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Pathogenicity of the fungus *Beauveria pseudobassiana* for *Popillia japonica* depends on the developmental stage of the insect

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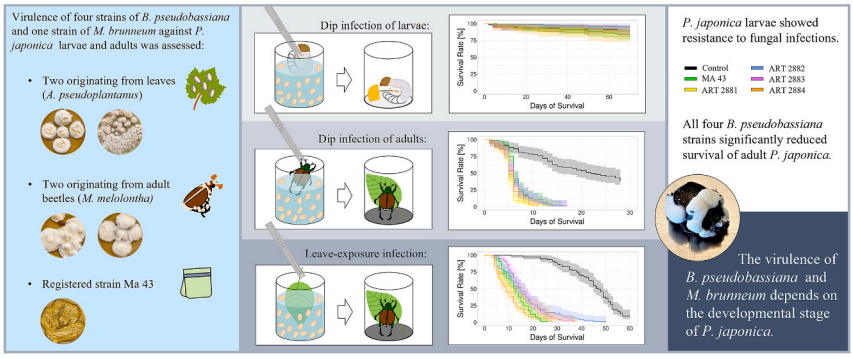
HIGHLIGHTS

- All the four tested *B. pseudobassiana* strains reduced adult *P. japonica* survival.
- Fungal strains did not reduce larval survival.
- Natural *B. bassiana* and *B. pseudobassiana* were obtained from field-collected beetles.
- Rearing beetles from larvae ensured absence of natural infections.

GRAPHICAL ABSTRACT



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ABSTRACT

Popillia japonica is an invasive, polyphagous beetle feeding on more than 400 host plants and responsible for major crop damage in infested regions in North America, northern Italy and southern Switzerland. Currently, control of *P. japonica* largely relies on synthetic insecticides. Recently, *Beauveria pseudobassiana* has been described as the dominant pathogen on *Melolontha melolontha* adults, a native scarabaeoid relative of *P. japonica*. *B. pseudobassiana* has been detected on several insect species, in soil but also on plant leaves indicating its adaptation to environmental conditions above ground.

We evaluated survival of *P. japonica* adults and larvae exposed to four strains of *B. pseudobassiana* (ART 2881, ART 2882, ART 2883, ART 2884) and a *M. brunneum* strain (Ma 43), registered as control agent against several Scarabaeidae species in Europe. All the fungal strains led to a significant three-to-five-fold reduction in the median survival of *P. japonica* adults, when dipped in conidia suspensions or exposed to fungus inoculated leaves, while ART 2884 was in both tests the most virulent strain. In contrast, none of the fungal isolates reduced larval survival, with mortality rates of 2–8 % after 70 days. From field-collected beetles, we obtained natural *Beauveria* isolates, some of which were identified as *B. pseudobassiana*, indicating a possible role of the fungus in natural infection scenarios. The high *in vitro* virulence of *B. pseudobassiana* together with its competence for above ground conditions and occurrence on *P. japonica* in the field indicates potential of this fungus as biological control agent (BCA) against adult *P. japonica*.

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1. Introduction

Popillia japonica Newman, 1841 (Coleoptera: Scarabaeidae) is native to Japan (Fleming, 1972) and has been inadvertently introduced to New Jersey, USA in 1916. From there, it extended its range to all the East Coast and the Midwest of the United States, up to some provinces of Canada (Borner et al., 2023; USDA, 2025). In Europe *P. japonica* was detected for the first time on the Azore islands in the 1970s and on mainland Europe in northern Italy, close to the international airport of Milan Malpensa International airport in 2014 (Nardi et al., 2024; Pavesi, 2014; Strangi et al., 2024). Both introductions have recently been demonstrated to originate from the USA most likely by unintended transport of the insect via air travel and goods (Nardi et al., 2024; Strangi et al., 2024). Since its introduction, the population in northern Italy has grown and expanded by approximately 5.5 to 13 km per year (Gotta et al., 2023), reaching southern Switzerland in 2017 (Poggi et al., 2023). In 2023, *P. japonica* has been reported for the first time north of the Alps where it has infested an area close to the international airport of Zurich (EPPO, 2023).

P. japonica is a polyphagous beetle, feeding on more than 400 host plants (Tayeh et al., 2023). The larvae of this beetle primarily feed on grass roots, leading to severe damage in agricultural grasslands, golf courses, and lawns (Potter & Held, 2002). Unlike many of its relatives of the family Scarabaeidae, such as *Melolontha melolontha* (Linnaeus, 1758) and *Amphimallon solstitiale* (Linnaeus, 1758), which cause most damages in their larval stage, *P. japonica* poses the greatest threat in the adult stage. Adult *P. japonica* feed on leaves, flowers, and fruits of many crops (Tayeh et al., 2023), including berries, maize, stone fruits or grapevine (Baker et al., 2019). *P. japonica* has been classified in 2019 as priority pest for agriculture, viticulture, and horticulture in the European Union (EU-Comission, 2019).

Control measures of *P. japonica* in the USA and Europe mainly rely on synthetic insecticides (Potter & Held, 2002; Santoiemma et al., 2021). However, due to environmental concerns, some of these products (Zuščíková et al., 2023) have been banned in the European Union (Anastassiadou et al., 2021) and therefore an urgent need for low-risk products and biological solutions to reduce and control *P. japonica* populations exists. Several biological control approaches have been tested in the USA and Europe during the past years (Gotta et al., 2023; Potter & Held, 2002). They include the use of bacteria such as *Paenibacillus popilliae, P. lentimorbus or Bacillus thuringiensis* (Potter & Held, 2002; Redmond et al., 2020), parasitoids such as *Tiphia vernalis* (Rogers & Potter, 2004), the microsporidium *Ovavesicula popilliae*, (Piombino et al., 2020) as well as parasitic nematodes of the genera *Steinernema* and *Heterorhabditis* (Simões et al., 1993). Furthermore, entomopathogenic fungi have shown great potential for *P. japonica* control (Barzanti et al., 2023; Behle et al., 2015; Benvenuti et al., 2019; Giroux et al., 2015; Graf et al., 2023; Lacey et al., 1995; Lacey et al., 1994). Species of the genera *Metarhizium* and *Beauveria* are of particular interest as these organisms are already successfully used for the control of larval stages of other scarab species like *M. melolontha* or *A. solstitiale* (EPPO, 2025).

