.771
(dilution 2)	Na	224 ± 141	85 ± 30	62 ± 54	3.6	2	0.168
Total							
(dilution 1)	881 ± 292	Na	Na	1 201 ± 463	0.7	1	0.408
(dilution 2)	Na	1 984 ± 496	1 210 ± 200	2 802 ± 1 258	4.2	2	0.121

[†]Dilution 1 corresponds to the 2013 background dilution.

[‡]Dilution 2 corresponds to the 2013 day 1 and 8 after fungi application dilution.

obtained by soil baiting, representing 35 soil subsamples, showed diagnostic 445 bp bands on agarose gels, while 119/124 isolates (96.0%) from selective media (SM), representing 61 soil subsamples, were PCR-positive. Two of the PCR-positive isolates came from control plots in June 2012.

In total, 84 isolates from soil baiting and selective media, representing 36 soil subsamples collected 43 weeks after application in April 2012, were PCR-assayed and gave 76 positives (90.5%). All 18 isolates obtained by soil baiting in April 2012, representing 7 soil subsamples, showed GHA diagnostic bands, while selective media isolates representing 29 soil samples resulted in 58 positives (87.9%), of which 4 were found in the control. Of 11 isolates from soil baiting 55 weeks after application in July 2012, representing 5 soil subsamples in treated plots, 10 (90.9%) showed positive PCR amplicons. On the same occasion, 8 of the 11 (72.7%) isolates, representing 11 soil samples obtained from selective media, showed GHA diagnostic bands.

In FE1 at the Kivik site, 15 *Metarhizium* spp. isolates were sequenced for 2011 and 29 isolates for 2012, representing 12 and 28 soil subsamples per year, respectively. In 2011 before application of *B. bassiana* GHA, 81.8% of the isolates ($n = 9$) were identified as *M. brunneum* and 18.2% were *M. robertsii* ($n = 2$), while in June after application 3 of 4 isolates were *M. brunneum* and the other *M. robertsii*. In April 2012, 71.4% of the isolates were identified as *M. brunneum* ($n = 10$), while 28.6% were *M. robertsii* ($n = 4$), and in July the corresponding proportions were 8.00% and 20.0% ($n = 12$ and 3, respectively). The overall relative proportions of the 2 species *M. brunneum* and *M. robertsii* were not different between the 2 years (17 : 3 vs. 22 : 7; $\chi^2 = 0.6$, df = 1, $P < 0.435$) and isolates of both *Metarhizium* species were obtained from selective media and *T. molitor* bait larvae. Furthermore, 6 isolates were morphologically identified as *M. flavoviride* in April 2012 and 10 in July 2012. In 2013 (Dammstorp site, FE2b), 11 isolates, of which 10 were isolated in April before *B. bassiana* GHA application, were identified as *M. majus* ($n = 10$) and a single isolate as *M. brunneum*.

Discussion

Our laboratory infection experiment showed a significant effect of *B. bassiana* GHA and indigenous *M. brunneum* on apple sawfly, but soil application according to common orchard practice and the highest recommended field dose

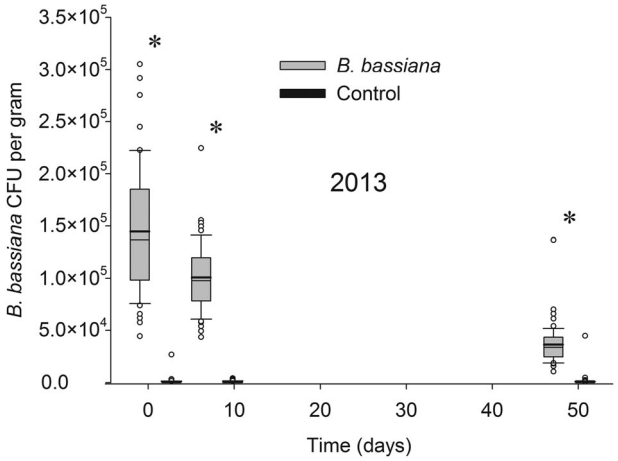

Fig. 3 Boxplot of *Beauveria bassiana* persistence in field experiment 2b (FE2b, 2013) at 1, 8, and 49 d after *B. bassiana* application. Bold lines represent average values and standard lines median values of number of colony forming units (CFUs) per gram dry soil). *Represents statistically significant difference (GLMM; Wald test, $P < 0.01$).

