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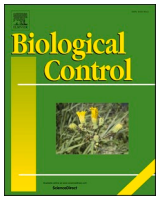

How to control nasty scarabs? Effectiveness of the generalist entomopathogenic fungus *Metarhizium brunneum* against garden chafer larvae

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HIGHLIGHTS

- Applying *Metarhizium brunneum* to the soil as granules is effective.
- *Metarhizium brunneum* shows variable control effect on garden chafer larvae.
- Naturally occurring antagonists control garden chafer populations effectively.
- The cockchafer pathogen *Beauveria brongniartii* infects garden chafer larvae.

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ABSTRACT

Several scarab beetles (Scarabaeidae) cause major damage in agriculture, horticulture, and forestry. Especially root feeding scarab larvae cause substantial economic losses on crops, forage plants and recreational areas. In Europe, the entomopathogenic fungi (EPF) *Beauveria brongniartii* and *Metarhizium brunneum* are applied against the most problematic, native scarabs, the common cockchafer (*Melolontha melolontha*) and the garden chafer (*Phyllopertha horticola*). While the control of cockchafer larvae with *B. brongniartii* is well-researched, conclusive results from field applications for the control of the garden chafer with *M. brunneum* remain elusive. We therefore assessed the performance of commercially available fungal strains of *M. brunneum* against garden chafer larvae in pot and large-scale field experiments.

The application of *M. brunneum* significantly increased the abundance of fungal propagules in the soil by approximately a factor of ten, irrespective of high levels of naturally occurring *Metarhizium* spp. Furthermore, the applied strains infected and propagated on the larvae and the mortality of garden chafer larvae was slightly increased due to the fungal treatments. We found three other EPF species frequently infecting garden chafer larvae (mean infection rates: 13–25%), including *B. brongniartii* which is considered to be a specific pathogen of the cockchafer. Thus, the applied fungal strains were only part of a consortium of natural enemies which reduces garden chafer populations strongly as a whole. Hence, we suggest that the application of EPF may be advisable on areas with reduced natural enemies such as golf courses but is probably redundant on meadows harboring a diverse consortium of antagonists.

1. Introduction

Scarab beetles (Scarabaeidae) within the order Coleoptera, represent a highly diverse group of insects and include several species causing major damage in the agricultural sector globally (Jackson and Klein, 2006). In addition, some scarab species, such as the European chafer, *Amphimallon majale* Razoumowsky (Coleoptera: Scarabaeidae) or the Japanese beetle, *Popillia japonica* Newman (Coleoptera: Scarabaeidae), are invasive, threatening the agricultural and horticultural sector in regions they have invaded (Tashiro, 1972; Potter and Held, 2002). While

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adult scarabs primarily cause above-ground feeding damage on crops and ornamental plants, larvae, commonly known as white grubs feeding belowground on plant roots, cause substantial economic losses on crops, forage plants and recreational areas (Jackson and Klein, 2006).

To control these subterranean insect pests, the conventional approach involves the use of soil insecticides (Morales-Rodriguez and Peck, 2009). However, the application of such chemicals raises concerns as they not only harm beneficial insects but also disrupt the balance of agroecosystems (Chagnon et al., 2015; Main et al., 2018; Siviter and Muth 2020). In Europe, many of these soil insecticides are therefore banned, leaving farmers without measures to control the pests (Directive 2009/128/EC). Thus, to reach a sustainable management of scarab pests, there is a need for environmentally friendly alternatives that can be readily applied and remain cost-effective.

Entomopathogenic fungi (EPF) are a promising alternative to soil insecticides and important biocontrol agents which are commercially applied worldwide against a variety of insect pests (de Faria and Wright, 2007; Hajek and Delalibera, 2010; Thakur et al., 2020). These biocontrol products are primarily based on fungal strains of the genera *Beauveria*, *Metarhizium*, *Isaria* and *Lecanicillium* and have demonstrated efficacy against a wide range of insect pests, encompassing Lepidoptera, Coleoptera, and Diptera (Lacey et al., 2015). As a result, EPF have become an integral component of integrated pest management (IPM) strategies, effectively combating insect pests (Skinner et al., 2014; Kumari et al., 2022).

A variety of EPF, particularly including species of the genera *Metarhizium* and *Beauveria*, are soil borne insect pathogens (Zimmermann, 2007a,b; St. Leger and Wang, 2020). Fungal spores attach to the insect's cuticle while the insects are moving through the soil (Ferron, 1967; Hajek and St. Leger, 1994; Bruck, 2005). Once adhered, the spores germinate and form an appressorium to penetrate the insect cuticle (Ortiz-Urquiza and Keyhani, 2013). The success of the infection depends, among other factors, on the number of adhering spores, as mortality of the insect is dose-dependent (Ansari et al., 2004; Nong et al., 2011). Inside the insect hemolymph, EPF form blastospores and exploit the insect's nutrients (Zhang and Xia, 2009). After the death of the host insect, the EPF proliferate by producing conidiospores on the surface of the insect's cadaver (Schrank and Vainstein, 2010; Moino et al., 2002). The speed of kill of the EPF varies from a few days to several months, depending on factors such as insect species, fungal virulence, and environmental conditions (Keller et al., 1997; Moino et al., 2002; Anderson et al. 2011; Kabaluk et al., 2023).

In Europe, the most problematic, native scarab pests are the cockchafer, *Melolontha melolontha* L. (Coleoptera: Scarabaeidae) and the garden chafer *Phyllopertha horticola* L. (Coleoptera: Scarabaeidae; Keller and Zimmermann, 2005). The larvae of those two species inhabit primarily meadows and turf, with heavy root feeding by second and third instars (Keller and Zimmermann, 2005; Strasser et al., 2005; Keller and Schweizer, 2007). Through their feeding activities, the larvae detach the sward from the soil, which is followed by the die-off of the grass from desiccation, secondary damage from predators searching for the grubs, and landslides of the instable upper soil layers after rain (Büchi et al., 1986; Benker and Leuprecht, 2005; Keller and Zimmermann, 2005).

A unique and efficient natural regulatory mechanism exists for the cockchafer, involving *Beauveria brongniartii* (Sacc.) PETCH (Hypocreales: Cordycipitaceae) which causes natural epizootics among cockchafer larvae (Dufour, 1894; Ferron, 1967; Fornallaz, 1992; Kessler et al., 2004). The presence of this pathogen in the soil often correlates with the presence of cockchafer larvae and *B. brongniartii* is recognized as specific pathogen of the cockchafer in central Europe (Keller et al., 2003; Kessler et al., 2004; Zimmermann, 2007a). To control cockchafer larvae, infective propagules of *B. brongniartii* are artificially augmented in the soil by cultivating the fungus on sterilized barley kernels which produces conidiospores on the barley's surface. These fungus-colonized barley kernels (FCBK) are then applied to cockchafer larvae infested grasslands using a no-till seeder (Keller, 2000).

Despite extensive studies on garden chafer populations in Great Britain, covering three decades, no epizootics of garden chafer larvae induced by EPF were observed (Milne, 1984). Likewise, Ritterhaus (1927), in her studies around Berlin in Germany, did not report any fungal pathogens as natural control agents of garden chafer larvae. To date, no specialist EPF is known to effectively control garden chafer populations. Consequently, for the control of the garden chafer, generalist fungal strains of *Metarhizium brunneum* (Hypocreales: Clavicipitaceae), i.e., strain Ma 43 (BIPESCO5 / F52), originally isolated from the codling moth, *Cydia pomonella* L. (Lepidoptera: Tortricidae; European Food Safety Authority, 2012) and a wireworm, i.e., strain ART212 (Ma714, *Agriotes* sp., Agroscope, Switzerland), are applied to infested grassland using the same method as employed for the cockchafer control (Keller and Schweizer, 2007; Strasser et al., 2005). Unlike *B. brongniartii*, *M. brunneum* is ubiquitous in soils, forming mutualistic relationships with plants by colonizing the rhizosphere (Zimmermann, 2007a; St. Leger and Wang, 2020). This allows *M. brunneum* to persist in soils independently of insect hosts and its occurrence is not associated to a specific insect species (St. Leger and Wang, 2020).

Although products based on the two *M. brunneum* strains Ma 43 and ART212 are registered in the European Union and in Switzerland for the control of garden chafer larvae, there are only few studies available on the effect of those products, such as the establishment of the applied fungi in the soil or the reduction of the larval population (Pernfuss et al., 2005; Strasser et al. 2005; Keller and Schweizer, 2007; Keller et al., 2008). While most studies found elevated levels of *Metarhizium* spp. colony forming units (CFU) in the soil after application (Strasser et al., 2005; Keller and Schweizer, 2007), control effects were moderate (Keller and Schweizer, 2007) or weak (Strasser et al., 2005). Other studies report qualitative observations instead of measurable impact of the FCBK treatments (Pernfuss et al. 2005; Keller and Schweizer, 2007). Thus, conclusive results from field applications remain elusive and the extent to which garden chafer larvae are effectively controlled by these generalist EPF under field conditions remains unclear.

To address this knowledge gap, we assessed the performance of the generalist fungal strains *M. brunneum* Ma 43 and ART212 in pot experiments under controlled greenhouse settings and in large-scale field experiments. Given the susceptibility of garden chafer larvae observed in laboratory inoculation experiments (Strasser et al., 2005), we hypothesized that a treatment with EPF leads to a reduction of garden chafer larvae in our pot and field experiments. Our aims were to (1) monitor the establishment of the applied fungal inoculum by assessing *Metarhizium* spp. colony forming units (CFU) in the soil, (2) to compare the survival of larvae in fungus treated and untreated soil, and (3) to assess the establishment of the applied fungal strains in the larval populations. We complemented this data with genetic analyses of the fungal isolates recovered from garden chafer larvae from one pot and one field experiment to check if the fungal infections can be attributed to the experimentally applied fungal strains or to other strains of *Metarhizium* spp., naturally occurring in the soil. Furthermore, we recorded the occurrence of EPF other than *Metarhizium* spp. in the larval populations to investigate possible competition among EPF.