Strains of *Metarhizium anisopliae*, *M. robertsii*, *M. brunneum*, *Beauveria bassiana*, and *B. brongniartii* have been tested in a number of studies against larval stages of *P. japonica.* However, results were inconsistent with some studies reporting high larval mortality (Barzanti et al., 2023; Behle et al., 2015; Giroux et al., 2015) while others found only small or no control effects (Graf et al., 2023; Ramoutar et al., 2010). The susceptibility of adult stages to fungal infection has been demonstrated using spray, dip or injection assays (Benvenuti et al., 2019; Graf et al., 2023; Lacey et al., 1994). Furthermore, experiments investigating horizontal transmission of the fungus among *P. japonica* adults under controlled conditions revealed high mortality suggesting potential for attract-infect-release strategies (Benvenuti et al., 2019; Wey et al., 2025). However, as for the control of larval stages, attempts to control adults with entomopathogenic fungi in the field so far have revealed inconsistent control of the pest (Graf et al., 2023; Lacey et al., 1995). Current research suggests that in general approaches targeting adult stages of *P. japonica* are more promising than approaches targeting larval stages when using entomopathogenic fungi as control agents (Graf et al., 2023). Environmental conditions above ground are challenging for fungal applications due to variable humidity and temperature or high UV- radiation, which may explain the inconsistent results obtained so far. Screening for species and strains that are adapted to above ground conditions and exhibit the required environmental competence is of major importance for achieving successful pest control (Quesada-Moraga et al., 2024).

In a recent study it was demonstrated that *B. pseudobassiana* is the dominant fungal pathogen of the adult stage of *M. melolontha*, a scarab species closely related to *P. japonica* and a pest in grassland and orchards in central Europe, (Pedrazzini et al., 2024). *B. pseudobassiana* has been recognized as a species rather recently (Rehner et al., 2011). It has a wide host range infecting species of different orders like Lepidoptera (Arias-Aravena et al., 2022), Hemiptera (Barrera-López et al., 2020) and in particular Coleoptera (Pedrazzini et al., 2024; Wang et al., 2020b). *B. pseudobassiana* has been detected throughout Europe and it has been isolated primarily from soil but recently it has also been reported in the phylloplane of several plant species (Garrido-Jurado et al., 2015; Howe et al., 2016; Mayerhofer et al., 2015a; Pedrazzini et al., 2025; Pedrazzini et al., 2024), which indicates adaptation to above ground conditions. There are no commercialized products based on *B. pseudobassiana* yet available. However, since this species infects scarab species like *M. melolontha* in Europe and is present in above ground habitats we hypothesize it may have the potential as a control agent against adults of *P. japonica*.

The aim of this study was to perform a series of laboratory experiments to assess the virulence of *B. pseudobassiana* to *P. japonica* adults and larvae. The specific goals were (1) to test the virulence of four *B. pseudobassiana* strains originally isolated from infected adults of *M. melolontha* or *Acer pseudoplantanus* leaves in a spore dip assay using the commercial strain *M. brunneum* Ma43 as a positive control. (2) to investigate whether *P. japonica* adults get infected by contact and/or feeding on leaves treated with the four fungal strains.

2. Material and methods

2.1. Popillia japonica adults and larvae

P. japonica adults were collected from grapevine leaves in a vineyard close to Landiona, Italy (45.5003, 8.4197) in July 2022. Beetles were transported to the laboratory in a plastic container filled with peat and blackberry boughs as feed and incubated at 24 °C and 70 % RH for one week to allow identification and selection of healthy and vivid beetles for the bioassay. Third instar larvae of *P. japonica* were collected from a maize field close to Mottalciata (45.525079, 8.205902), Italy in September 2022. Larvae were placed individually in 6 well plates containing moist peat, transported to the laboratory, fed with a carrot slice and maintained in quarantine at 10 °C for 7 weeks until further use. Three weeks before start of the virulence assay, the larvae were fed with fresh carrots and acclimatized at 24 °C and 70 % RH. To rear adult beetles, third instar larvae of *P. japonica* were collected from the locality in January 2023. After 3 weeks at 10 °C larvae were transferred individually to cylindrical polystyrol containers (∅ 47 mm h: 64 mm) containing peat substrate and incubated at 24 °C and 70 % RH for 9–12 weeks. Larvae were monitored and fed with carrot slices weekly until emergence of adults. For the bioassay two- to three-weeks old batches of adult beetles were selected.

2.2. Fungal strains

Four *B. pseudobassiana* strains collected (CH, 2021) and described by Pedrazzini et al. (2025) were used for the virulence assays. The strains ART 2881 / Mm_soil-1 and ART 2882 / Mm_soil-3 were isolated from

adult *M. melolontha* collected in Bristen UR, Switzerland. The other two strains, ART 2883 / Tree_lv-2 and ART 2884 / Tree_lv-1 were isolated from leaves of *A. pseudoplantanus* trees infested by *M. melolontha* in Laax GR, Switzerland. In addition, the commercialized strain *M. brunneum* Ma 43 (EFSA, 2012) was included as a positive control. All strains were maintained on semi-selective solid medium (SSM) at 22 °C and 70 % RH (Strasser et al., 1996).