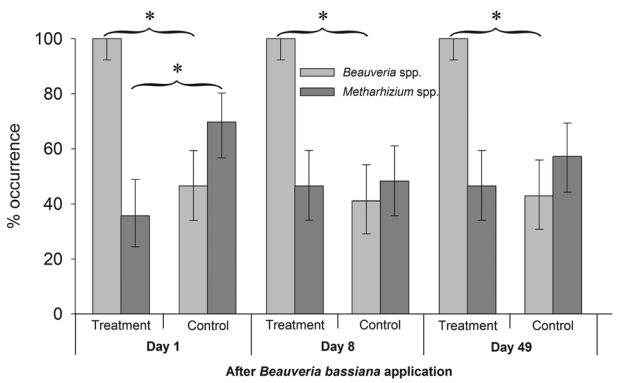

Fig. 4 Entomopathogenic effect of *Beauveria* spp. or indigenous entomopathogenic fungi *Metarhizium* spp. and *Isaria* spp. presented as occurrence (%) on selective media plates 1, 8, and 49 d after *B. bassiana* application in field experiment 2b (FE2b, 2013). Error bars show Agresti–Coull 95% confidence intervals. *Represents statistically significant difference (GLMM; Wald test, $P < 0.01$).

had only a limited effect on pest mortality, with a relatively low level of fungal infection detected. Persistence of *B. bassiana* during the week of application was high, while the medium- and long-term occurrence was low.

Apple sawfly mortality in field

Although the results showed that some *B. bassiana* infection was initiated, soil-incorporated fungus did not result in a high level of apple sawfly infection. These results contrast with findings reported by Jaworska (1981) showing 60%–80% infected apple sawfly larvae in a semi-field trial in Poland using *B. bassiana s.l.* However, that early study did not report the number of *B. bassiana s.l.* as CFU/g soil, so it is not directly possible to correlate this difference in mortality with the dose of the fungus. Since our study was performed during a single season, caution should be used when interpreting the results. However, larval mycosis as low as 17% indicates that modification of the application technique may be necessary. Although a higher dose of the fungus may lead to higher efficacy in sawfly control, the additional costs and increased non-target effects require further economic and environmental evaluation.

In the study by Jaworska (1981) the fungus was incorporated into the entire volume of the soil cages, forcing the larvae into continuous contact with the EPF. Since apple sawfly larvae are known to be able to penetrate down to 30-cm depth (Miles, 1932; Jaworska, 1981), it is reasonable to assume that they are capable of escaping exposure to fungal propagules in this way. Hence the fungus free amount of soil at the bottom of the cages in FE2a may partly explain the discrepancy between the studies.

The cool soil temperature of 16 °C recorded in FE2 is reported to be suboptimal for *B. bassiana s.l.* and *M. anisopliae s.l.* germination and growth (Jaronski, 2007). However, Jaworska (1981) showed high sawfly mortality due to mycosis at 17.3 °C soil temperature using other fungal isolates, thus low temperature does not necessarily explain the low proportion of infected sawfly larvae in the present study.

Another factor that influences EPF efficacy is humidity (Jaworska, 1981; Jaronski, 2007). A dry period commonly occurring during the time of apple sawfly descent into the soil, together with the limited soil humidity provided by drip irrigation, may have inhibited fungal germination and infection. Similarly, Gaugler *et al*. (1989) found that a high level of Colorado potato beetle (*Leptinotarsa decemlineata*, Say) mycosis only occurred after soil irrigation. However, studies on the impact of soil moisture on conidia viability and mycosis have produced contradictory results (Lingg & Donaldson, 1981; Studdert & Kaya, 1990; Hallsworth & Magan, 1995; Lanza *et al*., 2009; Cossentine *et al*., 2010). Studdert and Kaya (1990) showed a significant increase in *Spodoptera exigua* (Hübner) pupal mycosis by *B. bassiana* (strain ABG 6178) at water potentials of -37 to -200 bar compared with -0.3 to -15 bar. In contrast, Garrido-Jurado *et al*. (2011) reported maximum mycosis by 2 strains of *B. bassiana s.l.* in the Mediterranean fruit fly (*Ceratitis capitata* [Wiedemann]) at a water potential optimum of -2.8 to -4.7 bar, well over

the permanent wilting point of -15 bar. Those authors also showed varying effects depending on fungal strain. It appears that further studies are needed to find suitable strains, establish the humidity requirements for sawfly infection by EPF and determine whether these levels can realistically be achieved in commercial orchards.