In our pot experiments, we varied larval and inoculum densities, expecting to induce epizootics in pots with higher densities of both, larvae and inoculum, more efficiently. In our field experiments, we compared the effectiveness of Ma 43 and ART212 when applied as FCBK.

This study aims to bridge the existing knowledge gap regarding the efficacy of generalist EPF against susceptible scarab host larvae, as opposed to the highly specific interaction observed between the cockchafer and *B. brongniartii* which is already well researched. The outcome of this study has important implications for the biological control of scarab beetles and the development of sustainable control measures for subterranean insect pests in general.

2. Material and Methods

2.1. Fungal strains and inoculum production

For our experiments, we used two strains of *Metarhizium brunneum*, namely Ma 43 (BIPESCO5/F52; GenBank accession nr KR706489, European Food Safety Authority, 2012) and ART212 (Ma 714; GenBank accession nr KR706491, originating from *Agriotes* sp. isolated at Agroscope, Switzerland), both of which are commercially available, either in the European Union or Switzerland. To ensure the virulence of these fungal strains, they were passed through third instar *Popillia japonica* larvae. Conidiospores were subsequently isolated from infected cadavers on selective medium plates (SM – Sabouraud 2 % glucose agar supplemented with 0.05 g/L cycloheximide, 0.6 g/L streptomycin sulfate, 0.05 g/L tetracycline, and 50 mg/L dodine, modified from Strasser et al., 1996). The fungal cultures were grown for two weeks at 22 °C and 80 % relative humidity in darkness and were stored at 5 °C following full sporulation of the F2 generation on the plates.

To produce fungus colonized barley kernels (FCBK), the F2 generation of Ma 43 and ART212 was re-plated onto complete medium plates (CM – 10 g/L glucose, 0.36 g/L KH_2PO_4, 1.78 g/L Na_2HPO_4, 1 g/L KCl, 0.6 g/L $MgSO_47H_2O$, 0.6 g/L NH_4NO_3, 5 g/L yeast extract, and 20 g/L agar, Riba and Ravelojoana, 1984). Subsequently, 2 kg of unpeeled barley was soaked in 1.5 l of tap water in polypropylene zipper filter bags (Sac O2, Deinze, Belgium), autoclaved to prevent germination of the barley and contamination with saprophytes, and inoculated by adding one CM plate with sporulating F3 generation to each bag. The bags were heat-sealed, thoroughly mixed, and subsequently incubated in the dark for 6–8 weeks at 23 °C before being stored at 5 °C. Bags were mixed after two weeks of incubation to enhance spore production. The spore concentration per gram FCBK for each bag was determined by washing off spores from a subsample using a 0.1 % aqueous Tween 80 solution, followed by spore counting with a haemocytometer.

To evaluate the germination rate, we collected samples from all the inoculates after applications and rolled three kernels of each sample on CM plates. Following incubation for 24 h at 23 °C, we examined 100 spores from each sample for signs of germination. Germination rates exceeded 90 % in all samples.

2.2. Pot experiments

To assess the establishment of *M. brunneum* Ma 43 in the soil, its impact on larval survival and the disease rate in the larval population caused by Ma 43, we established two pot experiments in the greenhouse in October 2020 and 2021, respectively. We investigated the effects of a low and a high FCBK dose, three larval densities and two applications depths for FCBKs on the establishment abundance of *Metarhizium* spp. in the soil, the mortality of garden chafer larvae, and the establishment of Ma 43 in the larval populations.

We filled pots (28 cm diameter, 24 cm height) with field soil and sowed them with a fast-growing grass mixture comprising *Lolium perenne* 27 %, *Poa pratensis* 7 %, *Festuca rubra* 27 %, *Agrostis stolonifera* 13 %, *Cynosurus cristatus* 9 % and *Festuca arundinacea* 11 %. These grasses were allowed to grow for 5 months before starting the experiments. Garden chafer second and third instar larvae were collected in September 2020 and 2021 from natural populations in Swiss alpine areas and introduced into the pots a few days before treatments by gently placing the larvae into pierced holes in the soil. Larval densities encompassed 15, 7 and 3 larvae per pot. Due to natural mortality of field-collected larvae in 2020, half of the pots with high larval density contained between 10 and 14 instead of 15 larvae.

FCBKs were applied in a high (10^{14} spores ha^{-1}) and a low (2.5 x 10^{13} spores ha^{-1}) concentration which referred to 28 and 7 FCBK in 2020 and 21 and 5 FCBK in 2021, respectively. For the FCBK control, 28 and 21 non-inoculated and autoclaved barley kernels were used, respectively. To apply the FCBKs and the autoclaved barley kernels, we cut three slits in the soil of each pot and placed the kernels into the slits using tweezers. In 2020 we added application depth as a factor and applied FCBK and the barley control at 3 cm and 5 cm.

Each treatment combination was replicated in six pots and the pots were randomly distributed in two greenhouse chambers and irrigated with an automatic drip irrigation system controlled by sensors (Plant-Control CX, PlantCare AG, Russikon, Switzerland) to maintain an adequate moisture level throughout the 8 weeks duration of the experiments.

2.2.1. Sampling

We collected and pooled three and five soil samples per pot (1 cm diameter and approx. 10 cm deep) in 2020 and 2021, respectively, both before and 8 weeks after treatments. The samples were stored in plastic bags at 5 °C until processing. We determined the colony forming units (CFU) of *Metarhizium* spp. per gram soil following the method described by Kessler et al. (2003). For this a subsample of approximately 5 g soil was suspended in 25 mL aqueous tetra-sodiumpyrophosphate solution and plated on SM. After two weeks of incubation (22 °C, 70 % RH, darkness), we counted the colonies of *Metarhizium* spp. on each plate.

Larvae were retrieved from the pots eight weeks after treatments and categorized as follows: alive, dead without signs of fungal infection, dead with visible *Metarhizium* spp. infection, and dead with visible infection caused by other EPF. Living larvae were subsequently incubated in 90 mL insect tubes filled with moist peat and carrots as feed to detect further latent infections. In 2020, larvae were incubated at 22 °C, 70 % RH, for 10 weeks, whereas in 2021, larvae were incubated at 22 °C, 70 % RH for 2 weeks, then overwintered at 5 °C, and finally incubated at 22 °C, 70 % RH in spring for an additional 5 weeks, to mimic the natural life cycle of the larvae. Throughout the incubation periods, larvae were regularly inspected and fed, dead larvae removed and mycosed cadavers stored at 5 °C. In 2021, EPF growing on the cadavers were isolated on SM plates. The sum of mycosed larvae per pot found at the different time points, was used for statistical analysis.

2.3. Field experiments

To assess the establishment of *M. brunneum* Ma 43 and ART212 in the soil, its impact on larval survival and the disease rate in the larval population caused by the two fungal strains under field conditions, we conducted two large-scale field experiments at two different sites in 2020 and 2021, respectively.

Both sites were located in the alpine region of Switzerland and the experiments encompassed 24 plots, with experiment 2020 (46.6568°N, 8.2851°E, 1055 m a.s.l.) set up in May 2020 with a plot size of 16 × 15 m, and experiment 2021 (46.6778°N, 8.7571°E, 1435 m a.s.l.) initiated in May 2021 with a plot size of 12 × 20 m. The field site of the 2020 experiment was separated in two neighboring areas, referred to as field 1 and field 2. Each field harbored 3 replicates of each treatment.

Four treatments, each consisting of six plots randomly arranged, were established: (1) Ma 43 FCBK, (2) ART212 FCBK, (3) untreated control, (4) FCBK control. FCBK were applied with a no-till seeder at an equivalent concentration of 10^{14} spores ha^{-1}. For the FCBK control, the no-till seeder was used without seeding FCBK into the slits.

2.3.1. Sampling

We quantified CFUs of *Metarhizium* spp. in the soil both before and 1, 6 and 12 months after treatments. For this, we collected 20 soil cores per plot using a soil corer, each measuring 2.5 cm in diameter and ranging from 5 to 20 cm in depth, depending on the soil texture. The 20 samples were pooled, mixed, and stored in plastic bags at 5 °C until processing. From each pooled sample, three subsamples of 20–24 g each were processed as described by Kessler et al. (2003) and plated on SM plates. We measured the water content of the soil samples gravimetrically and calculated the CFU g^{-1} of dry soil weight. For statistical analysis, we took the mean of the three subsamples.

To examine the distributions of CFU among the 20 samples per plot, we analyzed the samples from one plot of each fungal treatment and one control plot in 2020 separately (see supplementary Fig. 1 for details). From these samples, a subsample of 20–24 g of each of the 20 samples per plot was processed as described above. The mean of the 20 separately processed samples was used for statistical analyses for these plots.

The larval density in the soil was assessed 6 and 12 months after treatments by counting all garden chafer larvae present in five soil blocks per plot, measuring 20 × 20 × 10–15 cm each. The sum of the number of larvae found in the soil blocks was used for statistical analysis. To assess the infection rate in the larval populations, we collected living larvae from the soil blocks in 90 mL insect tubes with moist peat and carrots as feed for further incubation in climate chambers. In 2020, the larvae collected six months after treatment were incubated for 70 days and larvae collected 12 months after treatment application for 46 days. In 2021, the larvae collected six months after treatment were incubated for 30 days at 22 °C and 70 % RH, subsequently overwintered at 5 °C and then incubated again for 40 days at 22 °C and 70 % RH. Larvae collected 12 months after treatment were incubated for 33 days 22 °C and 70 % RH. We checked all incubated larvae weekly, replacing carrot slices as feed at the same time. Dead larvae were removed, and mycosed cadavers were stored at 5 °C until EPF were isolated on SM plates. In 2021, we also recorded the mycosis of larval cadavers with EPF other than *Metarhizium* spp..

2.4. Identification of Metarhizium spp. isolated from larval cadavers

To determine the identity of *Metarhizium* spp. strains sporulating on larval cadavers of the pot experiment 2021 and the field experiment 2021, we conducted multilocus microsatellite marker genotyping as described by Mayerhofer et al. 2015a. We did this type of analysis for all available *Metarhizium* spp. isolates from various treatments, except for the pot experiment treatment with the high Ma 43 FCBK dose from which we randomly selected 10 isolates after incubation in autumn and spring, respectively.