2.3. Conidia suspensions

Conidia were washed off from three to four weeks old plate cultures using twice 10 ml of 0.1 % aqueous Tween 80 solution. After fifteen minutes of homogenization at room temperature using a magnetic stirrer, conidia density was determined using a Thoma chamber. Conidia suspensions were diluted to 10^3, 10^5 or 10^7 conidia ml^{-1} 0.1 % aqueous Tween 80, depending on the assay. Spore density and viability were verified for the different assays by plating 100 µl aliquots of 10^3 conidia ml^{-1} dilutions on SSM plates and counting colony forming units (cfu) after incubation at 24 °C for two weeks.

2.4. Dip assay

Field collected beetles were enclosed individually in a tea strainer and dipped twice for 10 s into 100 ml conidia solution (10^7 conidia ml^{-1}). Excess liquid was removed from the tea strainer using a paper towel before the beetles were placed individually into sterile petri dishes containing a moist filter paper and a freshly collected hazelnut leaf as feed. Treatments included the four *B. pseudobassiana* strains ART 2881, ART 2882, ART 2883, ART 2884, *M. brunneum* strain Ma 43 as positive and 0.1 % aqueous Tween 80 solution as negative control. Twenty-five beetles were used per treatment and treatments were repeated three times, each time with freshly prepared conidia suspensions. Beetles were monitored daily for 21 days. Every fourth day filter papers and hazelnut leaves were replaced.

The same infection procedure was used for the field collected larvae. After infection, they were transferred to cylindrical plastic containers (∅ 47 mm h: 64 mm) with a perforated polyethylene lid, moist peat substrate and fed with a piece of carrot. Larvae were monitored every second to third day and fed weekly for 70 days.

2.5. Leaf-exposure assay

B. pseudobassiana strain ART 2884 was used to assess the effect of conidia concentration on infection success from fungus contaminated hazelnut leaves. Hazelnut leaves were submerged and stirred in conidia suspensions of 10^3, 10^5 or 10^7 conidia ml^{-1}, or 0.1 % aqueous Tween 80 as control at room temperature for 20 min. Leaves were removed and excess liquid absorbed using a paper towel before placing single leaves in petri dishes containing a moist filter paper. Ten plates were prepared per treatment and one beetle was added to each plate. Plates were incubated at 24 °C and 70 % RH and infection development monitored daily for 50 days. Conidia-treated leaves were removed after one week and replaced with fresh leaves. Subsequently, leaves and moist filter papers were exchanged weekly. The experiment was repeated three times.

In order to verify conidial load on treated leaves two control experiments were performed: 1) Conidia were washed off from one leaf per treatment and repetition using 15 ml 0.1 % aqueous Tween 80 solution. Aliquots of undiluted stock (100 µl) and 1:10 and 1:100 dilutions were plated on SSM. 2) One leaf per treatment and repetition was homogenized in an extraction bag (BIOREBA) together with 15 ml 0.1 % aqueous Tween 80 solution and 100 µl aliquots of undiluted stock and 1:10 and 1:100 dilutions were plated on SSM. CFUs emerging on plates of experiment 1) and 2) were counted after incubation at 24 °C for two weeks.

The virulence assay including *B. pseudobassiana* ART 2881, ART 2882, ART 2883, ART 2884, *M. brunneum* Ma 43 and 0.1 % aqueous Tween 80 solution as control was performed as described above except hazelnut leaf treatments were performed with a single conidia concentration (10^7 conidia ml^{-1}) only and with 25 leaves per treatment. Incubation and monitoring were performed as described above for 60 days. The experiment was repeated three times.

2.6. Surface sterilization

Dead beetles and larvae were surface-sterilized in a tea strainer immersed in 1 % aqueous NaOCl for one minute, followed by dipping in ddH_2O three times for one minute each. Surface-sterilized individuals were transferred to sterile petri dishes, lined with a sterile and moist filter paper and monitored for two weeks for emergence of mycosis.

2.7. Isolation of fungal isolates and DNA extraction

Fungal isolates were obtained from mycosed individuals by transferring fungal tissue with a needle to SSM. Single colony isolations were performed for each isolate on SSM. Isolates were plated on potato dextrose agar medium PDA (Sigma-Aldrich, Merck, Darmstadt, Germany) overlayed with a sterile filter paper and the emerging mycelium harvested after incubation at 24 °C for three to five days. Mycelium was frozen at −80 °C, followed by lyophilization, homogenization, DNA extraction and DNA quality and quantity assessment performed as described by Pedrazzini et al. (2024).

2.8. Microsatellite markers

Multi-locus genotypes (MLG) of fungal isolates were determined using six microsatellite markers (SSR) for *B. pseudobassiana* (Pedrazzini et al., 2025) or *M. brunneum* (Mayerhofer et al., 2015b) in two sets of three primer pairs. *B. pseudobassiana* isolates were genotyped with markers BpsSSR01, BpsSSR02, BpsSSR03 (Set I) and BpsSSR10, BpsSSR11, BpsSSR12 (Set IV) using strain ARSEF 3405 as reference for allele sizing. *M. brunneum* isolates were genotyped with markers Ma 2049, Ma 2054, Ma 2063 (Set I) and Ma 2287, Ma 327, Ma 195 (Set II) using strains ARSEF 7524 as references for allele sizing. Marker amplification and allele size determination on a 3500xl Dx Genetic Analyzer (Applied Biosystems, Thermo Fisher Scientific, Waltham, Ma, USA) was performed as described by Pedrazzini et al. (2025) and Mayerhofer et al. (2015b).

