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Autodissemination of *Metarhizium brunneum*: a strategy for biological control of adult Japanese beetles

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Abstract
The Japanese beetle (*Popillia japonica*) is an invasive scarab beetle originating from Japan. In the European Union, it is listed as a priority quarantine pest. Currently, it is mainly controlled using synthetic insecticides. Here, we tested an environmentally friendly control alternative. We investigated whether Japanese beetle adults can be used as vectors to autodisseminate lethal doses of the European native entomopathogenic fungus *Metarhizium brunneum* ART 212 within adult populations. Additionally, we tested whether infested females could carry conidia into the soil environment during oviposition, increasing neonate larval mortality. We showed that inoculated adults can indeed transmit the fungal conidia horizontally for up to two days, significantly reducing the survival of both donor and recipient beetles in same-sex and opposite-sex couples. Furthermore, horizontal transmission among adults was verified under semi-field conditions. Another set of laboratory tests showed that beetles carried the inoculum to their oviposition sites, where larval survival was reduced at high concentrations ($\geq 1.11 \times 10^5$ conidia/g substrate). However, the release of inoculated beetles in semi-field cages resulted in soil fungal concentrations more than ten times lower, failing to provide larval control. Thus, carriage of *M. brunneum* ART 212 into the soil by female vectors does not seem to provide control of larvae outside the laboratory setup. However, our results suggest that lethal conidial doses can be autodisseminated among the more susceptible adults. This may be the basis for an environmentally friendly control strategy against invasive Japanese beetle adults, applicable in both agricultural and non-agricultural areas.

Keywords Invasive insect · Quarantine organism · *Popillia japonica* · Entomopathogenic fungus · Horizontal transmission

Key message

- Autodissemination of a fungal inoculum was tested to biologically control the Japanese beetle .
- Larval control after transmission of conidia to the soil was achieved under laboratory conditions.
- Adults horizontally transmitted the conidia, causing mortality among donors and recipients.
- This is the basis for a control strategy against adults in agricultural and non-agricultural areas.

Introduction

The Japanese beetle (*Popillia japonica* Newman (Coleoptera: Scarabaeidae)) is a significant plant pest originating in Northern Japan (Fleming 1972; Potter & Held 2002). Following its accidental introduction to New Jersey (USA) in the early twentieth century, the scarab beetle started to establish itself outside its native range (Fleming 1972). Since then, it has invaded vast areas of North America (Kistner-Thomas 2019). The Japanese beetle can cause damage to over 400 plant species including ornamental, horticultural and agricultural plants such as grapevine, stone fruit and soy (Tayeh et al. 2023). Due to the potential economic, environmental and social impact on European agriculture and ecosystems, the Japanese beetle is ranked as a priority quarantine pest in the European Union (European Commission 2019). In mainland Europe, it was first reported in Northern Italy in 2014 (Pavesi 2014). Since then, *P. japonica* has established stable populations in Italy and southern Switzerland (EPPO 2023; Poggi et al. 2022). From there, the pest

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is able to further expand its range as it encounters suitable environmental conditions in Central Europe (Borner et al. 2022). Usually, the beetle has a one-year life cycle, with third instar larvae overwintering in the soil and adults feeding, mating and ovipositing from around June to September (Fleming 1972).

Currently, pest management of Japanese beetle adults and larvae mainly relies on the application of synthetic insecticides (Potter & Held 2002; Santoiemma et al. 2021; Shanovich et al. 2019). However, this strategy is associated with environmental pollution, declines in biodiversity and reduced ecosystem functioning (Chagnon et al. 2015; Stehle & Schulz 2015; Thompson et al. 2020; Woodcock et al. 2016). Research on non-chemical alternatives should thus be a priority, especially in Europe, as the European Union aims to find strategies to reduce the use of synthetic pesticides (European Parliament 2009).

Promising alternatives to synthetic pesticides are biocontrol agents such as nematodes or entomopathogenic microorganisms (Inglis et al. 2009; Paoli et al. 2017; Ravensberg 2013). Among the latter, entomopathogenic fungi (EPFs) possess the unique ability to infect insects by penetrating their cuticle (Roberts & Hajek 1992). After germination, they combine enzymatic and physical mechanisms to penetrate the different cuticular layers (Mannino et al. 2019). Once the fungus has reached the hemolymph, it continues its growth by forming yeast-like cell types, called hyphal bodies or blastospores. These can multiply rapidly by division or budding, colonize the entire host body and, finally, kill the insect by, e.g., toxicosis (Inglis et al. 2009; Pedrini 2018).

Different EPFs (*Metarhizium* spp. and *Beauveria* spp.) have already been tested to control the larval stage of *P. japonica* (Behle et al. 2015; Fleming 1976; Graf et al. 2023; Potter & Held 2002; Shanovich et al. 2019). Yet, study results on field efficacy of *M. brunneum* (Petch) applied against third instar larvae are contradictory (Behle et al. 2015; Graf et al. 2023). On the one hand, Behle et al. (2015) and Ramoutar et al. (2010) found variable, but generally positive control effects of *M. brunneum* Ma 43 (BIPESCO5/F52) in small-scale field experiments. On the other hand, more recent studies conducted in Northern Italy state a low susceptibility of third instar larvae to *M. brunneum* Ma 43 and *M. brunneum* ART 212 (Ma 714, ARSEF 7524; Graf et al. 2023). Probably, third instar larvae are protected by their cuticular defense mechanism in combination with an efficient immune response (Graf et al. 2023).

In contrast to the contradictory findings on fungal biocontrol of larvae, studies agree that the adult stage is susceptible to several commercially available strains of EPFs, including the European native strain *M. brunneum* ART 212 (Behle & Goett 2016; Giroux et al. 2015; Graf et al. 2023; Lacey et al. 1994). Yet, to date, no effective biocontrol strategy against adult Japanese beetles using any EPFs is available for producers.

Recently, first leaf spraying tests with EPFs have been carried out in Italy to fight adult Japanese beetles (Graf et al. 2023). Even though recent formulation technologies can partially protect EPFs from environmental inactivation (Quesada-Moraga et al. 2023), foliar spray applications still face a number of environmental challenges (Braga et al. 2001; Fernández-Bravo et al. 2017; Jaronski 2010). Therefore, it is suggested to investigate alternative spore dissemination strategies, such as autodissemination (also called "attract-and-infest") with protected spore containers (Benvenuti et al. 2019; Graf et al. 2023; Klein & Lacey 1999).

Autodissemination is a pest management concept that uses inoculated insects as vectors. These insects carry lethal concentrations of an inoculum to their conspecifics via, e.g., mating, oviposition or aggregation (Gaugler et al. 2012). Currently, autodissemination is widely investigated as an opportunity to transfer chemical growth inhibitors to oviposition sites of mosquitoes (Gaugler et al. 2012; Thammavong et al. 2022; Unlu et al. 2017; Wang et al. 2014). However, the concept has also shown promising results in disseminating EPFs in several pest insect populations, e.g., *Ips typographus* (L.), *Ceratitis capitata* (Wiedemann), *Blatella germanica* (L.), *Plutella xylostella* (L.), *Pachnoda interrupta* (Olivier), *Delia radicum* (L.) or *Drosophila suzukii* (Mazumura) (Dowd & Vega 2003; Furlong & Pell 2001; Getahun et al. 2016; Kreutz et al. 2004; Meadow et al. 2000; Quesada-Moraga et al. 2004, 2008; Yousef et al. 2018).