The fungal isolates were cultured on filter paper placed on potato dextrose agar in petri dishes. Prior to sporulation, the mycelium was carefully scraped off from the filter paper and transferred to 2 mL Eppendorf tubes. Subsequently, the mycelia were frozen at −70 °C and lyophilized. Cells of the dry mycelia were disrupted with glass beads (3 mm and 1 mm) in the FastPrep-24 (MP Biomedicals, Eschwege, Germany) for 25 s at 6 m/s. Following this, DNA was extracted using the sbeadex™ plant DNA extraction kit (LGC Biosearch Technologies, Teddington, Middlesex, UK) and the King Fisher Flex Purification system (Thermo Fisher Scientific, Waltham, Massachusetts). DNA concentration was determined using a NanoDrop Microvolume Spectrophotometer (Thermo Fisher Scientific, Waltham, Massachusetts) and standardized to a concentration of 5 ng μL^{-1}.

Microsatellite multilocus genotypes (MLG) were determined using six markers (Ma 2049, Ma 2054, Ma 2063, Ma 2287, Ma 327, Ma 195; Oulevey et al., 2009; Mayerhofer, 2015a). Analyses included three reference strains freshly cultivated from the stock collection, i.e., ART212 and Ma 43 which have been used in this study, and a third strain, Ma 500, which is clearly differentiable from the applied strains with the markers indicated above. Multiplex PCRs were carried out using three primer pairs in two sets each, and fragment size analyses were conducted following the procedures described by Mayerhofer et al. (2015b) and Fernández-Bravo et al. (2021).

2.5. Identification of other EPF isolated from larval cadavers

Isolates with a morphotype different from *Metarhizium* spp. were taxonomically assigned by sequencing the ITS region using the primer pair ITS4/ITS5 and subsequent BLAST sequence similarity searches in the non-redundant nucleotide database of GenBank (Altschul et al., 1997). For the sample preparation and DNA extraction, we followed the same procedures as described above. PCR amplification of the ITS region and subsequent sequencing was performed as outlined in Mayerhofer et al. (2017).

The MLGs of isolates identified as *B. brongniartii* were determined by applying 6 microsatellite markers specifically designed for *B. brongniartii* (Bb1F4, Bb2A3, Bb2F8, Bb4H9, Bb5F4, Bb8D6; Enkerli et al. 2001). Protocols were followed according to Mayerhofer et al. (2015b). *Beauveria brongniartii* BIPESCO2 and BIPESCO4 were used as reference strains.

2.6. Data analysis

To analyze data of the pot and field experiments regarding larval mortality and mycosis, we employed quasibinomial generalized linear models. This approach allowed us to account for the effect of the pot or plot, which introduced a slight degree of overdispersion in the data. The results were then summarized in analysis of variance (ANOVA) tables.

For the CFU data obtained from the pot experiments, we calculated the change (slope) between the two time points, namely before treatment and 8 weeks after treatments, for each individual pot. To ensure data integrity and prevent data loss due to zero CFU counts, we added 1 to each data point and subsequently log-transformed the data to increase homoscedasticity. We then conducted ANOVAs using the transformed data. In these analyses, we prioritized the greenhouse chamber as a factor before considering other variables. This approach allowed us to account for any effects resulting from varying climatic conditions within the two chambers.

In both field experiments, we applied data transformations to enhance homoscedasticity. Specifically, we square root transformed data on the abundance of larvae and log-transformed the CFU counts. Subsequently, we modeled the variables in dependance of the applied treatments and the sampling date. To prevent pseudo-replication, we included plot as error term in our analysis. The results of these analyses were summarized in ANOVA tables.

For field experiment 2020, we introduced an additional variable to account for the separation of the study site into two fields characterized by different soil types. In the field experiment 2021, we incorporated variables labelled X and Y into the dataset. These variables were used to capture the order of the plots and account for any potential gradients within the field. In our analysis, we treated these variables as factors and included them before the applied treatments to account for their impact.

For all analysis, we decomposed the experimental treatments into a series of individual contrasts to thoroughly analyse the effects and potential interactions. For further details see the results section.

3. Results

3.1. Pot experiments

The application of *M. brunneum* Ma 43 FCBK, at both low and high concentrations, had a significant impact on the *Metarhizium* spp. population in the soil in both experiments (Fig. 1, Table 1). There was a modest population of native *Metarhizium* spp. present in the naturally grown field soil used in both experiments. Prior to the treatments, approximately 80 % of all pots in 2020 and 50 % in 2021 had less than 100 CFU per gram of soil. Moreover, only 3 % of all pots in both years exhibited elevated CFU levels of 1000 or more CFU per gram of soil, with none exceeding 10′000 CFU per gram of soil, before treatment.

The abundance of *Metarhizium* spp. CFU changed substantially after application in Ma 43 FCBK treated pots. In 2020, 47 % of pots treated with the low Ma 43 FCBK dose and 75 % treated with the high dose had more than 1000 CFU per gram of soil. A stronger effect was found in 2021, with 72 % of pots treated with the low dose and 100 % with the high dose exceeding 1000 CFU per gram soil. Further, 69 % and 47 % of the pots in 2020 and 89 % and 50 % of the pots in 2021 which were treated with the high and low Ma 43 FCBK dose, respectively, exhibited

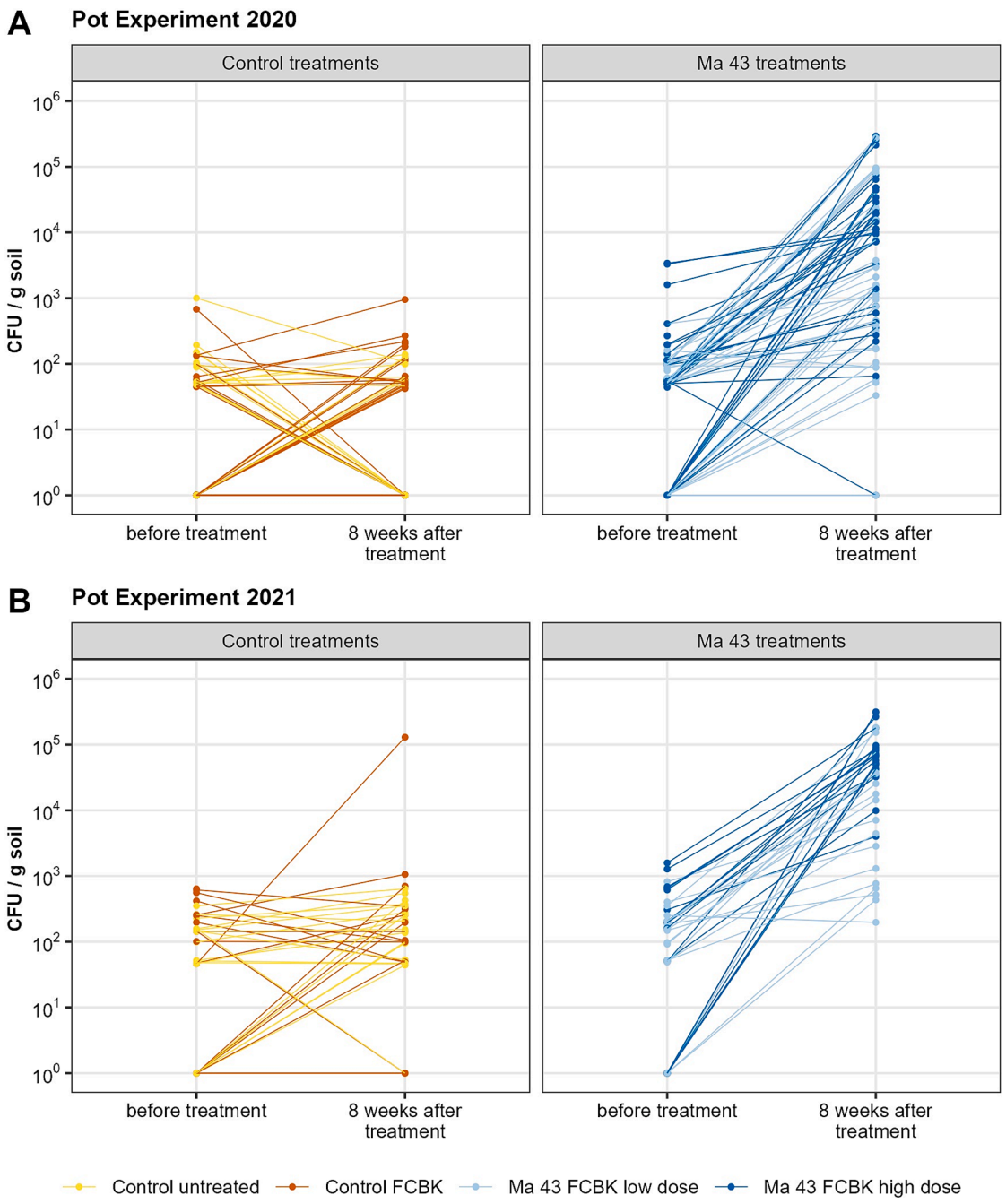

Fig. 1. Prevalence of *Metarhizium* spp. in the pot experiments in 2020 (A) and 2021 (B). Points represent CFU per gram dry weight for each pot on a log-transformed y-axis and the slopes visualize the change in CFU counts for each pot before and 8 weeks after treatment.

CFU counts above 10′000. In contrast, the control treatments showed minimal changes, with none in 2020 and only 2 pots in 2021 exceeding 1000 CFU per gram soil and approximately 80 % of the pots in 2020 and 40 % in 2021 of both control treatments with less than 100 CFU per gram soil.

The application of Ma 43 FCBK significantly reduced larval survival compared to the untreated control and the FCBK control in both pot experiments (Fig. 2, Table 1). Notably, this effect was more pronounced in 2021 than in 2020. In 2020, the mean larval survival rate for Ma 43 FCBK treatments with the high and low doses was 0.48 ± 0.03 and 0.49 ± 0.03, respectively (Fig. 2A). Slightly higher survival rates of larvae were observed in the control treatments, with rates of 0.56 ± 0.04 and 0.63 ± 0.05 for the FCBK control and untreated control, respectively. While there was a significant difference between the Ma 43 FCBK and the control treatments, no significant differences were observed among the control treatments or among the Ma 43 FCBK treatments (Table 1). Larval density did not affect the survival rate of larvae in 2020.

