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Efficacy of biological control agents against *Popillia japonica*: A strategy to reduce the risk of pest spread via potted plant substrates

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ABSTRACT

The international trade of potted plants represents a pathway for the unintentional spread of harmful organisms, leading to new infestations in pest-free areas. The Japanese beetle *Popillia japonica*, which is considered one of the most threatening insect pests in Europe, can evade detection at the larval stage by hiding within the substrates of potted plants. To reduce the risk of *P. japonica* dispersal via potted plants, we tested the efficacy of entomopathogenic nematodes (EPNs) and fungi (EPF) as biological control agents (BCAs).

Field trials with potted cherry trees were conducted in an Italian nursery in northern Italy, using preventive treatments applied before the beetle's flight period and curative treatments after larval establishment. Two *Heterorhabditis bacteriophora* (a native population, labelled as POP16, and a commercial product) significantly reduced larval survival, achieving mortality rates up to 97% after 60 days. By contrast, EPF treatments with *Metarhizium robertsii* and *M. brunneum* showed persistence in the substrate, without efficacy. EPNs proved to be much more effective when applied shortly before the adult beetle flight, rather than as a curative treatment targeting larvae already present in the pots.

These results suggest that, at present, the use of *H. bacteriophora* applied between 15 and 30 days before the commercialisation of the potted plants could represent the best trade-off between management effort and effectiveness in integrated pest management (IPM) programmes to reduce the unintentional spread of *P. japonica* through the nursery trade.

1. Introduction

Biological invasions represent a significant ecological challenge worldwide, primarily driven by the introduction of exotic species through international plant trade. Improper management of this trade can lead to the uncontrolled spread of non-native organisms, causing harm to local agriculture and ecosystems. These impacts range from substantial yield loss in specific agricultural crops and excessive costs for control measures, to competition with indigenous species for vital resources such as food and space, and to the transmission of diseases to plants (Pimentel et al., 2005; Straubinger et al., 2023). Several studies in this field focus on understanding ecological impacts of biological invasions, developing management strategies for invasive species, and investigating the pathways of introduction (Venette and Hutchison, 2021). Global trade plays a key role in the introduction of exotic species (Liebhold et al., 2012; Bonnamour et al., 2023) and addressing this issue necessitates a multifaceted approach, including preventive measures such as imposing stringent regulations on the trade of plants, actively monitoring trade routes, and promoting sustainable import–export practices. Equally important is international cooperation, as the rapid transboundary spread of invasive species demands coordinated global efforts for effective management and mitigation (Epanchin-Niell et al.,

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2021).

One of the invasive organisms which has travelled large distances by plant trade is *Popillia japonica* Newman (Coleoptera: Scarabaeidae), the Japanese beetle, native to Japan and eastern Russia. This highly polyphagous insect, feeding on more than 400 host plants, was inadvertently introduced into a New Jersey nursery in the early 1900s through the trade of plants from Japan (Fleming, 1972). Since then, it has progressively colonized large areas of the eastern and central United States (Althoff and Rice, 2022), and in the early 1970s *P. japonica* was detected for the first time in Europe, on Terceira Island (Azores archipelago) (Vieira, 2008). In 2014, it was intercepted in Italy, marking its arrival in the heart of continental Europe (Pavesi, 2014; Strangi et al., 2024). After initial introduction, this scarab beetle is able to expand its range on a local scale by natural flight. However, its spread to other regions is primarily driven by occasional and passive transportation (Poggi et al., 2022; Gotta et al., 2023).

Usually, *P. japonica* is a univoltine species, spending most of its life as a larva in the soil, feeding and damaging the roots of host plants (Potter and Held, 2002). The soil-dwelling larvae of *P. japonica* are difficult to detect in potted plants, so transportation of infested plant material or substrates has played a significant role in their long-distance dispersal. Plant nurseries and the trade of potted plants are therefore key pathways for the unintentional spread of *P. japonica* from infested areas to pest-free zones. Recent legislation in Europe has emphasized the importance of this issue (Commission Implementing Regulation (EU) 2023/1584, EFSA, 2018).

The aim of our study was to counteract the risk of *P. japonica* spreading through the trade of potted plants by testing the efficacy of phytosanitary measures implemented during plant production in nurseries. To date, there is no certified procedure in Europe available to ensure *P. japonica*-free plants in the trade chain. Therefore, effective management of Japanese beetle larvae in nurseries in infested regions is crucial to prevent the spread of this pest to new European areas vulnerable to future infestations. Mori et al. (2022) explored different management strategies for *P. japonica* in container-grown nursery stock in Italy and demonstrated that an integrated approach combining physical barriers to prevent oviposition with insecticide applications significantly reduces larval survival. Furthermore, their study emphasized the potential of BCAs as an effective alternative to chemical treatments, particularly in sustainable and integrated pest management programs.

Entomopathogenic nematodes (EPNs) and fungi (EPF), in particular, are promising tools due to their ability to infect and kill soil-dwelling insects and to persist for weeks or months in the environment (Koppenhöfer et al., 2020; Sharma et al., 2023). Entomopathogenic nematodes (EPNs) kill insects within 1–3 days by entering hosts and releasing symbiotic bacteria that produce toxins, reproduce inside the cadaver, and can persist in soil for several weeks depending on environmental conditions (Hazir et al., 2003). Entomopathogenic fungi (EPF) infect through the insect cuticle, kill typically in 4–14 days, reproduce via spores on the dead host, and can persist in soil for weeks to months (Inglis et al., 2001). Both groups are effective biological control agents of many different soilborne insect pests and have been used for *P. japonica* control (Glazer et al., 2022; Torrini et al., 2020; Benvenuti et al., 2019; Barzanti et al., 2023; Gotta et al., 2023).