2.9. Sequence analyses

Species identity of all the fungal isolates displaying a multi locus genotype (see below) different from the strains used for inoculation was verified by sequence analysis of the internal transcribed spacer (ITS1-5.8S-ITS2) region and/or the 5′ end of the translation elongation factor 1-alpha (5TEF) gene in case of isolates morphologically identified as *Metarhizium* spp. The ITS region and 5TEF sequences were PCR amplified in a 20 µl reaction volume containing 15 ng genomic DNA, 1x Phusion HF buffer containing 7.5 mM MgCl2, 0.2 mM dNTPs, 3 % dimethyl sulfoxide (DMSO), 0.2 U of Phusion Hot Start II High-Fidelity DNA polymerase (Thermo Fisher Scientific, Waltham, Ma, USA) and 0.2 µM of each primer ITS5: 5′GGAAGTAAAAGTCGTAACAAGG'3 (White et al., 1990) and ITS4: 5′TCCTCCGCTTATTGATATGC'3 (White et al., 1990), or EF1T: 5′ ATG GGT AAG GAR GAC AAG AC 3′ (Bischoff et al., 2006) and EFjmetaR: 5′ TGC TCA CGR GTC TGG CCA TCC TT 3′ (Mayerhofer et al., 2019), respectively. Amplification steps for the ITS region were: 30 s denaturation at 98 °C, followed by 36 cycles of 5 s denaturation at 98 °C, 20 s annealing at 60 °C, and a 30 s extension at 72 °C, followed by elongation for 10 min at 72 °C. The amplification of the 5TEF deviated from this protocol by two additional cycles (38), a lower annealing temperature (58 °C), and a longer extension time (1 min). Quality of PCR products was visually assessed on a 1.5 % agarose gel and

PCR products were purified using the NucleoSpin Gel and PCR Clean-up kit (Machery-Nagel, Düren, Germany). Sequencing was performed using the same respective primers as the PCR, using the BigDye™ Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's protocol. Sequences were determined using a 3500xl Dx Genetic Analyzer (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA) and forward and reverse sequences were assembled using the software DNABaser V5 (Heracle Biosoft, Pitești, Romania). Sequences were identified using BLAST similarity searches in the non-redundant nucleotide database of GenBank (Altschul et al., 1990). Sequences identified as belonging to the genera *Beauveria* or *Metarhizium* were aligned with reference strains (Bioedit v7.2.5) and a maximum-likelyhood tree was constructed (MEGAX v11.0.9) using Tamura-Nei substitution model and bootstrapping with 1000 pseudorepetitions. Reference sequences were obtained from Genbank.

2.10. Survival Statistics

The R packages readxl v1.4.3 and tidyverse v2.0.0 were used for data import and processing. Survival objects were created using the Surv function and the Kaplan-Meyer survival curve estimates were computed using survfit function, both from the R package survival v3.5–7. To compute Kaplan-Meyer curves of survival and mycosis rate, all three repetitions per treatment were included. Graphs were plotted using function ggsurvplot of the R package survminer v0.4.9. To calculate median survival and Cox proportional hazards regression models all three replicates were integrated. For the Cox proportional hazards regression modelling coxme function of the coxme v2.2–18.1 package was used with treatment as fixed and replicate as random variable and the control treatment was set as reference. To plot the hazard ratio the coxph function of the same package was used including only the fixed variable treatment. Pairwise comparison between the different treatment was performed using the function emmeans from package emmeans v1.10.2 and R base function pairs on the results from the Cox proportional hazards regression modelling. Barplots showing the percentage of beetles mycosed with either the strain of infection or infections with natural strains were plotted using ggplot2.

3. Results

3.1. Dip assay with P. japonica adults

All the four *B. pseudobassiana* strains tested in the dip-infection assays revealed three-to-five-fold reduced survival of adult *P. japonica* as compared to the not infected control beetles. (Fig. 1a). *B. pseudobassiana* ART 2881 and ART 2884 emerged as the most virulent strains, with median survival rates (5 days) that were significantly lower than the survival rates of strains *B. pseudobassiana* ART 2882, ART 2883, *M. brunneum* Ma 43 or the control (Table 1). The hazard rates in all the fungal treatments were significantly increased compared to the control (HR: 8.2–16.6) and the most virulent strains ART 2881 and ART 2884 revealed significantly higher values compared to the other two *B. pseudobassiana* strains and Ma 43 (Table 1**).** There were no significant differences among replicates within treatments (Supplementary Table S1). At the end of the experiment after 28 days, 100 % of all infected and 61.3 % of control beetles had died. Mycosis was observed in both, infected as well as control treatments. Beetles treated with *B. pseudobassiana* or Ma 43 revealed average mycosis ranging from 71 % to 92 %, while for the beetles in the control treatment the development of mycosis was lower with an average mycosis of 53 % (Fig. 1b). No significant differences were detected among the mycosis rates of treatments with the five fungal strains.