For the control of Japanese beetles, Klein and Lacey (1999) designed a first prototype of an autodissemination trap. Such traps need to attract the insect, inoculate it and enable the dissemination of the inoculum to the target habitat in order to ensure successful control (Gaugler et al. 2012). As inoculum, Klein and Lacey (1999) placed barley kernels colonized by the EPF *M. anisopliae* (Metschnikoff) into the trap. They demonstrated the presence of the fungal inoculum on adult beetles directly after passing through the trap. Additionally, they confirmed an earlier observed increased mortality after fungal treatment (Lacey et al. 1995). However, to determine the potential of autodissemination to control a pest insect population, it is fundamental to know whether EPFs are efficiently transmitted from inoculated to non-inoculated beetles (Furlong & Pell 2001). This was partly studied by Benvenuti et al. (2019) who found that beetles contaminated with *M. brunneum* Ma 43 transmit conidia to their coupling partners under laboratory conditions.

Thus, horizontal transmission of an EPF among Japanese beetle adults has only been observed among opposite-sex couples in the laboratory. Moreover, the time span for which fungus-inoculated donor beetles remain infective, i.e., can transfer lethal conidial doses to recipient beetles, is unknown. Furthermore, we lack information on whether

inoculated adults can be used as vectors to transport the inoculum to their oviposition sites. If so, it is unknown whether the subsequent presence of the fungal inoculum in the soil leads to an infestation of neonate larvae.

To address these gaps, we first investigated the mortality response of adult Japanese beetles to different doses of *M. brunneum* ART 212. In the same set of laboratory experiments, we studied mortality rates after horizontal transmission of conidia from donor to recipient beetles in response to the time span between donor beetle inoculation and contact with recipients. Finally, we verified horizontal transmission among adults in a semi-field study.

In another set of laboratory experiments, we first studied the early larval survival in substrates containing different concentrations of *M. brunneum* ART 212. Next, we determined the conidial dose that can be carried to the oviposition substrate by inoculated female vectors and assessed early larval survival in this vector-inoculated substrate. In the formerly mentioned semi-field study, we then investigated whether female vectors could carry conidia into the soil during oviposition. Finally, we assessed larval survival at these vector-inoculated oviposition sites.

Material and methods

Organisms

Japanese beetles

For laboratory experiments, third instar larvae of *P. japonica* were dug out of the soil at field sites in the infested zone in Piedmont, Italy. For experiments with adults, these larvae were fed with carrots weekly until they developed into adults. For more details, see Supplementals 1.1.1. Adults were sexed according to differences in their fore tibia (Fleming 1972). For mating, couples were formed and kept together for five days in round plastic cups containing blackberry leaves as a food source and a moist filter paper.

M. brunneum ART 212

In Switzerland, *M. brunneum* ART 212 (Ma 714, ARSEF 7524) is a commercially available strain originally isolated from *Agriotes* sp. L. (Coleoptera: Elateridae) at Agroscope (Switzerland). Infectivity was ensured by passaging the fungal inoculum through adult Japanese beetles and subsequent spore isolation from mycosed cadavers on selective medium plates (SM, Sabouraud 2% glucose agar (SDA) supplemented with cycloheximide (0.05 g/l), streptomycin sulfate (0.6 g/l), tetracycline (0.05 g/l) and dodine (50 mg/l); Strasser et al. 1996) as previously described (Graf et al. 2023). After incubation for 14–16 days at 22 °C and 70% RH in darkness, these F2 generation plates were stored at 5 °C.

To prepare conidial suspensions, F3 generation SM plates of *M. brunneum* ART 212 were freshly prepared from F2 plates. After incubation of 14–16 days (dark, 22 °C, 70% RH), conidia were washed off from plates using 0.01% Tween 80.

Conidia were counted with a hemocytometer, and the concentration of the suspension was set to $10^7 \pm 0.1$ conidia/ml by diluting with 0.01% Tween 80. The suspension was then further diluted to achieve the desired concentration.

Germination rates of the F3 conidia were determined by adjusting the suspension to 1×10^6 conidia/ml after counting with a hemocytometer and applying three 50-µl drops on complete medium plates (CM: 10 g glucose, 0.36 g KH_2PO_4, 1.78 g Na_2HPO_4, 1 g KCl, 0.6 g $MgSO_4.7H_2O$, 0.6 g NH_4NO_3, 5 g yeast extract, 20 g agar per 1 L distilled water; Riba & Ravelojoana 1984). After a 24-h incubation at 22 °C and 70% RH (darkness), the percentage of germinated conidia per drop was determined at 400 × magnification. Germination was validated based on the length of conidial germ tubes (Reinbacher et al. 2021a). Germination rates in all suspensions used were above 90%.

To prepare conidia-overgrown wheat bran, liquid medium cultures containing blastospores were produced. Wheat bran was inoculated with these cultures in 9-cm-diameter Petri dishes and incubated for two weeks (22 °C, 70% RH), in order to grow a conidia layer in the plates used to inoculate beetles. For more details on the liquid medium cultures, see Supplementals 1.1.2. Control plates were prepared using 20 ml of sterile water instead of the liquid medium culture.

To estimate *Metarhizium* spp. colony-forming units (CFUs) per g substrate or soil sample, subsamples were transferred to 200-ml Erlenmeyer flasks and suspended in distilled water containing 1.8 g/L tetra-sodiumpyrophosphate ($Na_4P_2O_7$) to prevent clumping as in Mayerhofer et al. (2017). Flasks were shaken as described in Kessler et al. (2003), and 100 µl of the supernatant was plated on SM. After incubation for two weeks (22 °C, 70% RH, dark), *Metarhizium* spp. colonies were counted on each plate and CFUs/g dry weight were calculated. To do so, the water content of each sample was measured gravimetrically.

To determine number of *Metarhizium* CFUs on beetles, beetles were transferred to Eppendorf tubes containing 1 ml of 0.1% Tween 80 directly after inoculation. To not miss already adhering conidia, Tween 80 was more concentrated than when preparing conidial suspensions for dip inoculation. The tubes were vortexed for 1 min to wash off conidia, and 100 µl of the supernatant was plated on SM plates. Plates were incubated (22 °C, 70% RH, dark) for 2 weeks. The number of colony-forming units (CFUs) counted on these plates was used as an estimate for the number of conidia washed off the beetles' surface.

Laboratory and semi-field assays

All laboratory assays were conducted under quarantine conditions in a climate-controlled room (22 °C, 60% RH, day–night cycle 16:8 h).

Response of Japanese beetle adults to different doses of *M. brunneum* ART 212

To determine the effect of different doses of *M. brunneum* ART 212 on adult survival, a series of five suspensions with concentrations of 10^3 to 10^7 conidia/ml was prepared as described (2.1.2). A solution of 0.01% Tween 80 served as the control. Into each of the six suspensions, five male and five female adults were dipped twice for two seconds. The conidial suspension was thoroughly mixed before each dip. After dip inoculation, beetles were individually transferred to round plastic cups (4.5 cm diameter × 6 cm height) with a moist filter paper and a blackberry leaf as food source. Mortality was monitored over the next four weeks.

