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# Using *Bursaphelenchus mucronatus* to demonstrate the potential nematicidal effect of *Beauveria bassiana* on pine wood nematode (*Bursaphelenchus xylophilus*) under in vivo conditions

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## Abstract

***Key message*** This study demonstrates the potential of *Beauveria bassiana* (Bals.—Criv.) Vuill. to control *Bursaphelenchus mucronatus* (Mamiya and Enda), which is close to *Bursaphelenchus xylophilus* (Steiner and Buhrer) Nickle but is a non-quarantine pathogen and, therefore, may be used as an alternative organism on which to perform in vivo assays without biological risk.

**Context** Pine wilt disease (PWD) is a serious threat for conifer forests worldwide. It is caused by *Bursaphelenchus xylophilus*, the pine wood nematode (PWN). In affected areas, eradication and subsequent disease containment measures are being implemented. The latter are, to date, based on control strategies for the insect vectors (*Monochamus* spp.) and on screening for genetic resistance in tree hosts. However, an integrated pest management strategy which also implements nematode control is still not fully developed.

**Aims** This study aimed to use *Bursaphelenchus mucronatus*, as an organism on which to demonstrate the nematicidal potential of *Beauveria bassiana*, an entomopathogenic fungus successfully tested on *Monochamus* spp., on PWN under in vivo conditions.

**Methods** To this end, a pathosystem was built to simulate these conditions and to bring the nematode *B. mucronatus*, the insect vector, and the fungus into contact.

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**Results** The results show (i) very similar responses of the two nematodes confronted to the fungus and its mycotoxin beauvericin under in vitro conditions and (ii) a remarkable antagonistic effect of *B. bassiana* on *B. mucronatus* also on the abovementioned pathosystem (in vivo conditions).

**Conclusion** Our findings have significant implications for the pine wilt disease control. In particular, this study demonstrates the potential of *B. bassiana* as a biological control tool to be implemented in a future integrated disease management strategy.

**Keywords** Pine wilt disease, Integrated management, Biological control, Nematophagous fungi

## 1 Introduction

*Bursaphelenchus xylophilus* (Steiner & Buhrer) Nickel, a member of the plant-parasitic nematode group (PPNs), threatens coniferous forests worldwide. This pathogen prompts gradual decay of affected trees, resulting in the development of pine wilt disease and ultimately leading to the death of the trees. While believed to have originated in North America, the first documented detection of PWN was in Japan during the early twentieth century. From there, the pathogen rapidly propagated to various other Asian countries (Kim et al. 2020). In the late 1990s, PWN was introduced to Europe through Portugal (Mota et al. 1999), causing substantial damage across more than 30% of the nation's forest area. Currently, several Spanish regions—specifically Extremadura, Galicia, and Castilla y León—are dealing with its impact (Zamora et al. 2015). The pathogen is classified as a quarantine pest in Europe (List A2) (EPPO 2016) due to its high pathogenicity and transmissibility. Furthermore, it is anticipated that the PWD will exacerbate under projected climate change scenarios (Hirata et al. 2017; De la Fuente et al. 2018; Tang et al. 2021).

The PWN needs an insect vector to translate from dead or dying trees to healthy ones, specifically beetles from the genus *Monochamus* (Coleoptera: Cerambycidae), *M. galloprovincialis* Olivier in Europe (Akbulut & Stamps 2012; Naves et al. 2016). The spread is mainly conducted by these insects through feeding (Mamiya and Enda 1972) and, once the nematodes have entered the tree, they lodge in the resiniferous canals and feed on epithelial and parenchymal cells, triggering a host response which leads to disruption of water transport and rapid death by cavitation (Fukuda and Suzuki 1988; Hara et al. 2006).