Fungal isolates were obtained from all mycosed beetles and genotyped with six microsatellite markers to verify strain identity. In the treatments with strains ART 2881, ART 2882, ART 2883 ART 2884, and Ma 43 on average 55 %, 48 %, 67 %, 71 % and 70 % of the mycosed beetles respectively, displayed the genotype of the isolates used for infection with no significant differences (Fig. 1b, Supplementary Table S2 and S3). One isolate in the ART 2883 treatment had the genotype of strain ART 2881 and one isolate in the control the genotype of Ma 43. All the remaining isolates, including isolates of the control treatment differed in genus, species or genotype from the strains used for treatment. These isolates were considered as natural infections acquired prior to the experiment (Supplementary Table S4, Supplementary Fig. S1). Double infections, i.e., beetles, from which two fungal isolates or species were obtained were observed in 4.3 % of the mycosed beetles. Among the 172 natural isolates obtained from all treatments, 10 isolates were identified as *B. pseudobassiana* and 2 as *M. brunneum* with genotypes different form strains used for infection (Supplementary Table S2 and S3). The remaining natural isolates were identified as *B. bassiana* (31), *B. brongniatii* (1) or *M. robertsii* (4), or as the genera *Beauveria* (13),

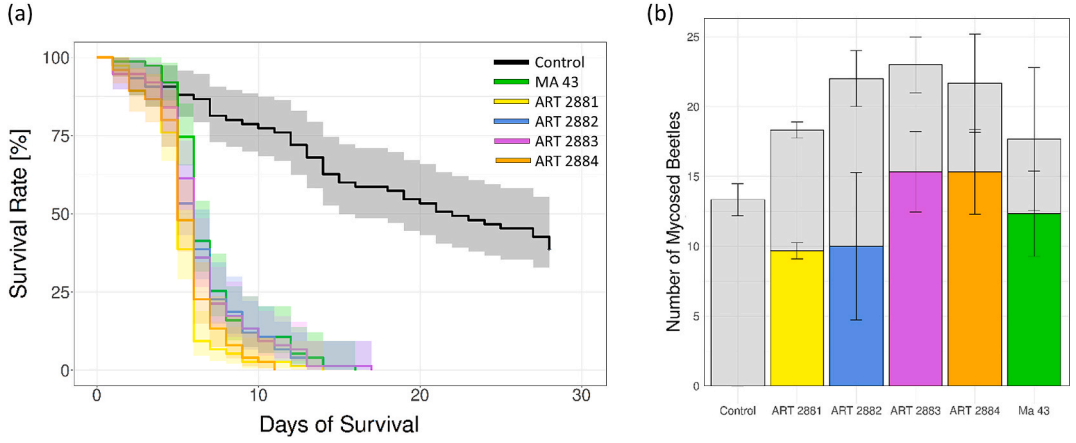

Fig. 1. Infection of field collected *P. japonica* adults dipped in suspensions of 10^7 conidial ml^{-1} of ART 2881, ART 2882, ART 2883, ART 2884, MA 43 or 0.1 % Tween 80 in water as control **(a)** Kaplan-Meier plot of beetle survival including three replicates of 25 beetles each. Shaded areas represent 95 % confidence intervals **(b)** Average number of mycosed beetles per treatment. Beetles were surface sterilized after death and monitored for two weeks for appearance of mycosis. Stacks from top to bottom: 1. Mean number of beetles (with SD) mycosed with natural fungal strains (light grey) 2. Mean number of beetles (with SD) mycosed with the strain of infection.

Table 1
Median survival times across all three replicates for different treatment groups in the adult and larvae dip as well as the leaf-exposure assays. The median survival is presented with 95 % confidence intervals (CI). p-values were calculated to assess the significance of the treatment effects compared to the control group, with hazard ratios (HR) indicating the relative risk of mortality associated with each treatment. Significance codes denote the statistical significance of the results for each experiment separately.

Experiment	Treatment	Median survival (95 % CI)	p-value	Hazard ratio (95 % CI)	p-value	Sign. Code[a]
Adult Dip	Control	22 (15, –)	–	–	–	a*
	Ma 43	6 (6,7)	<0.001	8.2 (5.22, 12.9)	<0.001	b*
	ART 2881	5 (5,6)	<0.001	16.6 (10.5, 26.5)	<0.001	c*
	ART 2882	6 (5,7)	<0.001	9.7 (6.16, 15.2)	<0.001	b*
	ART 2883	6 (6,6)	<0.001	9.3 (5.92, 14.6)	<0.001	b*
	ART 2884	5 (5,6)	<0.001	13.7 (8.57, 21.8)	<0.001	c*
Larvae Dip	Control	–	–	–	–	a**
	Ma 43	–	–	1.5 (1.08, 2.10)	0.996	a**
	ART 2881	–	–	1.0 (0.47, 2.12)	0.368	a**
	ART 2882	–	–	0.4 (0.17, 0.78)	0.174	a**
	ART 2883	–	–	1.0 (0.66, 1.49)	0.984	a**
	ART 2884	–	–	1.8 (1.20, 2.78)	0.131	a**
Adult Leaf	Control	47 (45, 50)	–	–	–	a*
	Ma 43	13 (12, 15)	<0.001	18.9 (11.9, 30.0)	<0.001	bc*
	ART 2881	12 (10, 16)	<0.001	9.8 (6.51, 14.8)	<0.001	cd*
	ART 2882	15 (12, 18)	<0.001	14.7 (9.42, 23.0)	<0.001	b*
	ART 2883	15 (13, 18)	<0.001	12.2 (7.76, 19.0	<0.001	b*
	ART 2884	9 (7, 12)	<0.001	26.2 (16.5, 41.4)	<0.001	d*

[a] Different letters indicate significant ($p < 0.05$) differences among treatments withing each experiment.
* Significance code based on median survival and hazard ratio.
** Significance code based on hazard ratio.

Metarhizium (7) *Epichloë* (4), *Lecanicillium* (1), *Liangia* (1), *Penicillium* (15), *Pochonia* (3), *Purpureocillium* (73), *Samsoniella* (6) or *Simplicillium* (1), based on morphology and/or ITS or 5TEF sequence analyses (Supplementary Table S1, Supplementary Fig. S4).