In 2021, we observed a more pronounced decrease in larval survival rates in Ma 43 FCBK treated pots (Fig. 2B). While larval survival dropped to 0.49 ± 0.07 and 0.51 ± 0.05 in the untreated control and the FCBK control, respectively, it was decreased to 0.42 ± 0.07 and 0.24 ± 0.04 in the low and high Ma 43 FCBK dose, respectively. High larval densities led to reduced larval survival, irrespective of the treatment (Fig. 2B, Table 1).

The application depth of Ma 43 FCBK did not impact larval survival or the abundance of *Metarhizium* spp. CFU per gram soil in 2020. Assessment of the effects of application depth was not repeated in 2021.

To estimate the infection rate of Ma 43 within the larval population,

Table 1
Effects of the different factors on survival of larvae and *Metarhizium* spp. prevalence in the pot experiments after 8 weeks. There were no interactions between the different factors, thus, they are not listed.

Exp.	Term	Larvae				CFU			
		df	ddf	*F*	*P*	df	ddf	*F*	*P*
2020	**Treatment**	3	121	2.59	>0.05	3	118	24.07	**<0.001**
	Untreated control, FCBK control ↔ Ma 43 FCBK low dose, Ma 43 FCBK high dose	1	123	7.24	**<0.01**	1	120	66.75	**<0.001**
	Ma 43 FCBK low dose ↔ Ma 43 FCBK high dose	1	121	0.01	>0.5	1	118	2.27	>0.1
	Larval density	2	122	0.73	>0.1	2	119	3.37	**<0.05**
	High ↔ medium, low					1	120	5.97	**<0.05**
	Application depth	1	120	0.21	>0.5	1	117	0.13	>0.5
	Interaction								
	Larval density × Treatment	6	113	0.27	>0.5	6	110	0.88	>0.5
2021	**Treatment**	3	67	7.82	**<0.001**	3	66	14.78	**<0.001**
	Untreated control, FCBK control ↔ Ma 43 FCBK low dose, Ma 43 FCBK high dose	1	69	13.28	**<0.001**	1	68	40.42	**<0.001**
	Ma 43 FCBK low dose ↔ Ma 43 FCBK high dose	1	67	5.36	**<0.05**	1	66	4.78	**<0.05**
	Larval density	2	68	5.42	**<0.01**	2	67	0.28	>0.5
	High ↔ medium, low	1	69	10.79	**<0.01**				
	Interaction								
	Larval density × Treatment	6	59	1.01	>0.1	6	58	0.59	>0.5

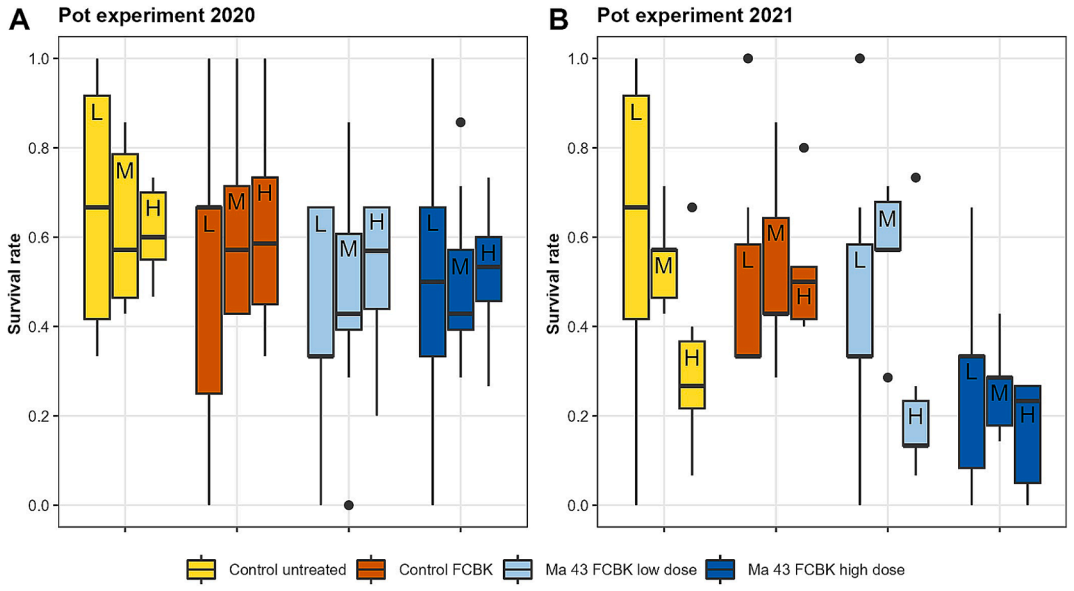

Fig. 2. Survival of larvae in the pot experiments performed in 2020 (A) and 2021 (B). The treatment groups are subdivided into the three larval densities applied to the pots (L = low, M = medium, H = high). The survival rate refers to the number of larvae inoculated in the pots.

we incubated larvae retrieved from the pots and assessed the mycosis rate of those larvae that died during incubation. In 2020, Ma 43 did not establish within the larval population. Only a limited number of larvae exhibited signs of mycosis with *Metarhizium* spp. after death (Fig. 3A). In contrast, in 2021, Ma 43 was successful in infecting a substantial proportion of larvae. The infection rate of Ma 43 was significantly higher in larvae retrieved from Ma 43 FCBK treated pots compared to those from the control treatments ($F_{1,\ 69} = 57.07$, $p < 0.001$, Fig. 3C). Furthermore, a dose-dependent effect was observed, with a substantially higher infection rate in the Ma 43 FCBK high dose treatment compared to the low dose treatment ($F_{1,\ 67} = 8.92$, $p < 0.01$). Additionally, we found that the infection rate with *Metarhizium* spp. was higher in the FCBK control compared to the untreated control ($F_{1,68} = 4.60$, $p < 0.05$).

Genetic analysis confirmed that Ma 43 was responsible for the elevated *Metarhizium* infection rates among larvae in 2021. All *Metarhizium* spp. isolates from the Ma 43 FCBK treatments exhibited the same multilocus genotype as Ma 43 (a total of 47 analyzed isolates, Supplementary Tables 1 and 2). MLG analyses of 10 isolates obtained from the control treatments revealed that eight were identical to the MLG of Ma 43, while two were different, each displaying a separate MLG.

In addition to *Metarhizium* spp., we identified other EPF accounting for larval mortality in both pot experiments. While they were found responsible for causing larval mortality across various treatments in 2020 ($F_{3,\ 123} = 1.88$, $p > 0.1$, Fig. 3B), they were more prevalent on larvae in the control treatments compared to the Ma 43 FCBK treatments in 2021 ($F_{1,\ 69} = 5.76$, $p < 0.05$, Fig. 3D). No significant difference was observed in the mycosis rate caused by other EPF between the low and high Ma 43 FCBK doses in 2021 ($F_{1,\ 67} = 1.36$, $p > 0.1$), but more larvae in the FCBK control group were affected by these EPF compared to the untreated control group ($F_{1,\ 68} = 5.94$, $p < 0.05$). ITS sequence analyses of five isolates representing the most prevalent morphotype in both experiments (Supplementary Table 6) assigned the isolates to the genera *Blackwellomyces* and *Liangia*.

3.2. Field experiments

Both experimental sites had high native *Metarhizium* spp. populations. In 2020, we detected approximately 1000 – 10′000 *Metarhizium* spp. CFU per gram of soil in all plots before treatment application. Field experiment 2021 exhibited even higher counts in all plots, ranging from 10′000 – 100′000 CFU per gram soil (Fig. 4).