To determine the actual number of CFUs ending up on the beetles' surface when dipping in the suspensions, three additionally dipped adults per concentration were transferred to Eppendorf tubes containing 1 ml of 0.1% Tween 80. The number of CFUs was determined as described in 2.1.2. Three independent experiments were performed.

Horizontal transmission of *M. brunneum* ART 212 among Japanese beetle adults

To study the horizontal transmission of *M. brunneum* ART 212, 48 square boxes (13 × 10.5 × 6.5 cm) each containing either one non-inoculated female or male recipient beetle were prepared for fungal and control treatments. In addition to the recipient beetle, the boxes contained a moist filter paper and a blackberry leaf.

On coupling day 0, the pronotums of 72 donor beetles of each sex were marked with Tipp-Ex (correction fluid, Lyreco, Marly, France) to be able to distinguish them from recipient beetles in same-sex couples. Thereafter, donors were inoculated with conidia (Fig. 1a) by allowing them to walk for 1 min on a Petri dish containing fungus-overgrown wheat bran (2.1.2).

A first batch of 24 female and 24 male fungus-inoculated donor beetles was transferred to the prepared boxes with recipient beetles to form 12 couples of each sex combination (m:f, f:m, f:f, m:m; Fig. 1b, coupling day 0). The remaining 48 inoculated donor beetles were transferred to round plastic cups (4.5 cm diameter × 6 cm height) with a moist filter paper and a blackberry leaf as a food source and stored for coupling on day 1 and day 2. On days 1 and 2 after inoculation of the donors (Fig. 1b, coupling days 1 and 2), the coupling was repeated exactly as on coupling day 0, using the inoculated donors that had been stored since day 0.

On each coupling day, eight donor and recipient beetles were used to determine the number of conidia on beetles' surfaces after coupling for 1 h (Fig. 1c1) as described in 2.1.2. The 40 remaining recipient beetles and 40 donor beetles were removed from the coupling boxes after 1 h, isolated singly in plastic cups with filter papers and blackberry leaves, and mortality was observed over the next four weeks (Fig. 1c2). All steps (Fig. 1a, b, c1, c2) were performed with control beetles, too. The experiment was performed three times over time.

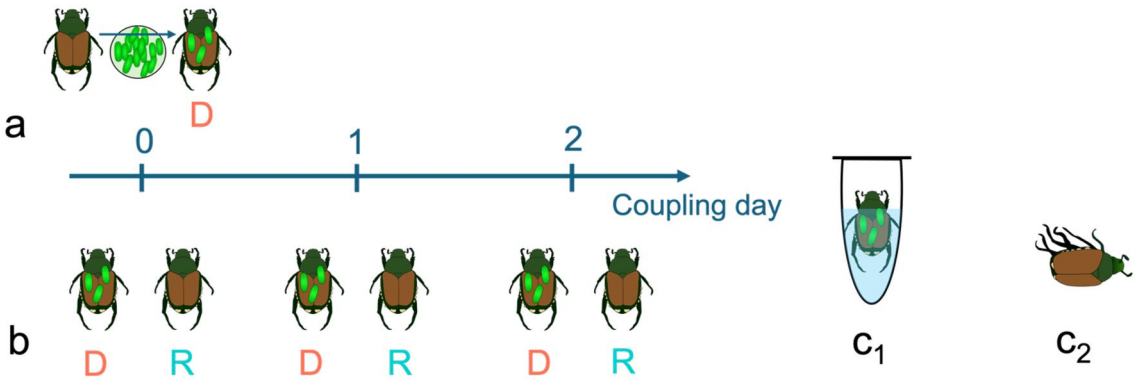

Fig. 1 Schematic representation of the experimental procedure to determine how long conidia of *Metarhizium brunneum* ART 212 can be transmitted horizontally among adult Japanese beetles, providing a control effect. Donor beetles (D) were inoculated with conidia **a** and coupled **b** with recipient beetles (R) on either the same day, one day after inoculation or two days after inoculation. The amount of conidia was determined on one set of donors and recipients $\mathbf{c_1}$. Mortality of another set of donors and recipients was observed over four weeks $\mathbf{c_2}$. Affinity Designer was used to create the schematic representation

Semi-field cage experiment: occurrence of horizontal transmission

Thirty pyramidal semi-field cages (250 cm^2 floor area × 50 cm height) were placed on a lawn next to an irrigated football field in Genestrerio (Ticino, Switzerland, 45°51′28.2" N 8°58′10.8"E) in the first week of July 2022. The occurrence of horizontal transmission was tested in ten of these cages (Fig. 3, Treatment 50%). The remaining twenty cages were used to test the transfer of fungal inoculum into the soil and the following survival of larvae (Fig. 3, Treatment 0% and 100%). Treatments were randomly assigned to cages to avoid position effects.

Twenty-five males and 25 females, collected in a vineyard in Genestrerio, were assigned to each of the ten semi-field cages designated to test horizontal transmission. Only the females were fungus-inoculated as described in 2.2.2 before all 50 beetles were set free inside the cages.

A shading net (Accura, Germany) was installed on top of the cages to prevent extreme temperatures inside. After one week, five males were collected from each of the ten cages, to check whether conidia had been horizontally transmitted from females to males. These males were transferred to cups halfway filled with moist peat to determine mycosis in the laboratory.

Survival of neonate *P. japonica* larvae at different doses of *M. brunneum* ART 212 in the substrate

Ten square oviposition boxes (13 × 10.5 × 6.5 cm) were filled with 100 g of oviposition substrate, consisting of a 2:1 mixture of peat and field soil. The components were sieved separately using a 2-mm sieve and dried at 100 °C for 24 h. After drying, components were thoroughly mixed. Two boxes each were inoculated with *M. brunneum* ART 212 suspensions to achieve the following five concentrations: 0 (control), 1.11×10^2, 1.11×10^3, 1.11×10^4 and 1.11×10^5 conidia/g substrate. The maximum concentration was based on the recommended field application rate (10^{14} conidia/ha), approximated for small-scale use by Reinbacher et al. (2021b). Gravimetric water content (GWC) in the boxes was set to 60%.

After inoculating the oviposition substrate, three to four mated females were transferred into each square box. One day later, blackberry leaves were provided to the females as feed.

Five days after starting oviposition, the eggs in the oviposition boxes were counted, and from boxes of each conidial concentration, portions of three eggs were transferred into ten round plastic cups (4.5 cm diameter × 6 cm height). Cups contained 20 g of substrate that was previously inoculated with conidial suspension to achieve the same conidial concentrations and GWCs in the substrate as in the oviposition boxes from which the eggs were collected. The eggs were placed into holes prepared with the tip of a knitting needle (adapted from George et al. (2007)). Fewer than ten cups were prepared in cases where fewer than 30 eggs were available. Germinated grass seeds (*Festuca rubra* L., *Lolium perenne* L.) were provided in the cups as feed for hatching larvae. Cups were randomly placed into boxes to avoid box effects. Moisture content of the substrate was monitored in three additional cups, also set to 60% GWC and integrated into the setup, but not containing eggs. If these "moisture control" cups indicated desiccation, water was added to all cups of the experiment. 24 days after transferring the eggs, hatched first instar larvae were counted in each cup.