Based on this background, our working hypothesis is that applying BCAs into the substrates of potted plants could represent an effective preventive measure to avoid the inadvertent transport of *P. japonica* larvae from infested regions to pest-free areas. To test this hypothesis we conducted nursery trials evaluating the efficacy of different treatments after time intervals of 15, 30, and 60 days. Biological control agents (BCAs), including commercial products and natural populations of entomopathogenic nematodes and fungi, were tested for their potential to control Japanese beetle larvae and to reduce oviposition in potted plants. By comparing the performance of these BCAs over time, we aimed to determine whether they are a practical and sustainable tool in an IPM strategy to impede the spread of this priority pest.

2. Material and methods

2.1. Study design

Experiments were set up in 2022 and in 2024 at the 'Vivai Furno' nursery in Biella (Piedmont region, Italy). This area has been infested with *P. japonica* since 2020.

Young cherry trees about one meter in height, grown in 4 L pots (20 cm diameter on the top) were selected as host plants for the experiments. Wire meshes were inserted on the bottom of the pots to prevent the larvae from escaping through the water drainage holes, and the pots were filled with Terflor® soil (Brescia, Italy) composed of dark peat and montmorillonite clay, blond baltic peat, and pumice (5.5–6.5 pH, bulk density 160 Kg/m^3). The pots were irrigated with a drip system for the duration of the experiments.

Two different sets of experiments were carried out. In the first one, application of the BCAs into the potting substrates was scheduled during the flight period of *P. japonica*, aimed at infecting newly hatched larvae after emergence from eggs (see 2.3 below). In the second one, the application of BCAs took place later in the season, with the aim to directly target *P. japonica* larvae developing in the potting substrates (see 2.4 below).

2.2. Biocontrol agents

Three formulations of different entomopathogenic fungi and two entomopathogenic nematodes were tested (Table 1).

2.2.1. Entomopathogenic fungi (EPF)

Two formulations of the native *Metarhizium robertsii* strain 17/T02, which proved to be virulent against *P. japonica* larvae in a previous study (Barzanti et al., 2023) were used: (i) Fungus Colonized Barley Kernels (FCBK) and (ii) Sodium Alginate Pellets (SAPs). A commercially available strain of *Metarhizium brunneum* (MA43, syn. BIPESCO5, Agrifutur, Italy, Alfianello), formulated as FCBK, was added as a third treatment.

SAPs were prepared at the laboratory of CREA (Research Centre for Plant Protection and Certification) in Florence (Italy) following a standardized procedure (Lewis and Papavizas, 1985) modified as follows: dried conidia of *M. robertsii* were obtained from colonies grown on quarter-strength Sabouraud Dextrose Agar (SDAY1/4, Liu et al., 2003), using SDA (VWR International PBI s.r.L.) plus 0.25% Yeast Extract (YE, Sigma-Aldrich Chemie GmbH) and overlayed with a sterile cellophane sheet. Approximately $1x10^8$ conidia/ml were suspended in 100 ml of a sterile 0.02% Tween®80 (BDH Laboratory Supplies, Poole, England) aqueous solution and added to 3.99 g of sodium alginate (BDH Laboratory Supplies, Poole, England) and 40 g of kaolin (Kaolin Washed, Carlo Erba Reagents) dissolved in 300 ml of sterile distilled water. The final preparation was stirred continuously and allowed to drip into a

Table 1
Treatments and doses of biocontrol agents applied to potted cherry trees in the nursery experiments in Biella, Italy, in 2022 and 2024.

	Treatments	Code	Dose/pot (4 L)
Control		CTR	
Entomopathogenic fungi	*Metarhizium brunneum* (MA43, Bipesco 5)	Bip5	$3x10^8$ conidia
	M. robertsii (isolate 17/T02)	SAPs	
	M. robertsii (isolate 17/T02)	FCBK	
Entomopathogenic nematodes	*Heterorhabditis bacteriophora* (native population)	POP16	47,100 infective juveniles
	H. bacteriophora (Commercial product – nematop)	HbCom	

0.18 M sterile calcium chloride gelling solution (ThermoFisher GmbH, Kandel, Germany). The alginate beads were let to gel for approximately 20 min, then removed, washed in sterile distilled water, dried on sterile filter paper, and transferred onto a sterile aluminum sheet for the final 24 h of air-drying phase under the airflow of a biological hood. Dried SAPs were stored in sterile glass bottles at approximately 4°C until use. The production process was completed with a post-production quality assessment, which included evaluating the number of fungal conidia per gram of SAPs and their germinability.

Fungus Colonized Barley Kernels (FCBK) were prepared at the laboratory of Agroscope in Zurich (Switzerland). Fungal isolates were cultivated on complete medium in Petri dishes (CM – 10 g/L glucose, 0.36 g/L KH2PO4, 1.78 g/L Na2HPO4, 1 g/L KCl, 0.6 g/L MgSO47H2O, 0.6 g/L NH4NO3, 5 g/L yeast extract, and 20 g/L agar, (Riba and Ravelojoana, 1984)) until forming a uniformly grown spore layer. Two kg of unpeeled barley was soaked in 1.5 L of tap water and autoclaved in polypropylene zipper filter bags (Sac O2, Deinze, Belgium). Bags were inoculated by adding the spore layers of one Petri dish into each bag. The bags were incubated for four weeks at 22°C and stored at 4°C until use. The production process was completed by a post-production quality check assessing the number of fungal spores per gram of FCBKs and their germinability.