Overall, the fungal treatments significantly increased *Metarhizium*

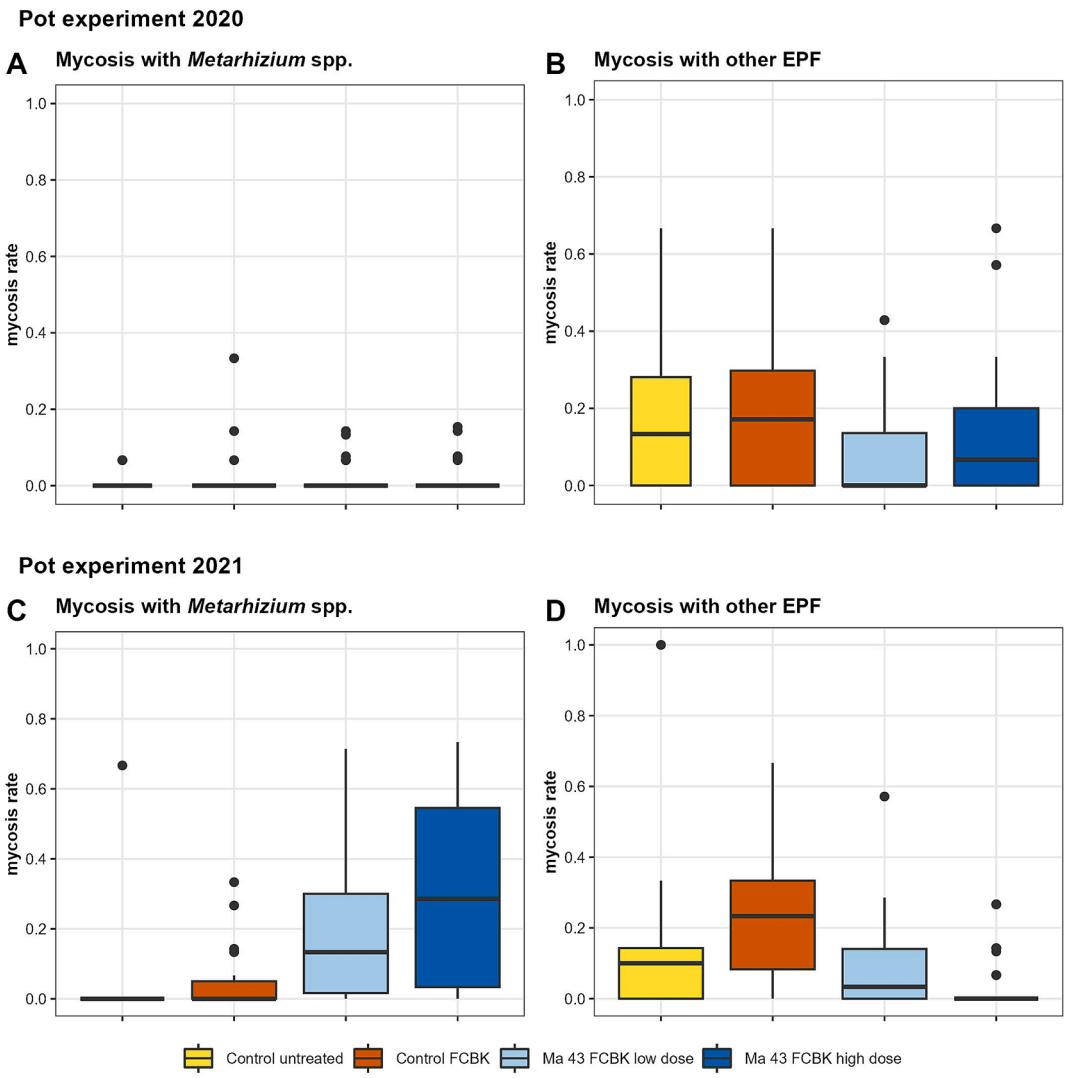

Fig. 3. Prevalence of *Metarhizium* spp. and other EPF among the larval populations in the pot experiments in 2020 (A, B) and 2021 (C, D). The mycosis rate refers to the total number of larvae placed into the pots.

spp. CFU in the soil in both experiments (Table 2 and Table 3). Furthermore, the difference between the fungal-treated and control plots persisted until the final sampling date, 12 months after treatments. We did not find differences between the Ma 43 and the ART212 FCBK application in both experiments. In field experiment 2020, which was spread across two neighboring fields, we observed differences between field 1 and field 2, with an interaction between treatments and fields (Table 2).

No differences in larval densities were observed between the fungal-treated and the control plots at both sampling dates in the 2020 experiment (Fig. 5A, Table 2). The fungal treatments reduced larval populations in 2021 six month after treatment ($F_{1,\ 20} = 7.37$, $p < 0.05$, Fig. 5B) but the effect diminished after 12 months ($F_{1,\ 20} = 1.86$, $p > 0.1$). It is important to note that both experiments witnessed high overwinter mortality among the larval populations independent of the various treatments (Fig. 5 and Table 3). In 2020 we did not find a higher *Metarhizium* spp. mycosis rate among larvae collected six or 12 months after treatment in the fungal treated plots compared to the control plots (Fig. 6A and B, Table 2). Furthermore, we found other EPF infecting larvae collected at both sampling dates, but we did not systematically record those findings.

In 2021, six months after treatment, we found a tendency towards elevated *Metarhizium* spp. mycosis rate in ART212 FCBK treated plots but not in Ma 43 FCBK treated plots (Fig. 6C), however, the effect is statistically non-significant (Table 3). Other EPF were more abundant on larvae than *Metarhizium* spp., with a mean mycosis rate of 0.18 ± 0.03 caused by *Metarhizium* spp. in the ART212 treatment, and a mean mycosis rate of 0.25 ± 0.03 caused by other EPF across all treatments (Fig. 6E). In spring, 12 months after treatment, no differences in the larval mycosis rates with *Metarhizium* spp. were detected (Fig. 6D). The mean mycosis rate with *Metarhizium* spp. across all treatments was 0.25 ± 0.03. Additionally, we observed a mean mycosis rate of 0.16 ± 0.03 attributed to EPF other than *Metarhizium* spp.

We genetically analyzed a total of 62 *Metarhizium* spp. isolates collected from larvae from field experiment 2021, with 33 originating from larvae collected six months after treatment and 29 from larvae collected 12 months after treatment. The majority of *Metarhizium* spp. strains recovered from the cadavers of these larvae had a MLG identical to *M. brunneum* ART212. This observation held true regardless of the treatment or the timing of larval collection (Supplementary Tables 3, 4 and 5). Another six isolates had an identical MLG to Ma 43, with only three of these isolates originating from larvae collected from Ma 43 FCBK treated plots. Additionally, 8 isolates did not match the genetic profiles of either Ma 43 or ART212. Four of these isolates were recovered from larvae collected in control plots.

The sequence analysis of the ITS region in three isolates of the most

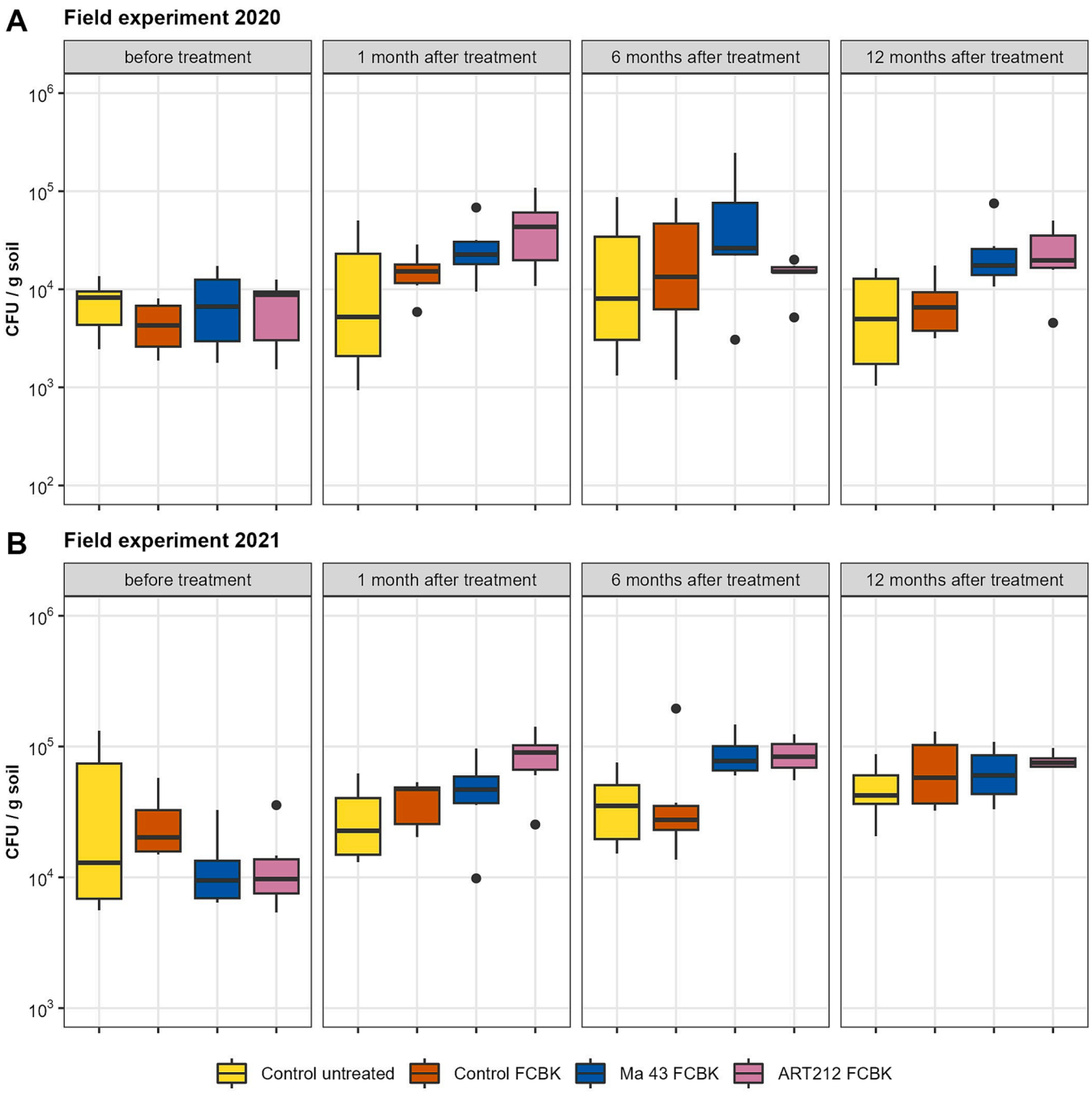

Fig. 4. Abundance of *Metarhizium* spp. CFU in the soil of the experimental sites in 2020 (A) and 2021 (B) prior, 1, 6, and 12 months after treatments. The scale of the y-axis is log-transformed.

frequent morphotype of EPF, which differed from *Metarhizium* spp. (Supplementary Table 6), allowed assignment of these isolates to *B. brongniartii*. Subsequent microsatellite marker analyses revealed distinct MLGs for each of the three strains (Supplementary Table 7).

4. Discussion

To control native scarab species in central Europe, the soil-born entomopathogens *B. brongniartii* and *M. brunneum* can be applied to larval habitats where they induce epizootics among their host insects. While extensive research has been conducted on *B. brongniartii* and its host, the European cockchafer, the same is not true for the generalist pathogen *M. brunneum*, which may have the potential to control several noxious scarab species. Thus, we focused our study on two commercially available strains of *M. brunneum*, Ma 43 and ART212, which are used to control garden chafer larvae. Besides assessment of the direct impact of the EPF on survival of the host larvae, we assessed the establishment of the fungal control agent in soil, and the disease prevalence in the host population in our pot and field experiments. Both are important prerequisites for the induction of epizootics in grub populations.

4.1. Establishment and persistence of fungal inoculum in the soil

In all our pot and field experiments, we found naturally occurring *Metarhizium* spp. in the soil, at low and medium densities in the pot experiments and high densities at both of our field sites. Our treatments consistently increased CFU densities by a factor of approximately ten in all experiments. This pattern of a tenfold increase in CFU per gram of

Table 2
Field experiment 2020. Effect of the different treatments on the dependent variables CFU per gram of soil and total number of larvae found per plot. To disentangle the effects of the different treatments, we applied contrasts shown here..