Up to now, the main eradication measure in the EU, if the presence of *B. xylophilus* is confirmed in a tree, is the felling of all individuals within the *clear-cut area* of 500 m radius (European Commission 2012; MAPA 2020). Subsequent management in the demarcated areas is based on strategies for mitigating the disease that have revolved around the pursuit of genetic resistance within the host trees and the management of disease transmission by reducing populations of the insect vector. The earliest breeding programs for genetic resistance were developed in Japan back in 1978, specifically targeting *Pinus thunbergii* Parl. and *P. densiflora* Siebold and Zucc. and obtaining high rates of post-inoculation survival for both species (Fujimoto and Ohba 1981; Toda and Kurinobu 2002). Since then, numerous similar programs have been implemented, spanning across various tree species like *P. massoniana* Lamb. in China (Liu et al. 2017; Zhu et al. 2021), *P. pinaster* Aiton in Portugal and Spain (Gaspar et al. 2017; Carrasquinho et al. 2018; Menéndez-Gutiérrez et al. 2018), and *P. radiata* D. Don in Spain (Zas et al. 2015; Menéndez-Gutiérrez et al. 2021). In terms of insect vector control, several strategies have been developed to reduce *M. galloprovincialis* populations starting by mass trapping combining pheromone and kairomones (Álvarez-Baz et al. 2016; Galloprotect 2D®), followed by the use of entomopathogenic fungi (Naves et al. 2008; Petersen-Silva et al. 2015; Álvarez-Baz et al. 2015) and finally the development of an auto-infection device based on the combined use of the attractant and the entomopathogenic fungi (Sacristán-Velasco et al. 2018; Sacristán-Velasco and Martín-García, 2024). Thus far, only the use of mass trapping has been implemented widely, but successful field trials have been performed combining this system with the auto-infection device and powdered formulations of an entomopathogenic fungus (J. Martín-García, unpublished data).

Despite progress in those areas, an effective integrated management technique for this disease including also nematode control has not yet been fully developed. Advances towards this goal have been made with studies on the effect of fungi on different PPNs (Mankau 1980; Askary 2015; Abd-Elgawad and Askary 2018). For PWN, the most studied antagonistic fungal species is *Esteya vermicola* J.Y.Liou, J.Y.Shih and Tzean, with a well-proven efficacy against this pathogen (Kubátová et al. 2000; Wang et al. 2008, 2009, 2018; Lin et al. 2013; Pires et al. 2022). Likewise, other fungal genera such as *Verticillium* spp. or *Trichoderma* spp. have exhibited nematicidal effect on PWN populations too (Maehara and Futai 2000). Especially noteworthy is the proven nematicidal effect of different species of the genus *Beauveria* on PWN populations (Sánchez-Gómez et al. 2023). This genus has also a

widely demonstrated entomopathogenic effect on *M. galloprovincialis*, insect vector of PWN (Naves et al. 2008; Petersen-Silva et al. 2015; Álvarez-Baz et al. 2015). This is why it might serve as an efficient contributor for the future integrated pest management. However, this potential control method has not been examined in depth, let alone with in vivo assays simulating forests conditions.

This paucity of studies under in vivo conditions, particularly in Europe, may be due to the risk involved when bringing PWN into contact with the insect vector that requires working in safety laboratories. Therefore, the option of working with a closely related pathosystem (Robinson, 1976; Gleason et al. 2013) which includes a similar nematode species but not classified as a quarantine pathogen is seen as a good option to minimize this risk. Within the genus *Bursaphelenchus*, *B. mucronatus* (Mamiya and Enda) is taxonomically very close to *B. xylophilus* (Braasch 2001, 2004; Bolla and Wood 2004; Pereira et al. 2013). Due to their morphological and biological similarities, both species belong to the *xylophilus-group*, according to the most widely accepted *Bursaphelenchus* spp. classifications (Ryss et al. 2005; Braasch and Schönfeld 2015). In fact, the two species are so close that they may even hybridize, although F1 hybrids failed to generate genetically separated offsprings in further generations (Liu et al. 2019; Li et al. 2021). Concerning pathogenicity, although *B. mucronatus* can also cause damage to pine seedlings in nurseries (Bakke et al. 1991; Tomminen 1993), it is much less pathogenic than its relative *B. xylophilus* (Mamiya 1999) and is not classified as a quarantine organism. For these reasons, *B. mucronatus* may be an ideal candidate for risk-free in vivo assays whose results could eventually be extrapolated to *B. xylophilus*.

Many nematicidal fungi excrete specific secondary metabolites known as mycotoxins (Anke and Sterner 1997, 2002; Li et al 2007; Anke 2011; Baazeem et al. 2021; Seong et al. 2021; Li et al. 2023). The most studied mycotoxin from genus *Beauveria* is beauvericin (BEA) and is probably its prime mechanism of action against nematodes. More specifically, it can be stated that the natural production of this mycotoxin by the strain of *B. bassiana* (Bals.—Criv.) Vuill. used in the present study was confirmed in previous experiments (Sánchez-Gómez et al. 2023). Chemically, BEA is defined as a cyclic hexadepsipeptide consisting of alternating D-α-hydroxy-isovaleryl-(2-hydroxy-3-methylbutanoic acid) and amino acid units, and its toxicity was first tested on the crustacean *Artemia salina* L. (Hamill et al. 1969) but soon found to have insecticidal (Grove and Pople 1980; Gupta et al. 1991; Ganassi et al. 2002; Leland et al. 2005; Fornelli et al. 2004) and nematicidal effects, demonstrated first on *Meloidogyne incognita* Kofoid and White (Mayer 1995) and later on *Caenorhabditis elegans* Maupas (Shimada et al. 2010) and PWN (Shimada et al. 2010).