2.2.2. Entomopathogenic nematodes (EPN)

A commercially available product based on *Heterorhabditis bacteriophora* (nematop, e-nema® GmbH, Schwentinental Germany) and a native population of *H. bacteriophora* isolated in the Ticino Valley, Piedmont region, Italy (POP16, Torrini et al., 2020) were used for experiments. Both nematodes were produced and formulated by e-nema® in liquid culture (Peters et al., 2017). Nematode products were diluted in tap water and kept at 18°C for 24 h prior to testing. Concentrations of EPF and EPN applied into pots are given in Table 1.

2.3. Preventive BCA-application during the flight period of Popillia japonica adults

Treatments with EPF were applied on May 31st, 2022, by manually mixing the potting substrates with SAPs, FCBK, or Bip5 to ensure homogenous fungal distribution. Treatments with EPNs were applied on June 13th, 2022, by distributing the nematode solution directly onto the surface of the soil. EPF applications were carried out two weeks earlier to let the fungal granules re-sporulate and allow the fungi to establish in the soil (Shah et al., 2022). Control pots remained untreated. Twenty-four pots were prepared for each treatment, for a total of 144 pots. On the same date, 6 cages (1.5 x 1.5 m; h:1.8 m) made of wooden frames covered with insect nets were set up in two rows of three. Inside each cage, 24 pots (4 pots/treatment) were placed and arranged following a randomized block design. To minimize variation in radiation and temperature within the cages, a shading cloth was installed above the cages and extended 1–2 m laterally from the external cages.

Popillia japonica adults were captured using Trécé Pherocon yellow/green traps (Trécé Inc., Adair, Oklahoma, US) baited with floral lure only, to attract both males and females in similar quantities. Traps were placed in a site close to the nursery (Mottalciata, Biella – 45°29′43.5″N 8°12′37.2″E). A preliminary sex ratio assessment was conducted with a subsample of 200 trapped specimens before each release (see below). Subsequently, 480 adults were introduced into each cage, which was immediately sealed to prevent escape. *Popillia japonica* adults were left in the cages until their death. This procedure was repeated three times during the flight season, (i) on June 14th (sex ratio: 49% females), (ii) on June 29th (sex ratio: 46% females), and (iii) on July 12th (sex ratio: 46% females).

On September 28th, 2022, pots were removed from the cages and turned over onto plastic trays and the soil was carefully inspected to record dead and live *P. japonica* larvae. Furthermore, the substrate from each pot was mixed and a sample (200 mL) was collected in a plastic bag for successive evaluation of EPF and EPN persistence in the pots, as described in section 2.5.

2.4. Curative BCA-application during Popillia japonica larval development

This experiment was carried out in 2022 and 2024. In the first year, 104 pots (16 pots/treatment and 24 pots for control) with young cherry trees were used. On September 7th, 2022, five 3rd instar larvae of *P. japonica* were introduced into each pot to a depth of 5 cm. These larvae had been collected the day before in Mottalciata, Biella (see above) in a field which has never been treated with BCAs before.

After one week, the treatments were applied as described in section 2.3, and the pots were arranged in a randomized block design. Fifteen days after treatment, 8 pots treated with EPNs (POP16 and HbCom) and 8 control pots were randomly selected for assessment of the number of alive and dead larvae. The same procedure was repeated 30 days after treatment. Pots treated with EPF (Bip5, SAPs, and FCBK), were also examined twice, 30 and 60 days after treatment. The delayed evaluation of EPF treated pots was necessary to account for the comparably slower mode of action of EPF. In addition, substrate samples were taken for assessment of EPF and EPN persistence in the pots (see above).

In September 2024, the curative experiment was repeated on a reduced scale. The experimental procedure was similar to the one in 2022, however, only EPN, that had produced optimal results in 2022, were tested, and evaluation dates were set to 30 days and 60 days after treatment, to allow more time for the EPNs to kill the larvae. A total of 48 pots (16 pots/treatment and 16 for the control) was included.

2.5. Evaluation of EPN and EPF persistence in treated soils

For the evaluation of EPN persistence, soil samples treated with EPNs and those taken from controls were analyzed. Free-living nematodes were extracted from each soil sample using Oostenbrink dishes (EPPO, 2013), collected in 1.5 ml vials, and centrifuged at 2000 x g for 5 min. DNA was extracted from the nematode pellets using DNeasy PowerSoil Pro Kit (QIAGEN) following the manufacturer's specifications using the QIAcube (QIAGEN) extractor. The final elution step was performed in 50 μl of sterile ddH$_2$O.

The number of *H. bacteriophora* in the samples was quantified by qPCR amplification using species-specific primers and a probe targeting the ITS2 region and subsequent comparison to a standard calibration curve. Primers and probes were designed based on an alignment of 241 *H. bacteriophora* ITS sequences, including sequences from public databases (GenBank, NCBI). Moreover, others were obtained from POP16 and other local *H. bacteriophora* populations (Supplementary file S1).