3.2. Dip assay with P. japonica larvae

Larvae of *P. japonica* showed substantial resistance to fungal infections, with no significant differences in survival rates between infected and control larvae (Fig. 2). After 70 days, more than 80 % of fungus treated larvae as well as control larvae were still alive and started to pupate. In each of the control, ART 2881 and ART 2883 treatments, 8 of the 75 larvae died across all three replicates, compared to 12 in the Ma 43, 14 in ART 2884 and only 3 in ART 2882 treatments. The hazard ratio of the fungus treated larvae was between 0.4 and 1.8 compared to the control and no significant differences were observed (Table 1, Supplementary Table S1). Four larvae each in the ART 2883, ART 2884 and Ma 43 treatments mycosed with the strain with which they were infected and no infections with natural isolates were observed. In the control, the ART 2881 and the ART 2882 treatment no mycosis was observed among the dead larvae (**Data not shown**).

3.3. Leaf-exposure assay with adult p. Japonica

One of the two most virulent *B. pseudobassiana* strains ART 2884 was used to assess success of infection when exposing/feeding hazelnut leaves inoculated with different conidia concentrations of the fungus to reared adult *P. japonica*. Hazelnut leaves were dipped in three different conidia suspensions including 10^3, 10^5 and 10^7 conidia ml^{-1}. Conidia concentration on dipped leaves was verified by either washing the conidia off or leaf homogenization, followed by determination of colony forming units in the wash solutions or homogenates. Both assays

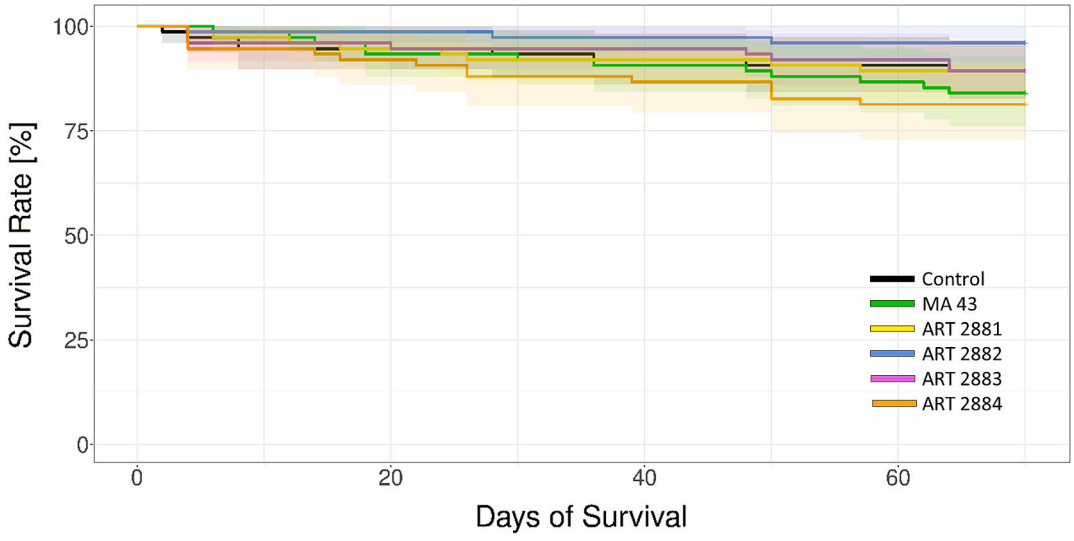

Fig. 2. Infection of field collected *P. japonica* larvae dipped in suspensions of 10^7 conidial ml^{-1} of ART 2881, ART 2882, ART 2883, ART 2884, MA 43 and 0.1 % Tween 80 in water as control. Kaplan-Meier plot of larval survival including three replicates of 25 larvae each. Shaded areas represent 95 % confidence intervals.

revealed presence of approximately 10^2, 10^4 and 10^6 conidia per leaf, corresponding to $3*10^0$, $3*10^2$ and $3*10^4$ conidia per cm^2 leaf (average leave size 34 cm^2). The median survival of adults exposed to leaves dipped in 10^7, 10^5 or 10^3 conidia ml^{-1} was 15 d (HR: 6.9), 26 days (HR: 2.7) or 27 days (HR: 1.6) compared to 36 days in the control (Supplementary Fig. S2). Twenty percent of the beetles exposed to the highest conidia concentration developed mycosis, whereas only 3 percent (one out of 30 beetles) developed mycosis in the middle concentration and none in the lowest concentration and the controls (**data not shown**).

Based on the results with different conidia concentrations of ART 2884, subsequent experiments including all four *B. pseudobassiana* strains and *M. brunneum* Ma 43 were performed with dipping solutions containing 10^7 conidia ml^{-1}. All the fungal strains significantly reduced the survival of reared *P. japonica* adults as compared to the controls (Fig. 3a). *B. pseudobassiana* ART 2884 revealed the lowest median survival (9 days) followed by ART 2881 (12 days), *M. brunneum* Ma 43 (13 days), *B. pseudobassiana* ART 2882 (15 days), *B. pseudobassiana* ART 2883 (15 days), and the control (47 days) (Table 1). Correspondingly, the hazard ratio ranged from 9.8 to 26.2 for all the treatments in relation to the control and was highest for ART 2884 followed by ART 2881, Ma 43, ART 2883 and ART 2882 (Table 1, Supplementary Table S1). In the control, the Ma 43 and the ART 2882 treatment all beetles were feeding on the inoculated leaves (at least ¼ of the leaf consumed), while in the ART 2881, the ART 2883 and the ART 2884 treatment 5.3 %, 5.3 % and 4 % of the beetles did not. At the end of the experiment, i.e., after 60 days, 100 % of the infected and 92 % of the control beetles were dead. No mycosis was observed in the control treatment. Treatments including strains ART 2881, ART 2882, ART 2883, ART 2884 or Ma 43 resulted in an average mycosis of 31 %, 24 % 37 %, 35 % or 27 % of the treated beetles, yet with no significant differences among treatments (Fig. 3b). All the isolates obtained from mycosed beetles displayed the genotype of the strain applied in the respective treatment **(data not shown)**.