All these in vitro proofs suggest that *Beauveria* spp. could be a potential control option against not solely the PWN insect vector, but additionally against the nematode itself. However, its nematicidal effect needs to be corroborated also under in vivo conditions. The goals of the work presented here were (i) to demonstrate that *B. mucronatus* can be used as a baseline to eventually extrapolate the results to *B. xylophilus*. To attain that objective, a similar behavior of *B. mucronatus* and *B. xylophilus* against *B. bassiana* and commercial BEA should be corroborated during in vitro experiments, especially in terms of reproduction and mortality and, if this assumption was confirmed, (ii) test the effect of *B. bassiana* on the pathosystem (tree-insect-nematode) based on *B. mucronatus* simulating natural conditions in laboratory, which could shed light on the potential of this fungus as a biological control tool against *B. xylophilus* populations.

## 2 Material and methods

### 2.1 Nematode, fungal and insect species used for the assays

*B. xylophilus* and *B. mucronatus* strains used were CSF-N-1 and CSF-N-17, respectively. Both strains come from the Forest Health Centre *Calabazanos*. The nematodes were grown using glass vials [28 mm (Ø), 43 mm (h), 25 mL (V)] with medium composed of hulled barley (autoclave sterilized 121 °C, 20 min) and non-sporulating *Botrytis cinerea* Pers.:Fr. Growing methods for *Bursaphelenchus* species are not standardized, but cultivation with *B. cinerea* grown on barley is the most widely used (Aikawa and Kikuchi 2007; Espada et al. 2016; Pimentel et al. 2020) and the one recommended by EPPO (2013). The vials were kept at 25 °C with the cover ajar, always with oxygen flow inwards, and under continuous darkness.

The fungal strain used as antagonist for in vitro and in vivo assays was *B. bassiana* EABps 11/01-Mg, cultivated using potato dextrose agar medium (PDA Scharlau, Spain). *EF1α and ITS* classification of several strains of *Beauveria* species (*B. bassiana and B. pseudobassiana* S.A.Rehner and R.A.Humber) were modified in GenBank a few years ago (see accession numbers AY531938.1 and AY531931). So, the isolate EABps 11/01-Mg was wrongly identified by Álvarez-Baz et al. (2015) as *B. pseudobassiana* based on the homology of the isolates AY531938.1 and AY531931 (previously identified as *B. pseudobassiana* and currently *B. bassiana*). However, we have analyzed the isolate EABps 11/01-Mg again (EF1α and ITS) and checked it in GenBank, and it should be classified as *B. bassiana*. In the case of in vivo trials, *Ophiostoma*

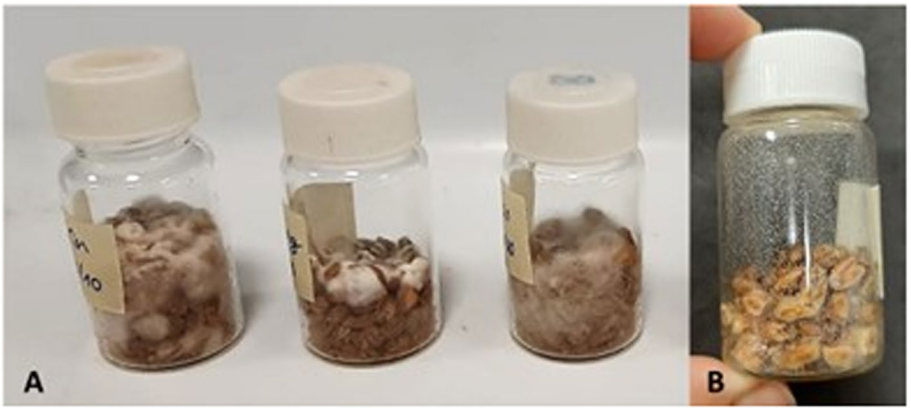


**Fig. 1** **A** Vials used for the in vitro tests on barley and mycelia. **B** Vials with *B. cinerea* before extraction, with the highest reproduction rate of nematodes

*minus* (Hedgc.) Syd. and P.Syd (strain 259 INIA, cultivated using PDA) was used to inoculate the artificial pupation chambers with the aim of facilitating the survival and distribution of the nematodes in the wood prior to the insect loading phase (Pimentel et al. 2020; Vicente et al. 2021).