Fungistatic effects caused by either antibiosis or pesticides such as sulphur, have been shown in laboratory studies, for example, (Lingg & Donaldson, 1981; Sterk, 1993; Shah *et al*., 2009; Demirci & Denizhan, 2010) and are correlated to low EPF levels in the field (Jabbour & Barbercheck, 2009). However, it appears that single applications of sulphur do not result in a decrease in insect mortality, for example, (Saito & Yabuta, 1996; Shah *et al*., 2009; Demirci *et al*., 2011). To our knowledge no study has investigated the fungistatic impact on EPF of the accumulation of several annual sulphur applications in a field situation.

Beauveria bassiana persistence and presence of other EPF

Mechanical weeding is common practice in intensively managed organic orchards and could affect the persistence of a fungal biocontrol agent, since tillage has been shown to have a detrimental effect on EPF (Bing & Lewis, 1993; Sosa-Gomez & Moscardi, 1994; Hummel *et al*., 2002). Several studies have reported a higher negative effect of cultivated soils compared with natural habitats on occurrence of *Beauveria* spp. than on *Metarhizium* spp., for example (Vänninen, 1996; Quesada-Moraga *et al*., 2007; Medo & Cagan, 2011). However, a single mechanical incorporation of the applied fungus into the soil has been shown to enhance its persistence (Gaugler *et al*., 1989). In addition, Shapiro-Ilan *et al*. (2008) found no significant difference in mortality or mycosis in pecan weevils with or without soil incorporation of *B. bassiana* GHA.

The rapid reduction in *B. bassiana* density in cultivated soils is an advantage, since it decreases the risk of damage to non-target organisms. However, as Scheepmaker and Butt (2010) have pointed out, no reference is available for the time within which the concentrations should return to the background level. Those authors suggest a reference background level of 830 CFU/g soil, defined as the 95th percentile of the geometric mean, representing the upper natural background level in other published studies.

At 43 and 55 weeks after application in FE1, the average density of *B. bassiana* CFUs (0.4% and 0.2% of the density at application, respectively) was still significantly higher than the negligible levels in the control. Fungal density reductions of the same order of magnitude over a comparable time span are reported in the literature (Müller-Kögler & Zimmermann, 1986; Inglis *et al*., 1997), although higher application rates than that used in our study resulted in higher densities a year after application. Storey *et al*. (1989) reported complete disappearance of applied *B. bassiana* already after 6 months. This is a faster decrease than in our study and may be attributable to warmer soil and different product formulation. Although a significant increase in fungal densities compared with the control was found in our study, no entomopathogenic effect could be shown using soil baiting with *T. molitor* larvae in these soil samples. Similar lower detection sensitivity was found when using bait insects compared with selective media isolation in a Slovakian study by Medo and Cagan (2011). However, Keller *et al*. (2003) reported higher sensitivity than SM using *Galleria mellonella* L. (Lepidoptera: Pyralidae) as insect baits, indicating that detection levels can depend on insect species and media used. In our study, the median density of *B. bassiana* in both treated and control plots was zero at week 55. Furthermore, the average *B. bassiana* density in treated plots was 14 fold lower than the *B. bassiana* reference level of 830 CFU/g soil defined by Scheepmaker and Butt (2010). Hence, it can be assumed that at the application dose used in our study, applying the fungus did not increase the non-target impact risk 1 year after application. However, caution is needed when adopting a reference level, since different laboratory procedures may result in varying amounts of CFU (Nilsson *et al.*, 2010; Nilsson *et al*., 2011).