After counting the larvae, substrate subsamples (5–6 g) were taken from each cup to check for the abundance of viable conidia or their absence (control), respectively. These subsamples were stored at 5 °C room temperature until they were suspended in 20 ml distilled water containing 1.8 g/L $Na_4P_2O_7$. They were further processed to determine CFUs/g dry substrate as described in 2.1.2. In total, four replicate experiments were performed over time.

Transfer of fungal inoculum into the substrate by female *P. japonica* vectors and subsequent survival of larvae in this substrate

To determine the amount of conidia that fungus-inoculated females transport to artificial oviposition sites (cups) and the survival of first and second instar larvae in these cups, mated female Japanese beetles were inoculated as described in 2.2.2.

On day 0 (Fig. 2, day 0a), the initial number of *M. brunneum* ART 212 conidia on five females per treatment (fungus/control) was measured as described (2.1.2). Another 20 either fungus-inoculated or control females were individually transferred to round plastic cups (4.5 cm diameter × 6 cm height), halfway filled with oviposition substrate (Fig. 2, day 0b). GWC of the substrate had been set to 60%, and a sample had been taken to validate the absence of *Metarhizium* spp. CFUs. After one day, blackberry leaves were provided to the females. On day 5, germinated grass seeds (*F. rubra*, *L. perenne*) were provided to all cups as food for the neonate larvae and, where needed, blackberry leaves for the females were renewed (Fig. 2, day 5).

After 29 days (Fig. 2, day 29), hatched first instar larvae (L1) were counted in ten randomly chosen cups of the fungal and control treatment. After counting the larvae, *Metarhizium* spp. CFUs per g substrate were determined in three experimental repetitions. On day 41 (Fig. 2, day 41), number of second instar larvae (L2) and *Metarhizium* spp. CFUs per g substrate were determined in the remaining ten cups. The numbers of L1 and L2 larvae were determined in two experimental repetitions.

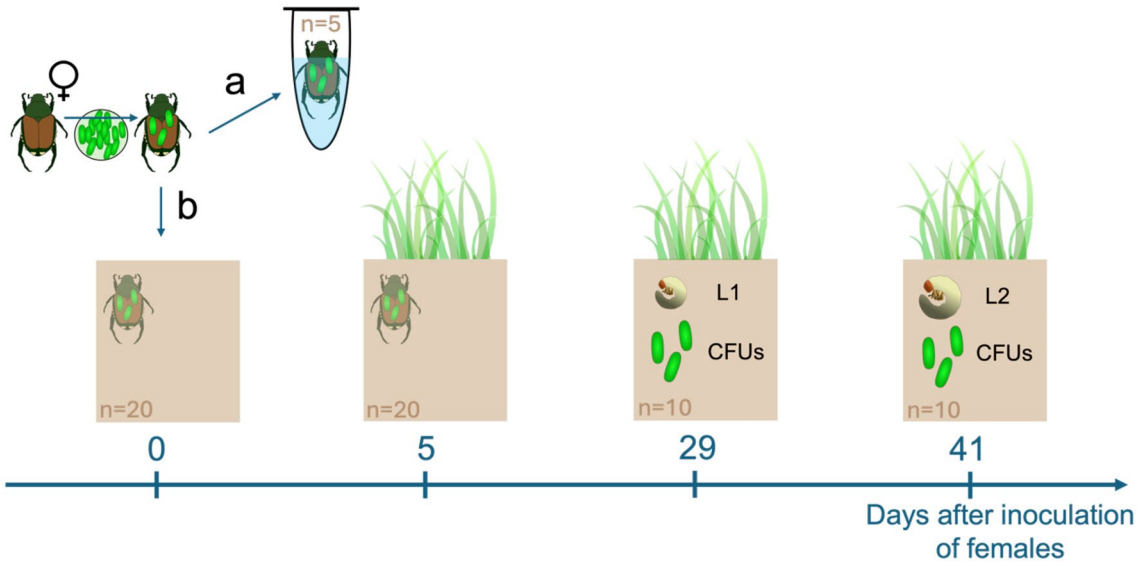

Fig. 2 Schematic representation of the experimental procedure to determine the amount of *Metarhizium brunneum* ART 212 that ovipositing fungus-inoculated females carry into the substrate and the subsequent survival of larvae in this substrate. Females were inoculated with conidia on day 0, and the number of conidia was determined on five beetles per treatment (day 0a). The remaining 20 inoculated females were transferred to cups (day 0b). On day 5, germinated grass seeds were provided to the cups. On day 29, the number of first instar larvae (L1) and *Metarhizium* spp. colony-forming units (CFUs) were determined in ten cups. On day 41, the number of second instar larvae (L2) and *Metarhizium* spp. CFUs were determined in the remaining ten cups. Affinity Designer was used to create the schematic representation

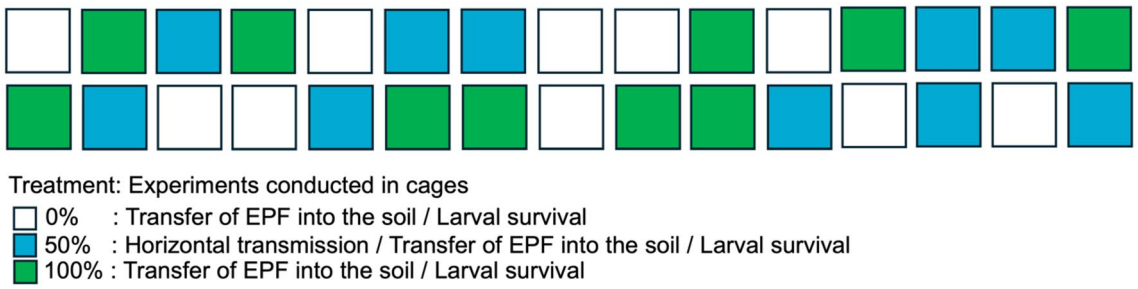

Fig. 3 Schematic representation showing 30 semi-field cages with three randomly assigned treatments. All 30 cages (Treatment 0%, 50%, 100%) were used to study the transfer of the entomopathogenic fungus (EPF) *Metarhizium brunneum* ART 212 into the soil by Japanese beetle vectors and the subsequent survival of larvae in the soil. Additionally, ten of the cages (Treatment 50%) were used to verify horizontal transmission of the EPF among adults

Semi-field cage experiment: transfer of fungal inoculum into the soil by adult vectors and subsequent survival of larvae

To investigate the transfer of fungal inoculum into the soil and the subsequent survival of larvae, thirty pyramidal semi-field cages were set up as described in 2.2.3 (Fig. 3). Before starting the experiment, three soil samples, each consisting of two pooled soil plugs (6 cm diameter × 10 cm depth), were taken per cage. Additionally, the lawn in the cages was watered with 3 L of water from a nearby water source to provide optimum conditions for egg deposition. 50 beetles, collected in a vineyard in Genestrerio, were assigned to each semi-field cage. In ten cages, 100% of the beetles were inoculated with *M. brunneum* ART 212 (Fig. 3, Treatment 100%) as described (2.2.2). In another ten cages, only 50% of the beetles were inoculated (see 2.2.3, Fig. 3, Treatment 50%). The remaining ten cages were used as a control (beetles without fungus inoculation; Fig. 3, Treatment 0%) to account for the fungus' natural soil cycle.