4. Discussion

Identification of effective and environmentally competent (Quesada-Moraga et al., 2024) isolates is a key factor for successful development of biological pest control approaches. In this study we aimed to examine the virulence of *B. pseudobassiana* to the invasive and polyphagous beetle *P. japonica*. This entomopathogenic fungus has recently been identified as a predominant natural pathogen of *M. melolontha* a scarabaeoid beetle prevalent in central Europe and a relative of the *P. japonica* (Pedrazzini et al., 2024). Given that *B. pseudobassiana* has been isolated with infection prevalence of 4.5–35.6 % from swarming *M. melolontha* (Pedrazzini et al., 2024) as well as from phylloplane (Garrido-Jurado et al., 2015; Pedrazzini et al., 2025) suggests its potential applicability for above-ground control of adult *P. japonica*.

In both of the infection experiments performed in this study, i.e., the dip- as well as the leaf-exposure assay, all four tested *B. pseudobassiana* strains demonstrated a high efficacy to infect and kill *P. japonica* adults. Two strains, one originating from *A. pseudoplantanus* tree leaves (ART 2884) and one originating from *M. melolontha* (ART 2881), were significantly more virulent than the other strains tested including *M. brunneum* Ma 43, which is registered for the control of several insects in Europe (EPPO, 2025). Overall, our data demonstrated that *B. pseudobassiana* genotypes isolated from *M. melolontha* are also virulent to *P. japonica*. Furthermore, they confirmed results reported by Pedrazzini et al. (2025), showing that *B. pseudobassiana* genotypes isolated from the phylloplane exhibited pathogenicity toward scarabaeoid beetles.

The median survival of *P. japonica* adults was generally lower in the dip-assay (one third to half) as compared to the leaf-exposure assay across all the five strains tested. Several factors may be responsible for this difference. First, adults in the dip-assay were field-collected with varied age and fitness, while those in the leaf-exposure assay were reared, young (2–3 weeks) and likely healthier as no infections in the control treatment were observed. Second, the infection pressure was higher in the dip-assay compared to the leaf-exposure assay. In the dip-assay insects were directly exposed to a high concentration of suspended conidia for twice 10 seconds, while in the leaf-exposure assay insects were in indirect contact with conidia that were deposited on leaves likely resulting in uptake of lower amounts of conidia although the exposure time was longer (one week). Furthermore, while in the dip-assay the whole body of the beetle was exposed to large amounts of conidia, infection in the leaf-exposure assay likely was restricted to the ventral body part. Despite the overall slower mortality, the leaf-exposure assay demonstrated that high infection rates can be achieved with 10^6 conidia per leaf. This conidia concentration corresponds to approximately 10^4 conidia per cm^2, a density attainable under field conditions (Clifton et al., 2020; Zottele et al., 2023), indicating feasibility of spray application.

In contrast to the adults, the larvae of *P. japonica* exhibited resistance to fungal infection, with no significant differences in survival rates observed between infected and control groups for all the strains tested. Over 80 % of both infected and control larvae survived the 70-day period of the test. Our findings align with recent work by Graf et al. (2023), which highlighted the resistance of *P. japonica* larvae compared

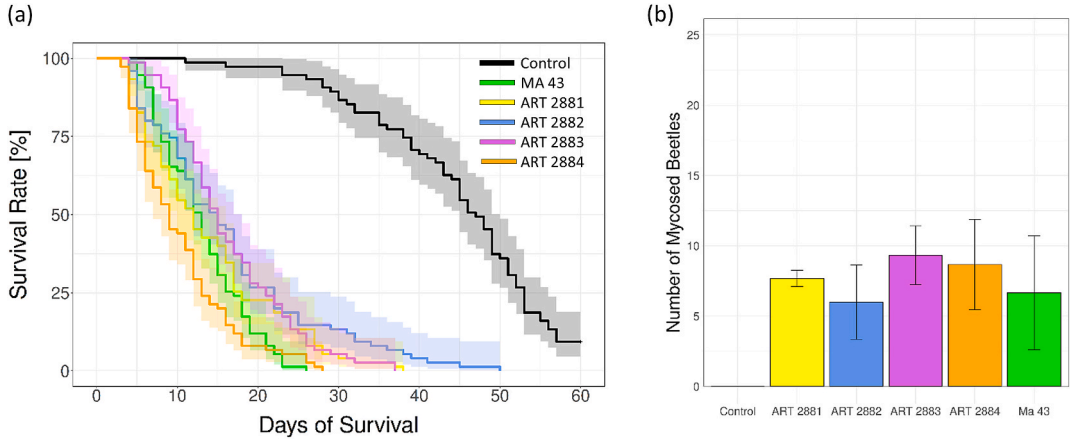

Fig. 3. Infection of reared *P. japonica* adults when exposed to / fed with hazelnut leaves treated with conidia suspensions (10^7 conidial ml^{-1}) of ART 2881, ART 2882, ART 2883, ART 2884, MA 43 or 0.1 % Tween 80 in water as control. **(a)** Kaplan-Meier plot of beetle survival including three replicates of 25 beetles each. Shaded areas represent 95 % confidence intervals **(b)** Average number of mycosed beetles per treatment. Beetles were surface sterilized after death and monitored for two weeks for appearance of mycosis. Mean number of beetles (with SD) mycosed with the strain of infection.