Amplification was performed in a total volume of 20 μl containing 0.3 μM of each primer H.bact581F (5′- GTC GAG CGG TGT ACT GTT GA −3′) and H.bact644R (5′- ATA GAA GAC ATG TTG CCC CAT −3′), 0.1 μM of probe H.bact601P (5′- FAM-AAG TAC CCC GTT CAA GTA TCT TT-BHQ1 −3′), 0.3 mg/ml non-acetylated BSA (ThermoFisher) and 1X Taqman Fast Universal PCR Master Mix (ThermoFisher). Amplification was performed in a LightCycler 480 II real-time thermocycler (ROCHE) following the subsequent thermal program: 2 min at 50°C as hot start activation followed by 3 min at 94°C as initial denaturation step, 40 cycles of denaturation at 94°C for 30 s and primer annealing/extension at 60°C for 40 s. Fluorescence data were acquired at the end of the annealing/extension step.

Primers and probes were experimentally tested for specificity and sensitivity using other nematode species and populations previously isolated in the experimental area.

The number of *H. bacteriophora* specimens present in each sample was estimated through comparison with a standard calibration curve. The calibration curve was obtained from a flask-reared colony of *H. bacteriophora* POP16. Specimens of both sexes in mixed stages were considered to account for biological variability. Specimens were

individually counted, setting the calibration range from 10 to 100 nematodes (calibration points 10 – 25 – 50 – 75 and 100 nematodes). Each calibration point was calculated as a mean C_p of 9 amplifications consisting of three biological replicates analyzed in three technical replicates. DNA extractions of standardized samples were performed by adopting the same protocol as previously described.

The soils treated with EPF were analyzed at Agroscope in Zurich (Switzerland) with a soil dilution method described by Kessler et al. (2003), to assess the amount of *Metarhizium* colony forming units (CFUs) present in the potting substrates. Twenty grams of well mixed soil were diluted in 100 ml of an aqueous tetra-sodiumpyrophosphate solution (1.8 g $Na_4P_2O_7$ / L). Flasks were shaken for a minimum of 3 h at 110 rpm. One hundred microliters (μl) of the supernatant of the solution were plated on selective medium (Strasser et al. 1996) and incubated for 2 weeks at 22°C and 70% RH in darkness. Afterwards, *Metarhizium* colonies (CFU) on plates were counted. One randomly selected soil sample per treatment was weighed before and after drying at 110°C for 24 h in order to be able to calculate the number of CFU per gram of substrate dry weight.

2.6. Statistical analysis

The Kruskal-Wallis and post hoc Conover-Iman tests were used in both preventive and curative BCA-application experiments to compare the number of living larvae among the untreated (control) and treated pots. These tests were also used to assess the persistence of nematodes (number of free-living nematodes among control and treatments with POP16 and HBcom) and fungi (number of CFU/dry weight among control and treatments with Bip5, SAPs, FCBK) in the potting medium. Benjamini-Hochberg correction was applied to the paired tests. Data from both the curative BCA-application experiments (2022 and 2024) were also analyzed using the Aligned Rank Transform (ART) procedure (Salter and Fawcett, 1993) with Treatment and Time as the independent factors and the number of alive larvae as the dependent variable to spot the best time of treatment application. A Tukey's HSD post-hoc test was conducted to determine which specific levels differed significantly when significant main effects were observed. The alpha level for all statistical tests was set at $p = 0.05$. All the tests were performed using R software (R Core Team, 2025); the ARTool R package was used for the ART analysis (Kay et al., 2025; R Core Team, 2025). The percentage reduction of living *P. japonica* larvae in treated pots at each sampling time was calculated using the Henderson and Tilton (HT) formula.

3. Results

3.1. Preventive BCA-application during the flight period of Popillia japonica adults

The number of living *P. japonica* larvae collected from the potting soils was different among treatments (chi-squared = 56.36; df = 5; $p < 0.0001$). Post hoc tests revealed differences between pots treated with EPNs compared to the control and to pots treated with EPF. No difference was found between the latter (Fig. 1).

In particular, only one single living larva of *P. japonica* was found in only one of the 24 pots treated with, the natural (POP16) and the commercial *H. bacteriophora* (HbCom).

Regarding the presence of *H. bacteriophora* specimens in the treated soil, quantified by qPCR as described in section 2.5, a difference was observed among the two EPN treatments and the control (chi-squared = 11.1; df = 2; p = 0.0039; Table 2A). The native population (POP16) was more abundant than the commercial one (Hbcom). However, the number of nematodes found in the pots three months after treatment was very low compared to the initial dose applied (47,100 IJs).

Significantly more CFU were found in potting substrates treated with EPF than in the control (chi-squared = 168.76; df = 3; p< 0.0001; Table 2B). In addition, the commercial *M. brunneum* (Bip5) was more abundant than the indigenous strain *M. robertsii*, irrespective of formulation (SAPs or FCBK).