Term	df	ddf	*F*	*P*
Log (CFU)				
Fungal treatments ↔ Control treatments	1	22	19.77	**<0.001**
Bip5 FCBK ↔ ART212 FCBK	1	20	0.31	>0.5
Control untreated ↔ Control FCBK	1	20	2.26	>0.1
Field 1 ↔ Field 2	1	19	21.27	**<0.001**
(Field 1 ↔ Field 2) × (Fungal treatments ↔ Control treatments)	1	18	6.87	**<0.05**
1 month ↔ 6 months ↔ 12 months after treatment	2	46	1.82	>0.1
√ total larvae				
Fungal treatments ↔ Control treatments	1	22	0.40	>0.5
Field 1 ↔ Field 2	1	21	1.57	>0.1
(Field 1 ↔ Field 2) × (Fungal treatments ↔ Control treatments)	1	20	2.13	>0.1
6 months ↔ 12 months after treatment	1	23	23.61	**<0.001**
Mycosis rate with *Metarhizium* sp. 6 months after treatment				
Fungal treatments ↔ Control treatments	1	20	2.65	>0.1
Bip5 FCBK ↔ ART212 FCBK	1	18	0.10	>0.5
Control untreated ↔ Control FCBK	1	18	0.001	>0.5
Mycosis rate with *Metarhizium* sp. 12 months after treatment				
Fungal treatments ↔ Control treatments	1	20	0.21	>0.5
Bip5 FCBK ↔ ART212 FCBK	1	18	1.14	>0.1
Control untreated ↔ Control FCBK	1	18	1.19	>0.1

Table 3
Field experiment 2021. Effect of the different treatments on the dependent variables CFU per gram of soil and total number of larvae found per plot. To disentangle the effects of the different treatments, we applied contrasts shown here.

Term	df	ddf	*F*	*P*
Log (CFU)				
Fungal treatments ↔ Control treatments	1	20	23.99	**<0.05**
Bip5 FCBK ↔ ART212 FCBK	1	18	2.93	>0.1
Control untreated ↔ Control FCBK	1	18	1.34	>0.1
1 month ↔ 6 months ↔ 12 months after treatment	2	46	3.05	>0.05
√ total larvae				
Fungal treatments ↔ Control treatments	1	20	2.21	>0.1
Bip5 FCBK ↔ ART212 FCBK	1	18	2.85	>0.1
Control untreated ↔ Control FCBK	1	18	0.09	>0.5
6 months ↔ 12 months after treatment	1	23	61.56	**<0.001**
Mycosis rate with *Metarhizium* sp. 6 months after treatment				
Fungal treatments ↔ Control treatments	1	20	1.19	>0.1
Bip5 FCBK ↔ ART212 FCBK	1	18	1.50	>0.1
Control untreated ↔ Control FCBK	1	18	0.39	>0.5
ART212 FCBK ↔ Bip5 FCBK, Control treatments	1	20	2.36	>0.1
Mycosis rate with *Metarhizium* sp. 12 months after treatment				
Fungal treatments ↔ Control treatments	1	20	0.01	>0.5
Bip5 FCBK ↔ ART212 FCBK	1	18	0.50	>0.1
Control untreated ↔ Control FCBK	1	18	2.11	>0.1
Mycosis rate with other EPF 6 months after treatment				
Fungal treatments ↔ Control treatments	1	20	0.01	>0.5
Bip5 FCBK ↔ ART212 FCBK	1	18	0.42	>0.5
Control untreated ↔ Control FCBK	1	18	0.31	>0.5
ART212 FCBK ↔ Bip5 FCBK, Control treatments	1	20	0.33	>0.5
Mycosis rate with other EPF 12 months after treatment				
Fungal treatments ↔ Control treatments	1	20	2.05	>0.1
Bip5 FCBK ↔ ART212 FCBK	1	18	6.23	**<0.05**
Control untreated ↔ Control FCBK	1	18	1.17	>0.1

soil, regardless of the natural abundance of *Metarhizium* spp. before treatment, was consistent with the findings of previous studies (Strasser et al., 2005; Keller and Schweizer, 2007).

In both pot experiments, we had a background level of 100 – 1000 CFU per gram of soil, which increased to levels of 1000-10′000 or even more CFU per of gram soil, in pots treated with Ma 43 FCBK at both low and high doses. In control treatments, CFU levels remained mostly below 100 CFU per gram of soil. Thus, the application of Ma 43 FCBK efficiently increased the fungal inoculum in the soil eight weeks after treatment.

We expected higher CFU levels in pots with higher larval densities, as epizootics are expected to develop faster at higher larval densities (Fornallaz, 1992) which could lead to an increase in CFU due to spore production on larval cadavers. However, we did not find this effect in either of the pot experiments. Given the relatively short eight-week duration of the experiments, we assume that epizootics could not fully develop, and consequently, the prevalence of fungal propagules was not further increased. Epizootics induced by *B. brongniartii* in cockchafer populations are measurable after several months or even over a year only, depending on the larval population and location (Fornallaz, 1992; Keller et al., 1999; Kessler et al., 2004).

In our field experiments we observed an increase in CFU levels from roughly 1000 – 10′000 to 10′000 – 100′000 spores per gram of soil, following the application of FCBK, using both Ma 43 and ART212. This effect persisted for at least 12 months without diminishing over time, despite high densities of naturally occurring *Metarhizium* spp. Our results provide evidence that the application of *M. brunneum* conidiospores is efficient and that the applied fungal strains can persist for months if not years in natural habitats of garden chafer larvae, opening up perspectives for long term control of the soil pest with a single application only. This aligns with the findings of Strasser et al. (2005) who reported stable or slightly increasing CFU levels for the duration of eight months after treatment and Keller and Schweizer (2007), who found a clear increase in CFU levels between six and twelve months after treatment using the same fungal strains in their experiments. It has been shown that *M. brunneum* can persist in soil for decades at enhanced levels, independent of a specific insect host (Scheepmaker and Butt, 2010). This stands in contrast to *B. brongniartii*, which declines more rapidly than *Metarhizium* spp. when its host disappears (Keller et al., 2003; Kessler et al., 2004; Scheepmaker and Butt, 2010).

Certain *Metarhizium* spp. strains can colonize the rhizosphere, form symbiotic relationships with plants (Hu et al., 2014; Chandler, 2017) and transfer nitrogen from insect cadavers to the plants (Behie and Bidochka, 2014). It has been shown that *Metarhizium* spp. stimulate the development of plant roots, and, in return, the fungi might profit from the interaction by receiving nutrients and shelter from the plants (Bruck, 2005; Sasan and Bidochka, 2012; Behie et al., 2015, Dara, 2019). This allows those fungal strains to persist in soils independently of insect hosts and their occurrence is not associated to a specific insect (St. Leger and Wang, 2020). Specifically for Ma 43, it has been shown that this strain is able to colonize plants endophytically and promotes plant growths (Jaber and Enkerli, 2016; Jaber and Enkerli, 2017). As we could show a clear and persisting increase in CFU level in the Ma 43 and ART212 treated plots, we suggest that the strains Ma 43 and ART 212 are rhizosphere-competent and can thus, effectively establish in the soil.

While it is estimated that 1000 *B. brongniartii* CFU per gram of soil are sufficient to induce epizootics in cockchafer larvae (Kessler et al., 2004), there is no available literature on the minimum effective concentration of *M. brunneum* CFU for garden chafer control. Keller and Schweizer (2007) achieved effective control of garden chafer larvae with approximately 2000 CFU per gram of soil, whereas Strasser et al. (2005) reported insufficient control with around 20′000 CFU per gram of soil. This suggests that there is no general “threshold” for CFU level which guarantees a control effect of these fungal biocontrol products. More likely, the number of CFU required to achieve significant impact on pest population depends on various abiotic and biotic factors, such as soil moisture and temperature, the soil microbiome or the larval stage (Keller, 1992; Hajek and St. Leger, 1994; Villani et al., 1994; Strasser and Erschbamer, 2003; Jaronski, 2007; Ortiz-Urquiza and Keyhani,