The insect species used for the in vivo pathosystem was *M. galloprovincialis*, the vector of PWN in Europe. Their colonies came from 110 logs 1.20 m long of *Pinus sylvestris* L., placed in groups of 10 logs in eleven different locations around Tabuyo del Monte (León, Castilla y León, Spain) in early summer 2021 and baited with the attractant GALLOPROTECT 2D® to ensure colonization. Five months later, in November 2021, they were moved to a fully enclosed mesh cage and left there until larvae were needed in spring 2022 (see Sect. 2.4). Larvae extraction from inside the wood was carried out, on a workbench, using an electric saw to cut off wood discs. Once the internal chamber was localized, the larva was carefully extracted with entomological forceps.

### 2.2 In vitro antagonism tests of *B. bassiana* on *B. xylophilus* and *B. mucronatus* populations

Thirty glass vials, with 3 g hulled barley and 3 mL distilled water were initially autoclave sterilized (121 °C, 20 min). Fifteen vials were used for the assay with *B. xylophilus* and the other fifteen for *B. mucronatus*. Within each trial, 3 treatments with 5 replicates each were established: *B. bassiana* EABps 11/01-Mg, *B. cinerea* (positive growth control) and mock inoculated (negative growth control). A mycelium plug of each fungus was introduced into each vial, except for mock inoculated treatment, in which a plug of PDA was added, and left to grow for 13 days. After this growth period, a 100-µL aliquot with 270 nematodes was added to each vial and they were kept for a further 13 days at 25 °C and continuous darkness (Fig. 1). Finally, the extraction of the nematodes from the vials was conducted according to a slightly modified version of the Baermann funnel technique (Baermann 1917). A tea filter (M-size, Finum®) was placed with the content of each vial on an autoclaved beaker. The content was covered with autoclaved distilled water, so that the nematodes pass through the filter and remain in a clean aqueous suspension. After 24 h, they were washed with distilled water using a nylon sieve of 18 µm mesh size (NY-0073-Labopolis, Spain). The nematodes were retained by the sieve, washed with a washing bottle, and finally resuspended into 55 mm (Ø) Petri dishes. Three 100-µL aliquots of each replicate were counted to determine nematode concentration, using a counting grid and a stereo microscope with dimmable bottom light.

### 2.3 Nematicidal effect of commercial BEA on *B. xylophilus* and *B. mucronatus* populations

The effect of commercial BEA (Merck Life Science S.L.U, USA) was tested on *B. xylophilus* and *B. mucronatus.* The assays were performed in Eppendorf tubes previously autoclave sterilized at 121 °C for 20 min, with eight replicates per treatment. The mycotoxin was tested at 1 mM concentration, using 5% dimethyl sulfoxide (DMSO) as solvent. For the control treatment, only 5% DMSO was used (Fig. 2). The mycotoxin concentration was chosen based on previous studies (Shimada et al. 2010). The solvent and its concentration were determined after several pre-trial tests. Once solutions have been prepared, a 75-µL aliquot of the nematode suspensions (~400 nematodes $mL^{-1}$) were added to each tube, and initial live nematodes were counted, taking this volume into account when adjusting the concentrations. A small hole was made in the top of each tube with a heated entomological pin, to ensure oxygen flow, and the rack was covered with aluminum foil for continuous dark conditions. The

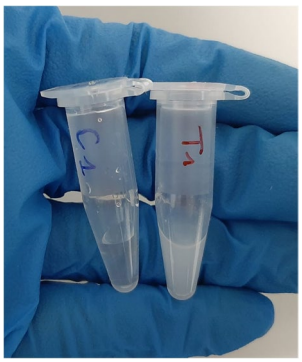

**Fig. 2** Control (DMSO 5%) and treatment (DMSO 5% + BEA 1 mM) solutions used in the BEA test on *B. mucronatus*

assay was incubated at 25 °C for 48 h, and each tube was vortex mixed once per day to resuspend the nematodes. After 2 days, the contents of each tube were poured on a grid and the live nematodes counted.