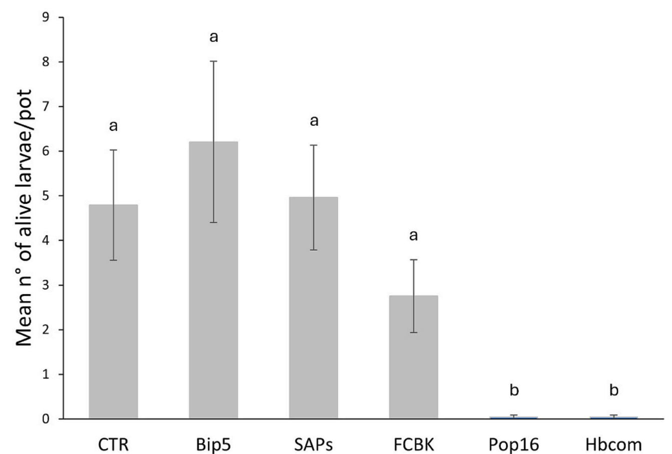

Fig. 1. Average number of alive *P. japonica* larvae (± SE) found in the soil of pots untreated (CTR) and treated with a commercial strain of *Metarhizium brunneum* (Bip5), two different formulations of *Metarhizium robertsii* (SAPs and FCBK), and with the *H. bacteriophora* native population (POP16) and commercial product (Hbcom). Different letters among treatments indicate significant differences in Conover-Iman tests after Benjamini-Hochberg correction. Bars indicate the standard error.

Table 2
(A) Average number (± SE) of *Heterorhabditis bacteriophora* specimens extracted from pots treated with the native population (POP16) and the commercial product (Hbcom). **(B)** Average number (± SE) of *Metarhizium* CFU/g dry weight isolated from pots treated with the commercial strain of *M. brunneum* (Bip5) and two different formulations of the native *M. robertsii* (SAPs and FCBK). Different letters in brackets indicate significant differences among treatments according to Conover-Iman tests.

(A)	Entomopathogenic nematodes			(B)	Entomopathogenic fungi		
	CTR	0.2 ± 0.2	(a)		CTR	13 ± 5	(a)
	Hbcom	1.0 ± 0.6	(a)		Bip5	18,457 ± 1,022	(b)
	POP16	32.8 ± 13.8	(b)		SAPs	14,105 ± 1,379	(c)
					FCBK	14,627 ± 668	(c)

3.2. Curative BCA-application during Popillia japonica larval development

In 2022, 15 days after treatment with EPNs the number of alive larvae in the treated pots was lower than in the control pots (chi-squared = 17.32; df = 2; p = 0.00017). In addition, the native *H. bacteriophora* population (POP16), caused 83% mortality, significantly higher than that caused by the commercial product (Hbcom, 60%, Fig. 2A).

A significant difference was also observed among treatments 30 days after treatment (chi-squared = 34.72; df = 5; $p < 0.0001$; Fig. 2B). At this time point, mortality of *P. japonica* larvae in EPNs treatments was significantly higher than that in the control and EPF treatments. The commercial and natural *H. bacteriophora* caused 87% and 79% larval mortality, respectively. Comparably low mortality rates were observed in pots treated with EPF with 18%, 11%, and 8% for SAPs, Bip5, and FCBK, respectively. None of these results was significantly different from the control. No difference in larval mortality was observed 60 days after treatment between the three EPF treatments and the control (chi-squared = 3.73; df = 3; p = 0.2919). Again, mortality rates of *P. japonica* larvae in EPF treatments were low (SAPs 17%, FCBK 11%, Bip5 3%; Fig. 2C).

In the experiment conducted in 2022 with both the natural population POP16 and the commercial product, a significantly higher efficacy