FE2b showed that the average number of CFU/g soil only decreased by 30.3% by day 8 after application. A limited decrease at this time is desirable, since it represents the period when apple sawfly larvae descend to the soil for hibernation. These findings were supported in a study by Thompson *et al*. (2006) where the viability of 2 *B. bassiana* strains with UV-protectant formulations applied to the soil surface was assessed using fluorescence microscopy. Although our study did not test for the effect of soil tillage on fungal persistence, other studies in which *B. bassiana* application was followed by soil tillage have reported similar density reductions (Gaugler *et al*., 1989; Storey *et al*., 1989). This may indicate that short-term persistence is not strongly influenced by tillage for formulated products. However, a comparison using the same strain is required. Under the common practices for intensive organic production used in our study, the average amount of *B. bassiana* in CFU/g soil decreased to 25.1% and the median amount to 24.8% over 49 d. Other studies have found similar or lower persistence over a comparable period (Müller-Kögler & Zimmermann, 1986; Gaugler *et al*., 1989; Storey *et al*., 1989). The average level of *B. bassiana* retrieved from our treated plots on day

49 was approximately 31 fold higher than in the control and 13 fold higher than the total average EPF CFU/g soil in the control. This corresponds to a 41 fold higher median amount compared with the upper natural background level proposed by Scheepmaker and Butt (2010) and could imply an increased risk to non-target organisms.

The majority of the *B. bassiana* CFUs molecularly characterized showed positive amplicons for GHA (Castrillo *et al*., 2003), indicating that they originated from the applied biocontrol product. Some occurrence was also documented in the control plots after treatment, indicating some degree of horizontal transfer within the orchard. However, levels of CFUs remained low in these plots, suggesting that horizontal dispersal is negligible. The applied *B. bassiana* strain still occurred in the soil at the Kivik Musteri field site in the following year, as revealed by the PCR assay, although at a low density below the upper natural background level of *B. bassiana* in the soil.

The relatively high occurrence of *Metarhizium* spp. in cultivated soil mentioned above was confirmed in this study, with *M. brunneum* and *M. majus* being the most frequent indigenous EPF in the soil of the 2 commercial orchards. *Beauveria* spp. and *Isaria* spp. were less commonly isolated. In contrast, an inventory in Polish apple orchards showed a higher frequency of *Metarhizium* only in sandy soil, while equal amounts of *Beauveria* spp. and *Metarhizium* spp. were detected in silty soils (Marjanska-Cichon *et al*., 2005). This indicates that fungus composition varies at different sites and is influenced by factors such as soil structure and management.

There were no significant differences in the amount of CFU/g soil over time in the control plots except for an increase in *B. bassiana* from April to August in FE2b. Although *B. bassiana* drift from treated plots to the control cannot be entirely excluded, it has been shown that *B. bassiana* levels increase during the season (Meyling *et al*., 2011).

Conclusions

This study demonstrated that the commercial strain *B. bassiana* GHA and an indigenous *M. brunneum* isolate were equally virulent to apple sawfly under laboratory conditions. However, field application of *B. bassiana* under common organic orchard practices did not provide a sufficient control effect against apple sawfly when evaluated in a single season. The relatively low mycosis level of 17% indicates that a modification in application technique may be necessary. The observed *B. bassiana* density after 49 d during the field season was higher than the upper natural background level of soil EPF, suggesting an increased risk to non-target organisms. On the other hand, the persistence of *B. bassiana* in the year following application at the highest recommended field dose was lower than the upper natural background level, hence posing a negligible risk. It remains to be investigated whether the elevated fungal densities caused by either annual or occasional applications negatively affect non-target organisms. There is an urgent need to find environmentally friendly control methods for this pest, as its population size increases rapidly if left untreated. Application of EPF to the soil could help reduce the sawfly population in the following year and facilitate efficient control with *Q. amara* at egg hatch. Further studies of the field efficacy against apple sawfly in different conditions are nonetheless required before entomopathogenic fungi can be included in plant protection schemes for apple orchards.

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Disclosure

The authors have no potential conflicts of interest, including specific financial interests and relationships and affiliations (other than those affiliations listed in the title page of the manuscript) relevant to the subject of the manuscript.

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