To determine the number of conidia on the beetles' surface directly after inoculation, conidia were washed off two additional beetles per treatment (2.1.2). Another two beetles per cage were inoculated and directly transferred to cups (4.5 cm diameter × 6 cm height) with moist peat to observe survival in the laboratory over five weeks.

Until mid-September, the lawn with the cages was irrigated three times a week. Weekly, fresh blackberry branches were put into the cages as feed. Four weeks after starting the experiment, three pooled soil samples were taken per cage.

In October, cages were removed, and the topsoil beneath the cages was examined for Japanese beetle larvae. In addition, another three pooled soil samples were taken per cage. All soil samples were stored in plastic bags at 5 °C room temperature until processing. From each sample, 20–24 g soil were suspended in 100 ml distilled water containing 1.8 g/L $Na_4P_2O_7$. Samples were further processed as described (2.1.2). Median CFUs/g soil dry weight was calculated for each cage based on these three pooled soil samples.

Genetic identification of *M. brunneum* ART 212

We selected fungal isolates from mycosed beetles of each laboratory experiment and of all mycosed recipient beetles from the semi-field cages to genetically confirm their identity as *M. brunneum* ART 212. For more details, see Supplementals 1.2. Identity of all fungal isolates from laboratory experiments was confirmed as *M. brunneum* ART 212. None of the control beetles showed fungal outgrowth.

Statistics

All analyses were performed using R (version 4.1.2). *P*-values were considered significant at $P < 0.05$. Whenever needed, data were transformed to meet the model assumptions (details in Sup. 1.3). Based on our experimental design, we corrected for the variability from the independent experimental repetitions by including them as random effect in mixed models. Emmeans (package emmeans, version 1.10.0) was used for post hoc pairwise comparisons. For details on the statistical analyses performed, see Supplementals 1.3 and Sup. Table 1.

Beetle survival was analyzed with mixed-effects Cox's proportional hazards models, followed by pairwise comparisons (package survival, version 3.5–5; package coxme, version 2.2–18.1). Median lethal time (LT50) was calculated using survminer (version 0.4.9). The proportional hazards assumption was validated based on Schoenfeld residuals, and if violated (coupling day 2), an accelerated failure time model with Weibull distribution was applied. Effects of couple type (same sex and opposite sex) and coupling day on number of conidia on beetles' surfaces were analyzed using linear mixed-effects models. Data were cube-root-transformed to meet the model assumptions.

Larval count data from the dose response experiment were analyzed using a Poisson mixed-effects regression model (package lme4, version 1.1–33). In the experiment where the substrate was inoculated by female vectors, overdispersion was present in the count data for the number of L1 and L2 larvae. Therefore, negative binomial regression (package glmmTMB, version 1.1.7) was used. To investigate differences in CFUs found per gram soil dry weight, linear mixed-effects models were fit. Data were log-transformed to meet the model assumptions.

For the semi-field experiment, numbers of larvae/m^2 in the differently treated semi-field cages were compared with an analysis of variance (ANOVA) and Tukey HSD post hoc testing. Data were square-root-transformed to meet the model assumptions. To investigate differences in CFUs found per gram soil dry weight, a linear model was fit. Data were log-transformed to meet the model assumptions.

Results

Response of Japanese beetle adults to different doses of *M. brunneum* ART 212

The time until death of Japanese beetle adults after dip inoculation in conidial suspensions of *M. brunneum* ART 212 was dose dependent. The higher the concentration, the shorter the time until 50% of the beetles died (LT50). More than 50% of the beetles dipped in suspensions containing fewer than 10^5 conidia/ml survived the observation period, but those dipped in suspension containing 10^5, 10^6 or 10^7 conidia/ml showed a LT50 of 23.5, 20.5 and 13 days, respectively.

Significantly reduced survival times were observed after dipping in suspensions with concentrations above 10^4 conidia/ml (Fig. 4). To determine the number of conidia on beetles' surfaces needed to achieve significant control effects, conidia were washed off beetles' surfaces. With an increasing concentration of the spore suspensions, there was a corresponding increase in the number of conidia found on the beetles' surfaces. Dipping beetles into suspensions with 10^3 or 10^4 spores/ml resulted in < 50 CFUs on their surfaces. The same procedure carried out with spore suspensions of 10^5, 10^6 or 10^7 spores/ml increased the number of CFUs on the beetles' surface to $6.3 \pm 5.1 \times 10^2$, $3.7 \pm 3.2 \times 10^3$ and $3.0 \pm 1.7 \times 10^4$ *Metarhizium* CFUs per beetle.

Horizontal transmission of *M. brunneum* ART 212 among Japanese beetle adults in the laboratory

Fungus-inoculated Japanese beetle donors and recipients of all coupling days (Fig. 5a coupling day 0; Fig. 5b coupling day 1; Fig. 5c coupling day 2) died significantly faster than the control groups (pairwise comparison, Sup. Table 2). Within the first seven days after coupling, 92.5–100% of all fungus-inoculated donor beetles died. Death of their coupling partners (recipients) occurred significantly slower (Fig. 5). The earlier recipients were

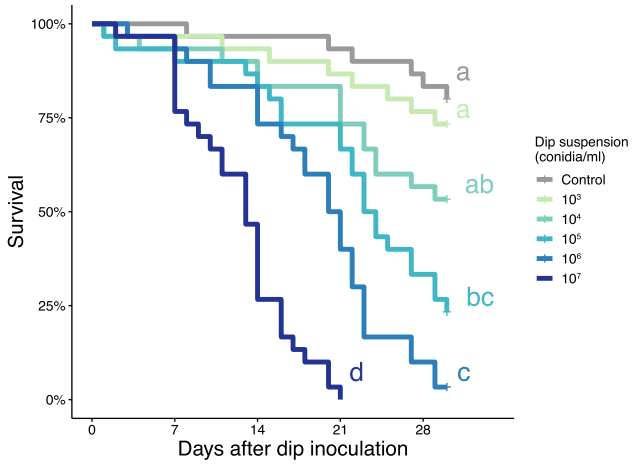

Fig. 4 Survival of Japanese beetle adults over time after dip inoculation in suspensions containing different concentrations of *Metarhizium brunneum* ART 212 conidia dissolved in 0.01% Tween. Survival was recorded over 30 days. The presented results derive from pooled data of three independent experiments with 10 beetles per concentration and experiment. Curves indicate the survival of beetles after dip inoculation. Curves with the same letter do not significantly differ. *P*-values were considered to be significant at $P < 0.05$

coupled with donor beetles, the earlier they died. In total, 70–95% of the recipients from coupling day 0 died within the first fourteen days after coupling. About the same degree of mortality was reached one week later for recipients from coupling day 1 and two weeks later for those from coupling day 2.