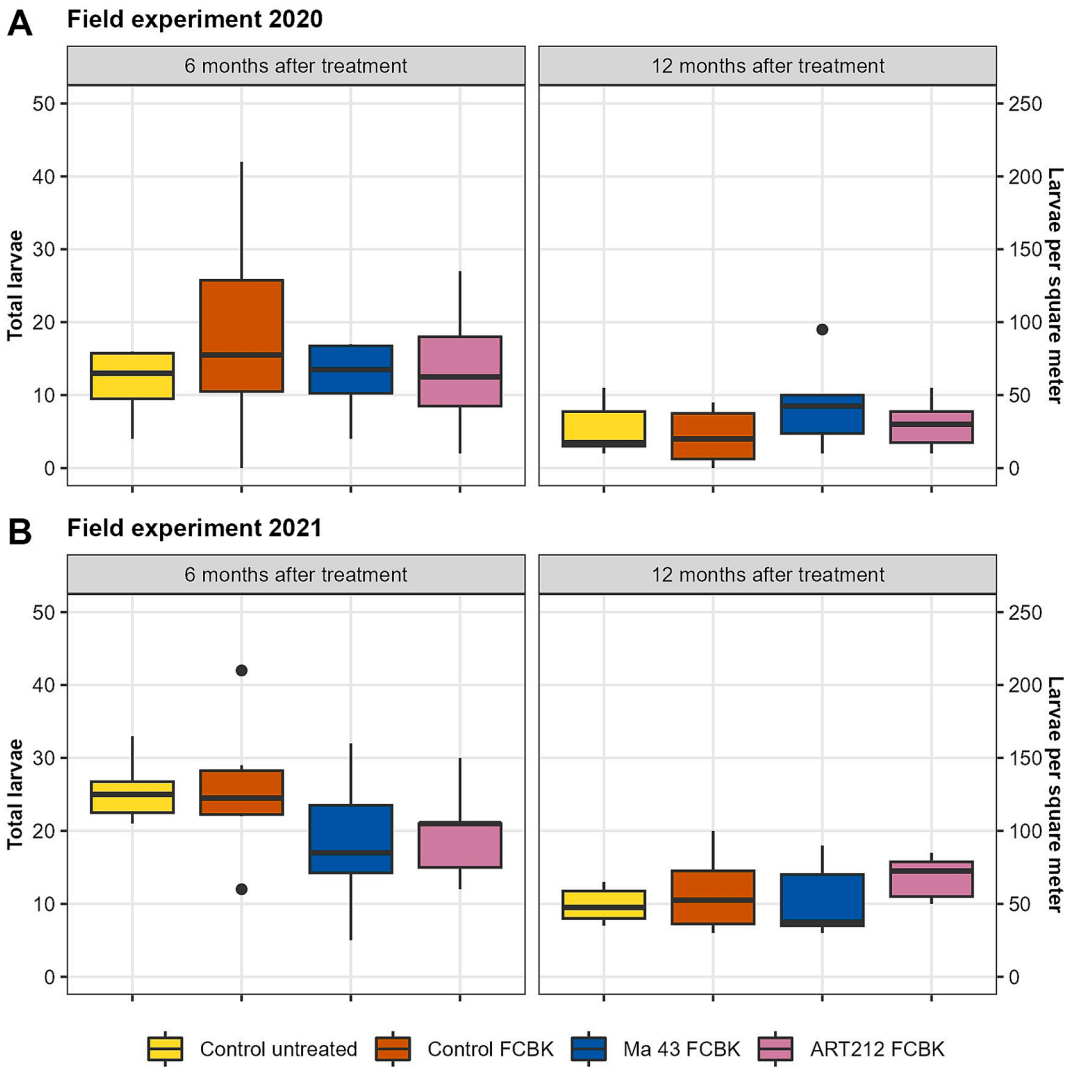

Fig. 5. Larval densities in experimental plots in 2020 (A) and 2021 (B) six and twelve month after treatment. Total larvae refer to the number of larvae counted in five soil blocks in each plot.

2013; Mayerhofer et al. 2017; Fernández-Bravo et al., 2021). Based on these studies we argue that the various fungal treatments in our pot and field experiments elevated CFU densities to a level which is appropriate for fungal infections of garden chafer larvae, provided that abiotic and biotic conditions in the soil support fungal infection. Furthermore, natural densities of *Metarhizium* spp. at both of our field sites were high and would likely have supported the control of garden chafer larvae if those native strains were virulent against them.

Metarhizium spp. are commonly found in various habitats (Scheepmaker and Butt, 2010) and are prevalent in Swiss grasslands (Fernández-Bravo et al., 2021). However, our field experiment sites harbored a remarkably high natural abundance of *Metarhizium* spp. which is rarely found across grassland in Switzerland (Fernández-Bravo et al., 2021). This may be attributed to high levels of garden chafer larvae present at these sites for several years (personal communication with landowners), which may have been subject to a natural increase of fungal inoculum in the soil before we started our experiments. The fact that we found garden chafer larvae infected with EPF in all our plots (treated and untreated) supports this hypothesis.

In contrast, Strasser et al. (2005) recorded only an average of 2000 CFU per gram soil in untreated plots on a golf course heavily infested by garden chafer larvae (~ 500 larvae per m^2) and Keller and Schweizer (2007) found only around 200 *Metarhizium* spp. CFU per gram soil despite high infestation levels. It has been shown that the presence and abundance of *Metarhizium* spp. may also be affected by the plant community composition and the land use type besides presence of a specific insect host (Fisher et al. 2011; Wyrebek et al., 2011; Fernández-Bravo et al., 2021). Since *Metarhizium* spp. can establish symbiotic relationships with plants in the rhizosphere (Jaber and Enkerli, 2016; Jaber and Enkerli 2017; St. Leger and Wang, 2020), there is evidence that different plant species foster symbioses with specific species and strains of *Metarhizium* (Sun et al., 2008; Fisher et al., 2011; Wyrebek et al., 2011). It follows that natural *Metarhizium* abundance may benefit from the diverse plant community on species rich meadows, as it was the case on our study sites, especially in our field trial 2021, and decrease in monoculture habitats such as golf courses, which were the study sites of Strasser et al. (2005) and Keller and Schweizer (2007). Besides a species-poor vegetation cover, golf courses are managed with high levels of pesticide applications which may additionally reduce the natural abundance of EPF and make the soils less conductive for *M. brunneum* (Scheepmaker and Butt, 2010).

4.2. Impact of the treatments on the larval populations

In both, pot experiments and in field experiment 2021, we found a reduction of the garden chafer population following the application of Ma 43 and ART212. Moreover, we found a tendency towards more dead larvae infected with *Metarhizium* spp. in the fungal treatments in the

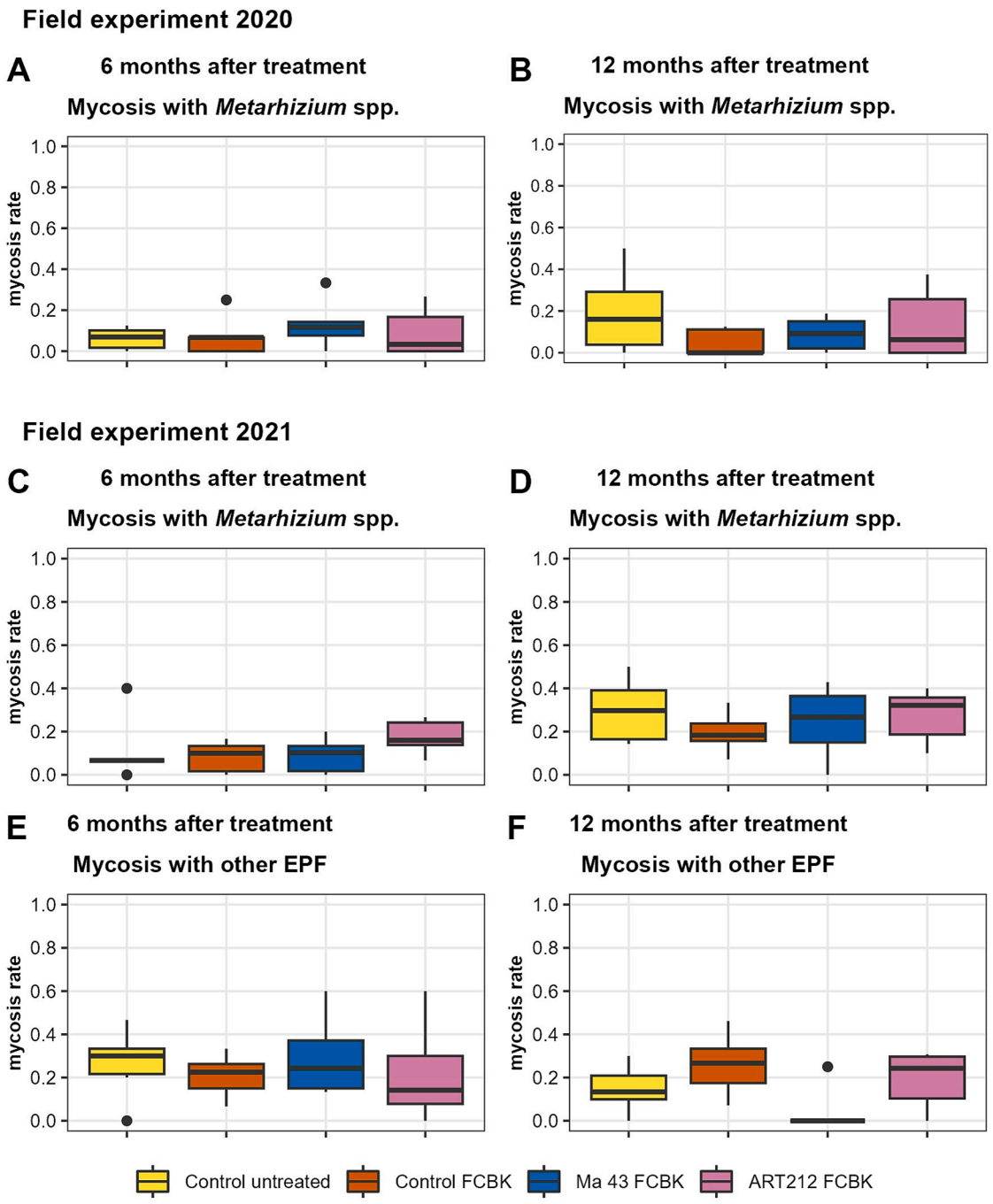

Fig. 6. Prevalence of *Metarhizium* spp. and other EPF in the larval populations six and 12 month after treatment in 2020 and 2021. The mycosis rate refers to the number of larvae collected at each sampling site and date.

field experiment 2021 and in the pot experiment 2021. This indicates that Ma 43 and ART212 affect the mortality of garden chafer larvae when applied in semi-controlled conditions and under field conditions.

In our pot experiments, the application of Ma 43 FCBK reduced larval survival during the eight-week incubation period. In 2020, larval survival rates were generally higher, and the application of Ma 43 FCBK only slightly increased larval mortality. Approximately 60 % of larvae survived in the control treatments, while around 50 % survived in the Ma 43 FCBK treated pots, with no difference between the high and low doses. In contrast, we found a clear effect of the high dose of Ma 43 FCBK on the larval mortality in 2021. Here, only about 25 % of the larvae survived in the high-dose Ma 43 FCBK treatment, while around 40 % survived in the low-dose Ma 43 FCBK treatment, and 50 % of the larvae stayed alive in the control treatments. Results of the latter experiment confirm that the number of spores adhering to and germinating on the larval cuticle influences the effectiveness of EPF as previously reported by Ansari et al. (2004) and Nong et al. (2011). Furthermore, this suggests that the lower dose of Ma 43 FCBK, which is generally used in commercial applications, may not be sufficient to effectively reduce larval populations within a short timeframe. However, this should not impair efficacy of field applications in general, since the inoculation period is considerably longer in practice, spanning the entire larval developmental phase of the garden chafer from early July to late October (Laughlin, 1957).