to adults when exposed to *M. brunneum* and *B. brongniartii*. In contrast, Barzanti et al. (2023) reported high mortality (up to 83 %) of third-instar larvae exposed to *M. robertsii* isolates from infested areas in Europe. Similarly, Giroux et al. (2015) demonstrated increased larval mortality with 13 isolates of *M. anisopliae*, *B. bassiana*, and *B. brongniartii* using dip infection method. However, the differences observed in larval mortality among the different studies may result from variations in fungal strains and protocols, with Giroux et al. (2015) using conidia concentrations 10^3 times higher than used in this study, and Barzanti et al. (2023) applying dry conidia powder to soil. Furthermore, field trials using *M. brunneum* Ma 43 have shown mixed results, from significant reduction of larval density depending on application method and turf conditions (Behle et al., 2015; Ramoutar et al., 2010) to no impact on larval population despite successful establishment of Ma 43 in soil (Graf et al., 2023). In general, larvae may be better equipped to resist fungal infections compared to adults. Possible factors contributing to this resistance include a robust cuticular defense combined with internal, immune system mechanisms, as suggested by Graf et al., (2023) or microbiome-related resilience, which has been shown to occur in other insects (Baur et al., 2024; Hong et al., 2022; Zhou et al., 2019). This underscores the need for further research into larval defense mechanisms and the development of alternative control methods targeting this life stage.

Among beetles collected from the field, 37.7 % were infected with natural fungal pathogens, resulting in 170 isolates across 13 taxa. *Purpureocillium* was most prevalent among natural isolates, a genus known to contain entomopathogenic species (Castillo Lopez et al., 2014). Among the natural *Beauveria* infections *B. bassiana* was the most frequently isolated. This species has been shown to be highly infectious to *P. japonica* adults with potential as BCA (Giroux et al., 2015; Lacey et al., 1994). Interestingly, natural *B. pseudobassiana* was detected in both control and treatment groups, indicating that *B. pseudobassiana* indeed might represent a relevant pathogen of *P. japonica* in Europe. Moreover, the entomopathogens *M. brunneum* and *M. robertsii* were identified, at low frequencies. Other natural infections included isolates from *Beauveria*-related genera, such as *Liangia*, *Lecanicillium*, *Samsoniella*, and *Simplicillium* (Wang et al., 2020a), as well as isolates from *Metarhizium*-related genera, such as *Pochonia* and *Epichloë* (St. Leger & Wang, 2020), along with some *Penicillium* spp. Our results demonstrate a substantial fungal pathogen diversity on *P. japonica* adults at the site where they were collected. However, a more comprehensive survey in different regions in recently infested areas in Northern Italy and Switzerland may be needed to fully describe the diversity and abundance of entomopathogenic fungi infecting *P. japonica* adults in Europe.

In this study we performed infection experiments with field-collected as well as laboratory-reared beetles. In contrast to the field collected beetles, the beetles reared from field collected larvae were healthier, as evidenced by absence of natural infections in the leaf-exposure assay. This method effectively mitigates the complexity of admixtures of intentional with natural infections, ensures age synchronization, and allows full use of their lifespan. Reared beetles had approximately two times longer median survival compared to field-collected adults, which greatly improves the possibilities for experimental design.

B. pseudobassiana has been isolated from insects of different orders from above and below ground but also from a range of different environments including soil and the phylloplane of several plant species (Arias-Aravena et al., 2022; Barrera-López et al., 2020; Garrido-Jurado et al., 2015; Howe et al., 2016; Pedrazzini et al., 2024; Wang et al., 2020b). In consequence, this implies that *B. pseudobassiana* is adapted to many different niches including above ground environments and conditions. Our results demonstrated that *B. pseudobassiana* is capable of infecting *P. japonica* through contact on inoculated leaves, even at lower conidia concentrations, as demonstrated in our dose–response assay, albeit with reduced efficacy. Together this supports the hypothesis that *P. japonica* beetles may get infected in the soil when emerging from the pupae but also by the fungus occurring in the foliage with which adults get in contact during swarming and feeding activity. The latter may be particularly interesting when considering *B. pseudobassiana* as potential BCA against *P. japonica* exerting above ground applications like spray or "trap and infect" approaches.

Furthermore, several studies have documented endophytic behavior of entomopathogenic fungi, particularly *B. bassiana* (Moonjely et al., 2016). Whether *B. pseudobassiana* is able to colonize plant tissue in particular leaves remains to be investigated. This behavior could help the fungus to resist or circumvent the harsh above-ground conditions and establish long-term presence in a plant, potentially controlling *P. japonica* through direct infection, in planta produced fungal toxins or endophyte activated plant defense as observed for other insect-plant-fungus interactions (Quesada-Moraga et al., 2023). To fully assess the potential of *B. pseudobassiana* as a BCA, it will be crucial to investigate its environmental distribution and competence. Resilience to environmental factors, such as solar radiation, moisture, and temperature is key for survival and establishment of the fungus above ground and require particular consideration (Quesada-Moraga et al., 2024).

In conclusion, the virulence of *B. pseudobassiana* depends on the developmental stage of the beetle. It is highly virulent against *P. japonica* adults, even when indirectly applied via their leaf feed, but not against their larvae. *B. pseudobassiana* has been reported from a range of climatic regions throughout Europe. It thus holds great potential as BCA for the development of protective strategies against *P. japonica* adults not only for areas currently affected by *P. japonica* but also for regions where populations are expanding or are at risk for invasion.

Credit authorship contribution statement

Noëmi Küng: Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Sara Boschi:** Writing – review & editing, Methodology, Investigation. **Franco Widmer:** Writing – review & editing. **Jürg Enkerli:** Writing – review & editing, Validation, Supervision, Methodology, Formal analysis, Data curation, Conceptualization.

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Declaration of competing interest

The authors 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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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biocontrol.2025.105887.

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