On each coupling day, fungus-inoculated donor beetles successfully transferred conidia to their coupling partners. This transfer was measured by washing conidia off the surfaces of beetles. The couple type (same sex and opposite sex) did not have a significant impact on the number of CFUs transferred to recipients ($p = 0.08$). However, the later the beetles were coupled, the fewer CFUs were found on both donors' and recipients' surfaces (Table 1). But, regardless of the coupling day, significantly more *Metarhizium* spp. CFUs were found on donor beetles in comparison to recipients (emmeans pairwise comparison, $p < 0.001$). On donor and recipient beetles of the control groups, no *Metarhizium* spp. CFUs were found.

Semi-field cage experiment: occurrence of horizontal transmission

Occurrence of horizontal transmission was tested in ten semi-field cages. In these ten cages, where only female donors were inoculated with *M. brunneum* ART 212, a mean of 26% of the male recipient beetles mycosed (min. 0% to max. 80% of males per cage sporulating with *M. brunneum* ART 212). Fungal isolates were genetically confirmed as *M. brunneum* ART 212.

Survival of neonate *P. japonica* larvae at different doses of *M. brunneum* ART 212 in the substrate

The number of living first instar larvae of *P. japonica* found in cups clearly depended on the dose of *M. brunneum* ART 212 conidia in the substrate (Fig. 6). The number of living larvae in cups containing the highest concentration of conidia (1.11×10^5 conidia/g substrate) was significantly lower than in the control cups ($z = -3.509$, $p = 0.0041$).

The abundance of *Metarhizium* spp. CFUs in substrate samples taken from cups after evaluation of the larval survival was consistent with the number of conidia that had been applied to the cups in the beginning of the experiment (Sup. Figure 1).

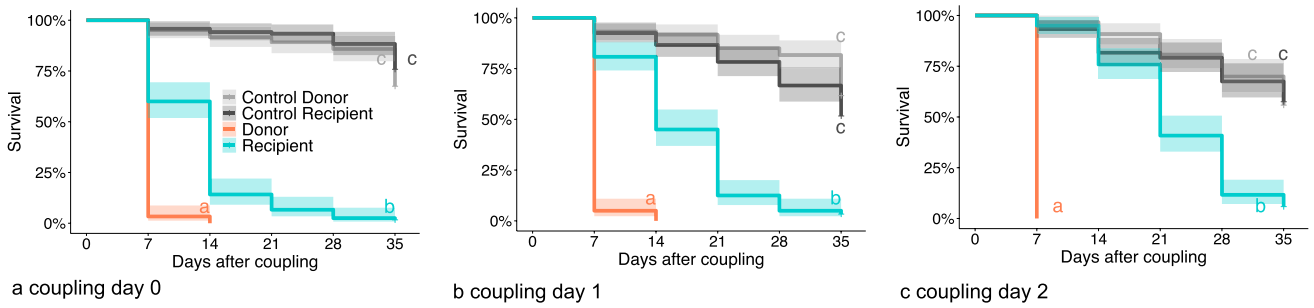

Fig. 5 Survival of Japanese beetle donors and recipients coupled to test horizontal transmission of *Metarhizium brunneum* ART 212. All donor beetles were inoculated with conidia of *M. brunneum* ART 212 on coupling day 0. One set of donor beetles was coupled for one hour with recipient beetles on coupling day 0 **a**, while two other sets of inoculated donor beetles were coupled for one hour on day one (**b**, coupling day 1) or on day two (**c**, coupling day 2) after inoculation. The survival curves derive from pooled data of three independent experiments with 40 beetles per treatment, coupling day and experiment. Shadows indicate the 95% confidence intervals. Different letters to the right of the survival curves indicate significant differences between beetle groups among pooled data. P-values were considered to be significant at $P < 0.05$

Table 1 Colony-forming units (CFUs) of *Metarhizium* spp. washed off from Japanese beetle donors and recipients right after coupling for one hour on coupling day 0, coupling day 1 or coupling day 2 of a horizontal transmission experiment. All donor beetles were inoculated with conidia on coupling day 0. One set of donor beetles was coupled for one hour with recipient beetles on coupling day 0, while two other sets of inoculated donor beetles were coupled for one hour on day one (coupling day 1) or two (coupling day 2) after inoculation with the fungus. The table shows pooled data of three individual experiments, with eight donor and eight recipient beetles per coupling day and experiment. Different letters indicate statistically significant differences between CFUs washed off from either donors or recipients on different coupling days. Differences were determined with pairwise comparison (package emmeans) after running a linear mixed model for donors and recipients individually. *P*-values were considered to be significant at $P < 0.05$

Coupling day	CFUs washed off from donor beetles (mean ± SD)		CFUs washed off from recipient beetles (mean ± SD)	
Coupling day 0	$10.7 \pm 8.1 \times 10^5$	a	$8.0 \pm 8.5 \times 10^4$	a
Coupling day 1	$1.9 \pm 1.7 \times 10^5$	b	$1.4 \pm 2.1 \times 10^4$	b
Coupling day 2	$0.88 \pm 1.2 \times 10^5$	c	$3.9 \pm 3.8 \times 10^3$	b

Transfer of fungal inoculum into the substrate by female *P. japonica* vectors and subsequent survival of larvae in this substrate

At counting time point L1, the number of living larvae did not significantly differ between the control and fungus treatment (Fig. 7, counting timepoint L1). Mean numbers of 3.25 ± 4.12 larvae (control) and 2.9 ± 3.63 larvae (*M. brunneum* ART 212) were found in the cups (pooled data). However, at the second evaluation (counting timepoint L2), significantly more larvae (mean = 1.55 ± 3.32) were found in the control cups compared to cups with fungus-inoculated females (pooled data: mean = 0.1 ± 0.31, z-value = 2.766, *p*-value = 0.00568).

Females carried a mean of $1.55 \pm 1.06 \times 10^6$ CFUs/g dry substrate ($= 3.1 \times 10^7$ CFUs per cup) into the cups. The abundance of *Metarhizium* spp. CFUs in the substrate did not differ based on counting time point (L1, L2; t-ratio = −0.262, $p = 0.7945$). In control cups, no *Metarhizium* spp. CFUs were detected.

Metarhizium spp. CFUs determined on the surface of freshly inoculated *P. japonica* females ranged from 1×10^6 to 2.28×10^7 per female. This was thus comparable to the number of CFUs that they carried into the cups (one female per cup).