The difference in the results between our pot experiments from 2020 and 2021, may be explained by a difference in the activity of the host

larvae. Although both experiments were started in the first week of October, the larvae used in experiments were not in the same developmental stage. Garden chafer larvae spend approximately 110 days actively foraging in the root zone, after which they move to deeper soil strata and rest in an earth cell for hibernation (Laughlin, 1957). This behavior is not determined by falling temperature but rather by the number of days the larvae spent feeding (Laughlin, 1957). This implies that larvae stemming from an adult population that experienced an early flight period due to warm spring temperatures cease their feeding activities earlier in autumn than larvae stemming from an adult population with a delayed flight period resulting from cold spring weather (Laughlin, 1957). In spring 2020, warmer and drier conditions favored an early flight and consequently an early start of the feeding period of garden chafer larvae (Meteo Schweiz, 2020). Many larvae collected for the pot experiment in autumn 2020 therefore, may have been at the end of their feeding period and entered their resting phase at the beginning of the experiment. Conversely, spring 2021 was one of the coldest in the past 30 years (Meteo Schweiz, 2021), causing a delay in larval development. Most of the larvae collected in autumn 2021 were therefore still in an active feeding phase at the beginning of the experiment. Since fungal spores do not move themselves but are passively transported through the soil either by water, other microorganisms, arthropods, or earthworms (Zimmermann, 2007b), as well as by the movement of the host itself, we assume that larvae of the 2021 experiment collected more spores from the inoculated pot substrates than the ones from 2020.

In addition, garden chafer larvae show aggressive behavior and tend to harm each other when they meet by chance while moving through the soil (Laughlin, 1957). This may explain higher mortality in 2021 in the control pots with high larval densities for the same reasons as mentioned above.

In field experiment 2021, larval densities were reduced in Ma 43 and ART212 treated plots six months after treatment. However, the effect was small with a reduction of the larval population by approximately 25 % and diminished 12 months after treatment. In field experiment 2020, we did not find an effect of the fungal treatments on the larval density. One explanation for this surprisingly low efficacy of the fungal treatments in our field experiments may be that natural mortality factors have masked effects of the applied EPF. Especially, we found high winter mortalities of larvae in both of our field experiments irrespective of the various treatments. Milne (1984) has shown that garden chafer populations fluctuate strongly and are highly sensitive to climatic conditions. Early autumn frost and wet or dry conditions during the egg hatching phase greatly impact larval survival, ultimately influencing population densities (Milne, 1984). Similarly, Keller and Schweizer (2007) found a substantial decrease of garden chafer populations over winter in their field experiment. Contrary to our results, however, they were able to show a strong effect of the EPF treatment 12 months after application. On the other hand, Strasser et al. (2005) did not observe any larvae infected with *Metarhizium* spp. in their field experiment. Thus, effects are variable with a strong control effect shown by Keller and Schweizer (2007), a moderate to weak control effect found in this study and no control effect reported by Strasser et al. (2005).

4.3. Prevalence of EPF infections in garden chafer populations

Besides abiotic factors, garden chafer larvae are vulnerable hosts or prey to a variety of different bacteria, nematodes, viruses, and animals (Milne, 1984). In addition to those garden chafer antagonists, we found a variety of EPF killing a substantial proportion of larvae in all our experiments.

In the pot experiment 2020, we identified EPF of the genera *Blackwellomyces* and *Liangia* which were responsible for approximately 10 % of larval mortality across various treatments. Moreover, we assume that these EPF may have exhibited greater competitiveness in cases of mixed infections (Uma Maheswara Rao et al., 2007; Guzmán-Franco et al., 2011; Pauli et al., 2018) since only an insignificant number of larval cadavers were infected with *Metarhizium* spp. in our 2020 pot experiment. As we introduced larvae into the pots a few days before application of the treatments, EPF already occurring naturally in the soil might have had an advantage when competing with Ma 43 for host resources (Uma Maheswara Rao et al., 2007; Staves and Knell, 2010).

In the pot experiment 2021, larvae from Ma 43 FCBK treatments were predominantly infected by Ma 43, whereas mycosis of larvae from the control treatments was dominated by *Blackwellomyces* spp. Moreover, Ma 43 induced an additive effect to the mortality caused by the naturally occurring *Blackwellomyces* spp. and seemed to outcompete the latter in mixed infections, especially in the high-dose Ma 43 FCBK treatment. This is in line with the study of Guzmán-Franco et al. (2011) who found that the competition of EPF in mixed infection is often won by the species with the higher number of spores on the insect host. However, this finding cannot be generalized as Li et al. (2021) showed that faster growing EPF strains are able to outcompete other strains when applied in a ratio as low as 1:9.

In our field experiments, we found a tendency towards a higher infection rate of garden chafer larvae with *Metarhizium* spp. in fungus treated plots compared to control plots. This gives evidence that the applied fungal strains were able to infect garden chafer larvae despite high natural abundance of other *Metarhizium* spp. which may compete with the applied strains.

In addition, there is evidence of competition between the two applied strains of *Metarhizium*. We detected the multi-locus genotype of ART212 on larvae from plots that were not treated with ART212, which was not the case for Ma 43. It has been shown that competitive exclusion is common among different EPF species and strains (Li et al. 2021). Thus, this finding suggests that ART212 might be more competitive in infecting garden chafer larvae than Ma 43. Since this effect increased over the investigation period, we assume that ART212 was passively spread to other plots over time. However, ART212 is a widely distributed genotype in Swiss grassland soils (Enkerli, unpublished data), thus, we cannot exclude that it occurred naturally at our field site.

We also identified other EPF on larvae in both field experiments and quantified the prevalence of the dominant morphotype in field experiment 2021 which was identified as *B. brongniartii*. Six months after treatment, infection rates with *B. brongniartii* even exceeded *Metarhizium* spp. infections. This is surprising, considering that *B. brongniartii* is described as a specific pathogen of the cockchafer in Europe (Scheepmaker and Butt, 2010). To the best of our knowledge this is the first record of *B. brongniartii* infecting up 30 % of larvae of a garden chafer population and may have contributed to a high winter mortality of the larvae. Since field sampling did not reveal the presence of any cockchafer larvae, it is unlikely that the population of *B. brongniartii* was established in previous years by infection of cockchafers, thus, garden chafer larvae appear to be the primary hosts of the *B. brongniartii* strains in the field investigated. Notably, *B. brongniartii* was not detected in our soil samples when we cultured suspensions on SM plates. However, tests involving mixed cultures of *M. brunneum* and *B. brongniartii* on SM plates revealed that *M. brunneum* is more competitive on this artificial medium and therefore may have suppressed the growth of *B. brongniartii* (unpublished data).

It is important to note that by collecting larvae from the field and incubating them in the laboratory, we may have added a stress factor and, thus, may have overestimated infection rates. More larvae may have survived latent infections with *Metarhizium* (and other EPF) if they had remained in their natural habitat in the field.

5. Conclusions

To conclude, our experiments have demonstrated that the application of two commercially available strains of *M. brunneum*, Ma 43 and ART212, can significantly increase the abundance of fungal propagules in the soil by approximately a factor of ten, irrespective of high levels of naturally occurring *Metarhizium* spp. Furthermore, those increased CFU

levels persisted over 12 month which highlights the potential of *M. brunneum* strains for long-term control of soil pests. This robust performance underscores the suitability of Ma 43 and ART212 for soil application, even when high numbers of other EPF are already present in the environment.

In three of our four experiments, the application of Ma 43 or ART212 led to slightly increased mortality of garden chafer larvae. However, larvae succumbed to death for a variety of other reasons and the applied fungal strains were only one component of a consortium of natural enemies of garden chafer larvae. In addition, the influence of adverse environmental conditions during the winter months proved to be stronger than the effect of the treatments. In the end, densities of *P. horticola* larvae decreased significantly in all plots – not only the treated ones – over the experimental period of 12 months. On the one hand, it is therefore clear that the applied fungi have contributed to larval mortality. On the other hand, however, their contribution seems of minor importance since a whole array of naturally occurring antagonists (including naturally occurring EPF) together with abiotic stress led to a general and significant decrease of the whole garden chafer population.

In addition to the experimentally applied and naturally occurring *Metarhizium* spp., we found three other genera/species of EPF frequently infecting garden chafer larvae. Notably, in the field experiment 2021, *Metarhizium* spp. and *B. brongniartii* together were responsible for killing nearly 50 % of all incubated larvae. These findings indicate that naturally occurring EPF play an important role in controlling garden chafer populations in Swiss mountainous regions. This has been largely underestimated so far, probably also because previous research in other habitats has shown contrasting results. For example, in his long-term study on population fluctuation and natural control mechanisms, Milne (1984) did not identify EPF as important natural enemies of garden chafer larvae in the UK.

We have to point out that it is unclear if the community of soilborne fungi found at our study sites was the exception rather than the rule, both in a qualitative and quantitative way. However, the application of Ma 43 or ART212 for the control of garden chafer larvae seems to be redundant in meadows already harboring a diverse consortium of EPF in high abundances. Such interventions might be advisable in other environments such as golf courses, where diversity and abundance of natural biocontrol agents are lower and even low damage levels in the turf are unacceptable. Furthermore, our study comprised only one grub pest species, and future studies on other abundant soil pests, such as cockchafers, *Melolontha* spp., may lead to different results. However, our study shows clearly that more research on the role of naturally present biocontrol agents in different habitat types, and on a broader spatial scale, is necessary to determine situations, where an application of generalist rhizosphere competent EPF is justified, and when it is not required at all.

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CRediT authorship contribution statement

Tanja Graf: Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Tabea Koch:** Investigation, Formal analysis. **Jürg Enkerli:** Writing – review & editing, Validation, Supervision, Resources, Methodology. **Giselher Grabenweger:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Tanja Graf reports was provided by H2020 Food Security Sustainable Agriculture and Forestry Marine Maritime and Inland Water Research and the Bioeconomy. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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During the preparation of this work the authors used ChatGPT in order to improve the language of the paper. After using this tool/service, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biocontrol.2024.105625.

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