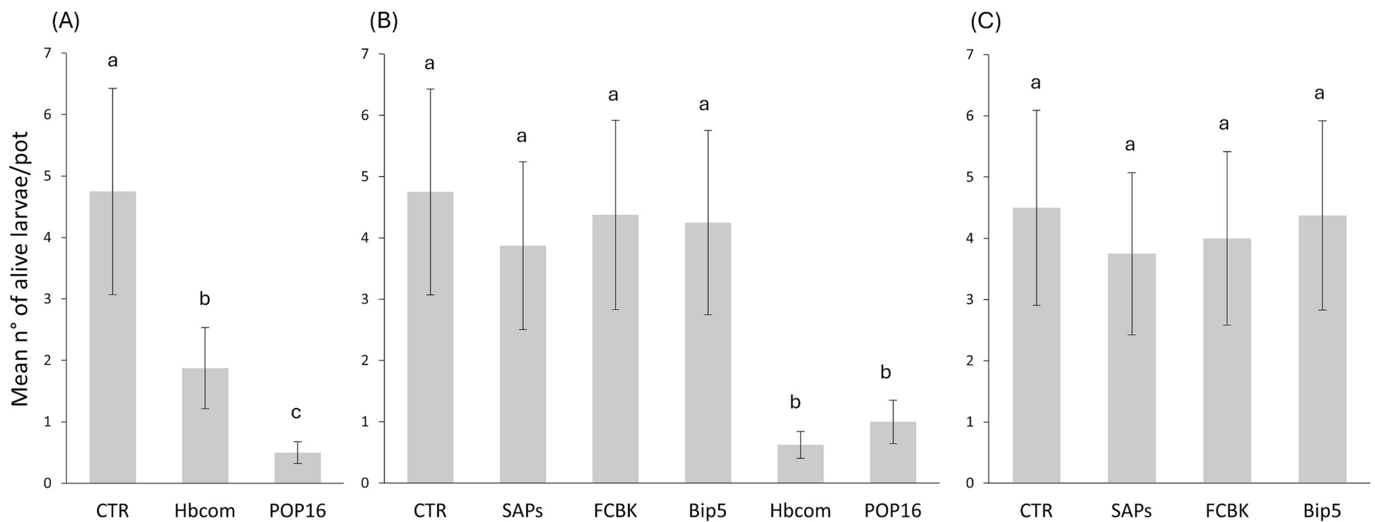

Fig. 2. Average number of alive *P. japonica* larvae (± SE) found in the soil of untreated pots (CTR) and of pots treated with the commercial strain of *M. brunneum* (Bip5), two different formulations of the native *M. robertsii* (SAPs and FCBK), and the native population (POP16) and the commercial product (Hbcom) of *H. bacteriophora*. Figures show *P. japonica* mortality (A) 15 days, (B) 30 days, and (C) 60 days after treatment in 2022. Different letters among treatments indicate significant differences according to Conover-Iman tests.

compared to the control group at both 15- and 30-days post-treatment (Treatment: $F = 50.75$; $df = 2$; $p < 0.0001$ – Time: $F = 3.43$; $df = 1$; $p = 0.07095$) was highlighted also by the ART procedure. However, no significant differences were observed between or within the two EPN at any time point during the trial.Regarding EPF, no significant efficacy at either 30 or 60 days after treatment (Treatment: $F = 1.87$; $df = 3$; $p = 0.14503$ – Time: $F = 0.22$; $df = 1$; $p = 0.63832$) was recorded.

The application of EPNs in the second repetition in 2024 yielded similar results as in 2022. Entomopathogenic nematodes significantly reduced the abundance of *P. japonica* larvae both at 30 (chi-squared = 16.65; $df = 2$; $p = 0.00024$; Fig. 3A) and at 60 days (chi-squared = 18.08; $df = 2$; $p = 0.00012$; Fig. 3B) after treatment. After 30 days, *P. japonica* larvae corrected (HT) mortality reached 82.4% and 88.2% in the Hbcom and POP16 treatments, respectively. *Popillia japonica* larval mortality further increased 60 days after treatment, reaching mortality rates of 97% and 90.9% for Hbcom and POP16, respectively. The ART procedure conducted to assess the effectiveness of EPNs over time revealed a significant reduction in larval numbers compared to the control group at both 30- and 60-days post-treatment (Treatment: $F = 43.82$; $df = 2$; $p < 0.0001$ – Time: $F = 1.50$; $df = 1$; $p = 0.22768$), but no significant differences were detected between or within the two EPN treatments at any time point during the experiment.

All EPNs and EPF used in different treatments were re-isolated from the soil of the treated pots in 2022 and 2024. While no *H. bacteriophora* were isolated from the soil of untreated pots (CTR), few *Metarhizium* CFUs (an average number of 97,7 CFU/g dry weight) were isolated from the untreated soil 60 days after treatments.

In 2022, the average number of *H. bacteriophora* specimens extracted from the soil of the pots treated with the two EPN treatments was not different 15 days after application (chi-squared = 0.28; $p = 0.599$), nor at 30 days (chi-squared = 2.82; $p = 0.0929$). In 2024, the average number of *H. bacteriophora* specimens extracted from the soil at 30 days after treatment showed a significant difference between the two EPNs treatments (chi-squared = 4.87; $df = 1$; $p = 0.0273$), with a higher number of nematodes present in the soil of the pots treated with the native population (POP16) compared to the commercial one (Hbcom).

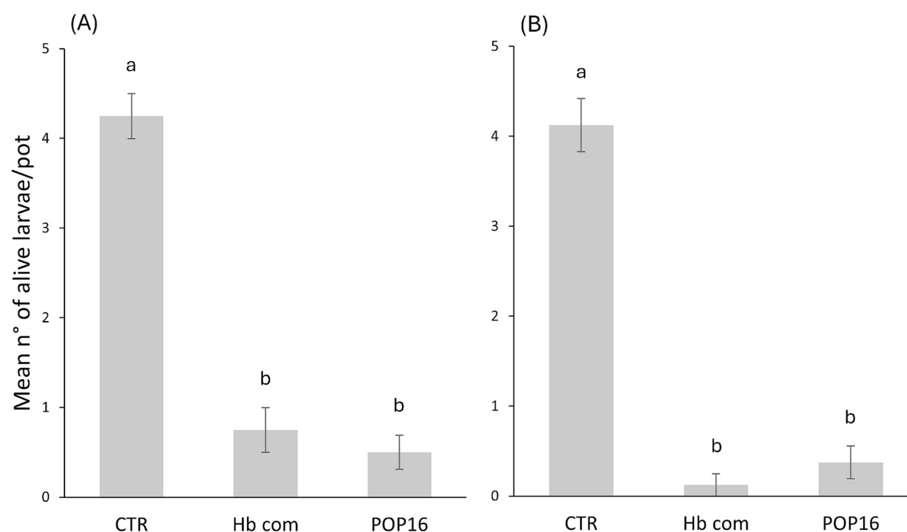

Fig. 3. Average number of alive *P. japonica* larvae (± SE) found in the soil of untreated pots (CTR) and of pots treated with the native population (POP16) and the commercial product (Hbcom) of *H. bacteriophora*, (A) 30 days and (B) 60 days after treatment in 2024. Different letters among treatments indicate significant differences according to Conover-Iman tests.

However, no significant difference was observed 60 days after treatment (chi-squared = 3.19; df = 1; p = 0.07399) (Table 3).