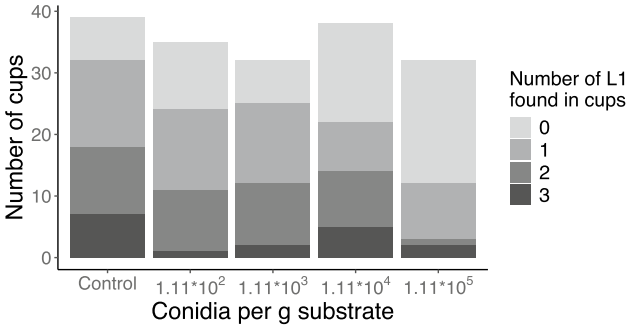

Fig. 6 Number of cups (3.5 cm diameter × 6 cm height) containing 0, 1, 2 or 3 living first instar (L1) Japanese beetle larvae that developed from three eggs. The cups contained 20 g of oviposition substrate (2:1 mixture of peat and field soil) that was treated with conidial suspensions to achieve different doses of *Metarhizium brunneum* ART 212 conidia per g substrate and a gravimetric water content of 60%. The graph shows pooled data from four individual experimental repetitions

Semi-field cage experiment: transfer of fungal inoculum into the soil by adult vectors and subsequent survival of larvae

Metarhizium spp. were naturally occurring in low numbers in soils of semi-field cages before the fungal treatment in July 2022 (mean = 5.2×10^2 CFUs/g soil).

Fungal inoculation of Japanese beetle adults significantly increased the soil abundance of *Metarhizium* spp. CFUs in the semi-field cages (Fig. 8a, 100 vs C t = 5.303, $p < 0.0001$; 50 vs C t = 4.767, $p < 0.0001$). The increase did not significantly differ between cages with 50% and 100% inoculated beetles (t = 0.537, $p = 0.8536$) or between the two sampling time points after treatment (t = −0.208, $p = 0.8364$). In October, a mean of $0.96 \pm 1.19 \times 10^4$ CFUs/g soil (Treatment 50) and $7.0 \pm 8.01 \times 10^3$ CFUs/g soil (Treatment 100) were detected in the soil.

Despite the significant increase in numbers of CFUs found in the soil, larval density in cages was not influenced by the treatment (Fig. 8b, F = 0.89, $p = 0.423$).

Fungus-inoculated adults that were taken back to the laboratory to determine survival time died within seven days, while control beetles gradually died within the observation period of 35 days (Sup. Table 3). Directly after inoculation, 8.8×10^3–6×10^5 *Metarhizium* spp. CFUs (mean 2.1×10^5 CFUs) were determined on fungus-inoculated beetles (n = 10).

Discussion

The Japanese beetle is a highly polyphagous pest insect threatening crop and non-crop hosts in regions where it is not native. Eradication as well as containment measures

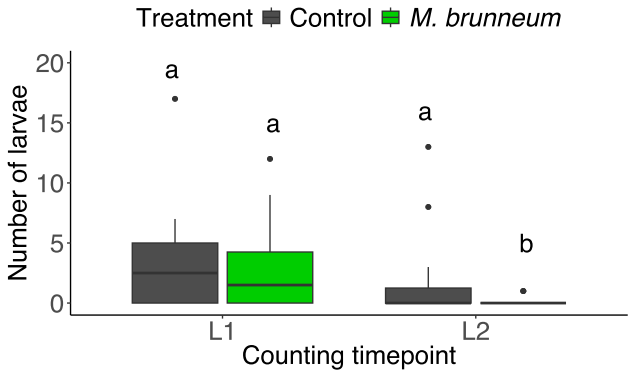

Fig. 7 Number of living Japanese beetle larvae that were found 29 days (counting timepoint L1) and 41 days (counting timepoint L2) after the addition of ovipositing Japanese beetle females to cups. Females placed in cups to lay eggs had either been treated with conidia of *Metarhizium brunneum* ART 212 or not (control) and were used as vectors to transfer *M. brunneum* ART 212 into the substrate of the cups (20 g, 2:1 mixture of peat and field soil). Different letters above the boxplots indicate statistically significant differences in the number of larvae found per treatment at each counting time point. The graph shows pooled data from two independent experiments with 10 cups per treatment, counting time point and experiment

against this invasive pest rely mainly on the use of synthetic insecticides. Here, we investigated an environmentally friendly alternative to pesticide use. In this autodissemination approach, we tested the use of adult Japanese beetles as vectors to spread the European native EPF *M. brunneum* ART 212 in adult populations and to oviposition sites. While it only provided control of neonate larvae in the laboratory setup, we found significant control effects after horizontal transmission of the inoculum in adult populations under both laboratory and semi-field conditions.

Potential to control adults

Our laboratory experiments with adult *P. japonica* have clearly shown that conidia are readily transferred between beetles, leading to high mortality rates among both donors and recipients. These mortality rates can be attributed to the high susceptibility of adults against *M. brunneum* ART 212, as stated by Graf et al. (2023), and confirmed in our dose response experiment. Here, we found that already beetles carrying a mean of $6.3 \pm 5.1 \times 10^2$ conidia on their surface die significantly faster than the control.

Japanese beetles that had passed an autodissemination device in a previous study carried an average of $11.3 \pm 1.3 \times 10^7$ M. *anisopliae* conidia per beetle (Klein & Lacey 1999). Our data demonstrate that the presence of even lower amounts of *M. brunneum* ART 212 conidia on donors (e.g., coupling day $0 = 10.7 \pm 8.1 \times 10^5$) might be sufficient to transmit lethal doses to recipients for up to two days.

However, with more time having passed since inoculation, fewer conidia were available on donors' surfaces for transmission, which might be a result of spore adhesion (Vega et al. 2012) or post-contact responses such as grooming (Zhukovskaya et al. 2013). As a consequence, fewer conidia were transmitted to recipients, what led to comparably lower but still significant mortality rates among them. This aligns perfectly with the dose-dependent

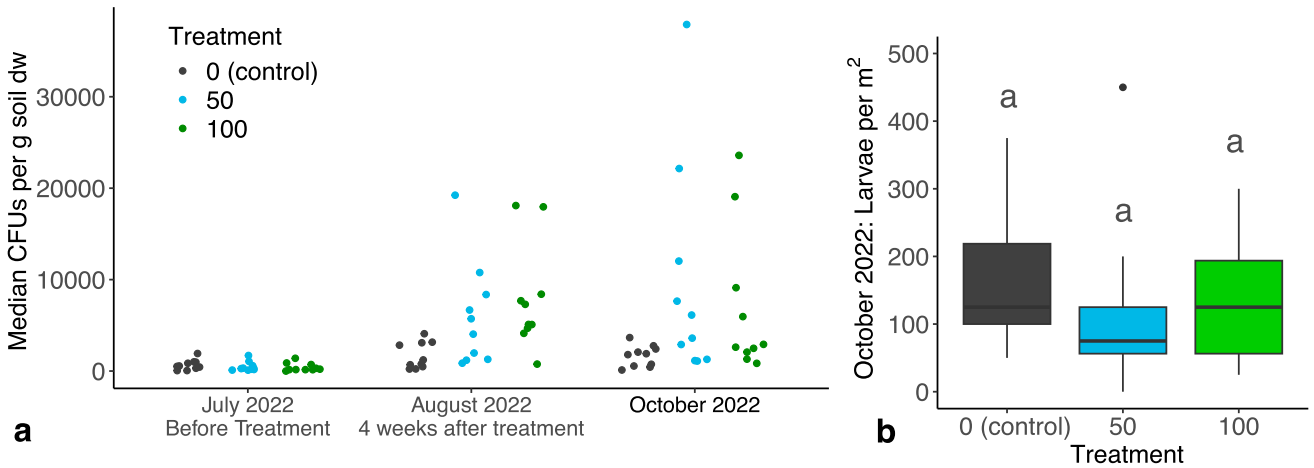

Fig. 8 **a** Colony-forming units (CFUs) of *Metarhizium* spp. per gram dry weight (dw), showing median values based on three soil samples for each semi-field cage (250 cm^2 floor area × 50 cm height, ten cages per treatment) in Genestrerio, Switzerland, and **b** number of Japanese beetle larvae found per m^2 in these semi-field cages in October. In ten cages each, either 100%, 50% or 0% (control) of the adult beetles were inoculated with conidia of *Metarhizium brunneum* ART 212 and were used as vectors to carry the inoculum into the soil. Soil samples were taken in July before treatment, four weeks after treatment (August) and in October of the same year. Larval density was determined in October. In **a** and **b**, values represent n = 10 semi-field cages for each treatment and sampling date. Different letters above the boxplots in **b** indicate statistically significant differences in number of larvae found per treatment in October

mortality effects that we determined in adults. It is further supported by the mortality effects observed in other insects after exposure to different doses of EPFs (Dogan et al. 2017; Shrestha et al. 2015).

Unlike previous research that only studied horizontal transmission in opposite-sex couples of Japanese beetles (Benvenuti et al. 2019), we also verified EPF transmission from inoculated to non-inoculated adults in same-sex couples. We assume that transmission of conidia within couples of the same sex is a result of the aggregation behavior of adult Japanese beetles (Fleming 1972; Potter & Held 2002). This behavior has been observed in other insects, too, and seems to generally enhance the occurrence of horizontal transmission in insect species (e.g., *I. typographus* or overwintering sap beetles (Dowd & Vega 2003; Kreutz et al. 2004)).

We were able to reproduce the successful laboratory results on horizontal transmission of EPFs among adults under semi-field conditions. After the release of inoculated donor females into field cages, we confirmed mycosis in 26% of the male recipients in these cages. To the best of our knowledge, this is the first study to report successful horizontal transmission of an EPF between adult *P. japonica* in a semi-field setup. These promising findings align with the positive results of others, indicating great potential to disseminate lethal doses of EPFs in adult pest insect populations (e.g., *C. capitata*, *B. germanica*, *P. xylostella*, *P. interrupta*, *D. radicum*, *D. suzukii* (Furlong & Pell 2001; Getahun et al. 2016; Meadow et al. 2000; Quesada-Moraga et al. 2004, 2008; Yousef et al. 2018)).

Limitations to control early instar larvae of Japanese beetles

Until today, only a few have tried to control early larval instars of the Japanese beetle (e.g., George et al. 2007; Power et al. 2009; Régnière et al. 1981). Some have even focused on controlling them by applying EPFs with preventive state-of-the-art application techniques (Behle et al. 2015; Graf et al. 2023). Nevertheless, autodissemination of EPFs to Japanese beetle oviposition sites has not been investigated before. However, studies with, e.g., *Oryctes rhinoceros* (L.) or *Rhynchophorus ferrugineus* (Olivier) suggested that adult insects can effectively disseminate EPFs to breeding habitats, thereby increasing larval mortality (Matveev et al. 2023; Moslim et al. 2011).

In our laboratory experiments with controlled moisture conditions, female vectors were indeed able to carry conidia into the substrate ($1.55 \pm 1.06 \times 10^6$ CFUs/g substrate). In this substrate, significant control effects were observed at the L2 stage. After direct substrate inoculation (1.11×10^5 conidia per g substrate), control effects were even significant to the 1st larval instar. The faster effect after direct inoculation might result from the presence of a nonionic surfactant in the conidial suspensions. This surfactant could have enhanced the insecticidal activity of the EPF by interacting with the hydrophobic insect cuticle (de Santos et al. 2012).

When upscaling the autodissemination experiments with female *P. japonica* to the semi-field level, we were able to significantly increase *Metarhizium* spp. abundance in the soil. The number of *Metarhizium* spp. CFUs found in soil samples beneath field cages reached levels around 10^4 CFUs/g soil which were comparable to those achieved by Graf et al. (2023) using a state-of-the-art application method with fungus colonized barley kernels (FCBKs). In their field study in Italy, they applied FCBKs with *M. brunneum* Ma 43 (BIPESCO5/F52) against *P. japonica* larvae preventively in May. Given the marginal control effects they observed, Graf et al. (2023) supposed that positive results could only be achieved in moist soils. Such intermediate soil moisture has been found to be favorable for EPF infections of *P. japonica* larvae (Krueger et al. 1991).

Similarly, despite the increase in *Metarhizium* spp. abundance, we could not determine any significant reduction of larval density in the soils of our semi-field cages, even though the lawn was irrigated regularly to ensure favorable soil moisture conditions. However, the conidial doses transported into the soils of the semi-field cages by female *P. japonica* were more than tenfold lower than those which achieved a significant control effect in our laboratory studies. The failure of our autodissemination experiment under semi-field conditions is therefore likely a matter of spore concentration. Additionally, we suppose that the concentration needed for successful control under semi-field and field conditions might even be higher than in the laboratory. Here, the complex interactions with abiotic factors (Jaronski 2007) and adverse effects of the soil microflora may further reduce efficacy of EPFs (Douglas Inglis et al. 1998).

In summary, we were not able to reproduce the encouraging findings from our laboratory experiments under semi-field conditions. It seems that ovipositing female vectors are not able to autodisseminate an adequate dose of conidia to their oviposition sites to provide control of neonate larvae. We therefore doubt that this approach makes sense for the control of *P. japonica* larvae in the soil, even more so since other biocontrol agents, such as entomopathogenic nematodes, have shown promising results for the control of larvae (Paoli et al. 2017; Torrini et al. 2020).

Future recommendations

Based on the results presented in this study, we argue that autodissemination can be a valuable concept to spread lethal doses of the European native *M. brunneum* ART 212 in adult populations of the invasive Japanese beetle. While it seems inefficient for providing control of neonate larvae outside the laboratory setup, it is a promising strategy in biological

control of the more susceptible adults. Inoculated donor beetles successfully transferred conidia to recipients for up to two days in both same-sex and opposite-sex couples, resulting in significantly increased mortality. By decimating the adults, we might control the stage that is mainly responsible for the spatial spread of the population. Thus, this could slow down further natural spread of the invasive insect.

As a next step, field trials studying horizontal transmission of EPFs in Japanese beetle populations are necessary to prove that the concept of autodissemination is applicable under realistic conditions and at a larger spatial scale. In addition, an autodissemination device should be designed which guarantees a continuous supply of freshly inoculated adults during the flight period of the pest. Nevertheless, the ongoing invasion process and the current lack of efficient control measures against adult Japanese beetles in agricultural and especially in non-agricultural areas underline the importance of our study and the need for further research into this versatile and environmentally friendly biocontrol concept.

Author contributions

MW, GG and MM conceived and designed research. MW, HN and EH collected laboratory and field data. MW analyzed data and wrote the first draft of the manuscript. All authors gave feedback on the manuscript and approved the final manuscript.

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Data availability The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Conflict of interest We declare that no competing interests exist.

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