Regarding EPF, no statistical differences among treatments were obtained 30 days after treatments (chi-squared = 0.30; df = 2; p = 0.8587). At 60 days, a higher abundance of the native EPF strain formulated as FCBK was observed compared to the other two treatments (chi-squared = 20.06; df = 2; p < 0.0001) (Table 4).

4. Discussion

One possible pathway of introduction and spread of *P. japonica* to so far un-infested areas is the unintended transport of the pest's subterranean life stages in the substrate of potted plants (EFSA, 2018). Since the arrival of *P. japonica* in Italy in 2014, proactive measures have been implemented to address this challenge of passive transport (Gotta et al., 2023). Nurseries within the affected area have been subjected to restrictive measures for the production and trade of potted plants. In particular, the movement of potted plants from the infested zone outside the demarcated area is prohibited resulting in an economic threat to the nurseries within this area. The Regional Phytosanitary Service may authorize such movement under certain conditions, most importantly when the risk of unintended transport of the invasive pest is negligible. Building on Mori et al. (2022), our study tested the hypothesis that the application of biological control agents (BCAs) into pot substrates can significantly reduce the survival of *P. japonica* larvae and thereby limit their unintentional movement through plant trade.The results of this study confirm that *H. bacteriophora* is highly effective in controlling *P. japonica* larvae in potted plants, particularly when applied preventively, before oviposition. It is important to note that our experiments were conducted under extreme conditions, where 1,410 adults were artificially confined during the flight period in 4.05 m^3 cages and forced to oviposit into the pots of cherry plants without any choice. Despite the extremely high artificial oviposition pressure applied in the experiment, only one larva was found in a single pot of the POP16 and Hbcom treatments, respectively. In contrast, more than 100 larvae (a mean of 3–6 larvae/pot) were found in control pots (CTR) and in those treated with entomopathogenic fungi. Under this artificially high population pressure, it is highly unlikely that adults avoided ovipositing in EPN-treated pots. The exact mode of action of the EPN treatments against *P. japonica* during the oviposition period remains unclear. A straightforward explanation is that oviposition itself was not influenced, but neonate larvae hatching from eggs in EPN-treated pots were quickly infected and killed by nematodes in the substrates. Although EPNs can alter belowground chemical cues, including plant mediated VOCs in natural systems (Helms et al., 2019), such mechanisms are unlikely to have influenced oviposition here, where adults had no choice of substrate. Rather, the results reflect high EPN persistence and activity throughout the experiment, consistent with previous reports of their ability to recycle within hosts and maintain infective populations over time (Griffin, 2015). Our persistence data support this interpretation. In 2022, nematode abundance did not differ between the two EPN treatments at 15 or 30 days after application, and in 2024 POP16 showed higher persistence only at 30 days, with no difference at 60 days. Overall, both populations of *H. bacteriophora* remained active throughout the oviposition period, providing sustained infective pressure sufficient to kill early instars shortly after hatching. This correspondence between EPN persistence and nearly complete larval suppression suggests that the effect of a preventive *H. bacteriophora* application resulted from infection of neonate larvae rather than reduced oviposition.

Table 3
Average number of *H. bacteriophora* specimens extracted from pots treated with the native population (POP16) and commercial (Hbcom) product at 15, 30, and 60 days after treatment in 2022 and 2024. Different letters in brackets indicate significant differences among treatments according to Kruskal-Wallis test. The data were analyzed separately for each year.

Entomopathogenic nematodes						
Days after treatment	2022 POP16	HBcom	2024 POP16		HBcom	
15	189.4 ± 43.4	339.8 ± 113.6				
30	319.4 ± 48.7	246.6 ± 136.1	129.9 ± 18.2	(a)	82.6 ± 10.7	(b)
60			77.3 ± 9.0		105.0 ± 11.2	

Table 4
Average number of *Metarhizium* CFU/g dry weight isolated from pots treated with the commercial strain of *M. brunneum* (Bip5) and two different formulations of the native *M. robertsii* (SAPs and FCBK) at 30 and 60 days after treatment in 2022. Different letters indicate significant differences among treatments according to Conover-Iman tests.

Entomopathogenic fungi			
Days after treatment	SAPs	Bip5	FCBK
30	135,101 ± 13,266	132,260 ± 15,814	120,164 ± 15,383
60	11,047 ± 1,278 (a)	38,889 ± 7,855 (a)	93,876 ± 11,963 (b)

In the second experiment, cherry tree pots were treated with EPN and EPF only after an artificial inoculation of the pot substrates with *P. japonica* 3rd instar larvae. The best results were again obtained with the application of EPNs. Larval mortality after curative treatment increased from 60% at 30 days to 97% at 60 days for the commercial product, Hbcom, whereas for the native population POP16 it increased from 79 to 90.9% over the same period. A comparison of these results with the ones from the preventive treatment above indicates that *P. japonica* larvae in the first and second instar are more susceptible to EPNs than those in third instar. This hypothesis is in line with results from previous studies (Koppenhöfer and Fuzy, 2004; Power et al., 2009).

Moreover, the extended interval between the treatment and pot assessments in the preventive experiment may have favored the proliferation of nematodes within *P. japonica* larvae, thereby sustaining their efficacy as long as susceptible hosts were present (Modic et al., 2020).

Contrary to EPNs, the application of EPF did not substantially affect the abundance of *P. japonica* in the substrates of potted plants, no matter if applied preventively or curatively. The number of living larvae found in the pots available for adult oviposition was not different from that of the control and in some cases even tended to be slightly higher. One might expect that the failure of the fungal treatments might derive from poor establishment of EPF in the pot substrates. However, our results (Tables 2 and 4) show clearly that EPF established nicely in pots, irrespective of applied EPF species or formulation. We therefore conclude that the subterranean developmental stages of *P. japonica* larvae may be resistant to *Metarhizium robertstii* and *M. brunneum*. Graf et al. (2023) have shown in lab experiments that *P. japonica* 3rd instar larvae are not susceptible to different strains of *M. brunneum* and *Beauveria brongniartii*. Even an injection of spores into the haemolymph of *P. japonica* 3rd instar larvae did not lead to high infection levels in their study, although they were significantly elevated when compared to EPF surface applications. By contrast, Ramoutar et al. (2009) and Behle et al. (2015) found clear effects of EPF applied against *P. japonica* larvae in turf grass experiments. To explain these contradicting results, Graf et al. (2023) proposed that sub-optimal moisture levels may have impaired their own results, and that younger larval instars of *P. japonica* may be more susceptible to fungal attack than the 3rd one. However, substrates in our experiment did never fall dry over the entire season, since pots were equipped with a drip irrigation system. Moreover, we treated the first experiment in May before start of the flight season, and evaluated it only by end of September, when *P. japonica* had run through most of the pre-imaginal developmental stages despite the presence of fungal inoculum

in the treated substrates. Our results therefore indicate that the EPF treatment did not noticeably affect oviposition, eggs production, neonate, or the development of early larval instars, suggesting that *P. japonica* pre-imaginal stages generally show considerable resistance to EPF infection. Furthermore, mortality in treated pots after release of the larvae into the soil did not exceed 17–18% for *M. robertsii* formulated as SAPs, 3–11% for the commercially available product of *M. brunneum* (Bip5), and 8–11% for *M. robertsii* formulated as FCBK. Thus, an application of EPF into the substrate of potted plants is unlikely to achieve a level of efficacy which is sufficient to significantly reduce the phytosanitary risk associated with the trade of potted plants. By contrast, EPNs have proven to be a promising option for controlling *P. japonica* larvae in environments at risk of oviposition by *P. japonica* adults, such as the pot substrates of host plants in the nursery. We assume that the constant humidity of the soil in the pots, maintained through irrigation systems, creates an optimal environment for both the vitality and activity of the EPNs. Additionally, this arrangement has optimal conditions for *H. bacteriophora* reproduction, which makes this BCA a viable method for controlling *P. japonica* larvae (Marianelli et al., 2018; Torrini et al., 2020).

In conclusion, the use of *H. bacteriophora* was effective in reducing *P. japonica* larvae in substrates of potted nursery plants, particularly when applied before the adult beetles' oviposition period. Although the EPN treatments did not completely eliminate larvae from the pot soil, as would be required for a "pest-free" status by plant health authorities, the observed levels of larval suppression were impressive. It is worth noting that even chemical insecticides rarely achieve 100% control of *P. japonica* larvae under similar conditions (Mannion et al., 2001; Oliver et al., 2009). While this study did not include a direct comparison with chemical insecticides, the results indicate that EPN treatments may offer a promising biological control approach that could complement integrated pest management strategies. Keeping in mind the demand to reduce the input of chemical synthetic insecticides, the application of EPNs is the best trade-off between phytosanitary requirements and environmental safety and should be included into an integrated strategy against *P. japonica*. Combining data from the 2022 and 2024 experiments, we found that EPNs are already effective 15 days after application, with no significant improvement observed at 60 days. Therefore, a 15–30-day interval appears to offer an optimal balance between biological efficacy and practical feasibility for nurserymen. This period is likely sufficient to target both pre-existing larvae in the potting soil and newly hatched first instars from eggs laid during the application window.

Future research should focus on optimizing application methods and exploring the use of *H. bacteriophora* combined with other control methods in nursery environments, to ensure its broader adoption as part of the integrated pest management strategy of the invasive pest.

Data availability statement

Data are available upon request to the corresponding author.

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CRediT authorship contribution statement

Giulia Torrini: Writing – original draft, Investigation, Data curation, Conceptualization. **Gian Paolo Barzanti:** Writing – review & editing, Investigation, Formal analysis, Data curation, Conceptualization. **Fionna Knecht:** Resources, Investigation, Data curation. **Giselher Grabenweger:** Writing – original draft, Supervision, Project administration, Conceptualization. **Immacolata Iovinella:** Writing – review & editing, Investigation, Formal analysis, Data curation. **Francesco Paoli:** Writing – review & editing, Investigation, Data curation, Conceptualization. **Claudia Benvenuti:** Investigation. **Giuseppe Mazza:** Writing – review & editing, Investigation, Conceptualization. **Giuseppino Sabbatini Peverieri:** Writing – review & editing, Resources, Investigation. **Agostino Strangi:** Writing – review & editing, Investigation. **Chiara Sciandra:** Writing – review & editing, Investigation. **Stefania Simoncini:** Resources, Investigation. **Arne Peters:** Writing – review & editing, Resources, Investigation. **Francesco Barbieri:** Investigation. **Leonardo Marianelli:** Writing – original draft, Supervision, Project administration, Investigation, Conceptualization.

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

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