### 2.4 In vivo tests to corroborate the nematicidal effect of *B. bassiana* on *B. mucronatus* under natural conditions

For the in vivo tests, 100 artificial pupal chambers were built using *Pinus sylvestris* branches from 4 to 6 cm diameter. They were cut to 7.5 cm in length, and a hole 1 cm in diameter and 5 cm in deep was drilled in the center (Aikawa et al. 2003). This central hole was then used for inoculations with fungal spore and nematode suspensions and finally for introducing the larvae. These chambers were autoclaved at 121 °C for 20 min and then inoculated with 1 mL of a spore suspension ($10^6$ spores $mL^{-1}$) of *Ophiostoma minus* (strain INIA 259) to favor de survival of the nematodes. This suspension was prepared by adding 3–4 mycelium plugs in an Erlenmeyer with PDB and stirring at 130–150 rpm for 7 days at room temperature.

Chambers inoculated with *O. minus* were kept in a box with high relative humidity until pycnidia started to appear on the surface (Fig. 3A). At this moment, nematode inoculation was carried out by introducing 2500 nematodes from a fresh suspension into each chamber. In all cases, the volume introduced ranged from 0.7 to 1 mL, depending on the concentration of the particular suspension (Fig. 3B). Once the nematodes and water were absorbed into the wood, the larva was placed inside the artificial pupal chamber, head downwards (Fig. 3C). Finally, the chambers were covered with black cardboard to achieve total darkness. It should be noted here that the larvae were previously quick washed with 70% ethanol and distilled water to remove any external contamination they might bring from the logs of origin.

The insects continued their development inside the artificial chambers, which were placed in glass jars with the lid pierced, and these jars in turn inside boxes with high relative humidity. Imagoes emergence was gradual, always lasting between 15 and 30 days from larva

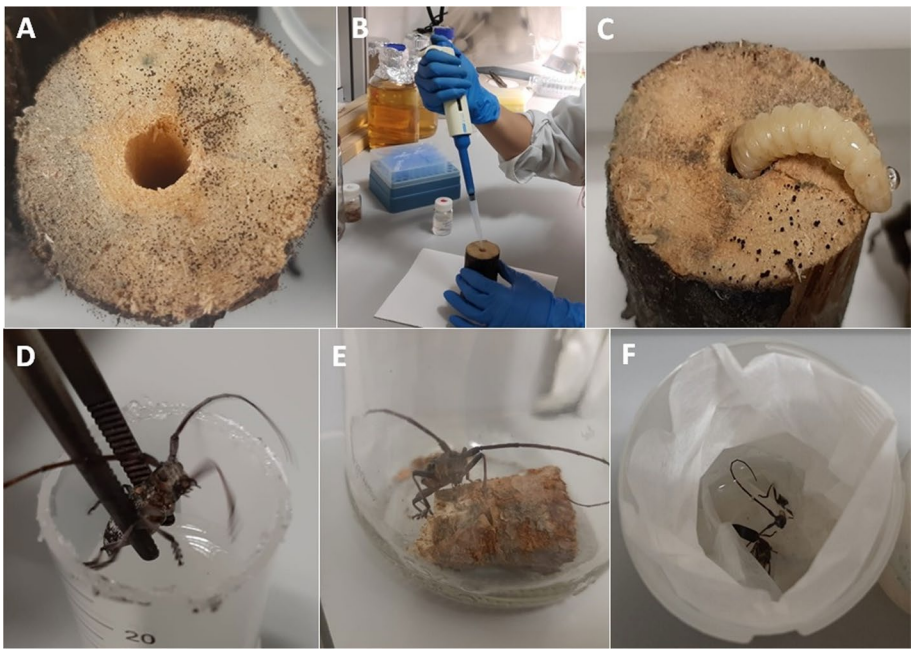


**Fig. 3** **A** Artificial pupal chamber inoculated with *Ophiostoma minus*. **B**. Inoculation with the nematode suspension **C** Introduction of the larvae. **D** Inoculation of the insect-nematodes pathosystem with *Beauveria bassiana* EABps 11/01-Mg spore suspension. **E** Maintenance and feeding of the insect. **F** Extraction of final live nematodes from the imago (modified version of the Baermann funnel technique)

**Fig. 4** Insect dissecting for nematode filtration (separation of head, thorax, abdomen and elytra)

introduction. For the trials, the insect pairs with the closest emergence dates were chosen, one of them serving as control and the other as treatment with *B. bassiana* EABps 11/01-Mg. To check if imagoes emerged loaded with nematodes, the first 10 insects were taken, and extractions were carried out. In all cases, we obtained a large number of nematodes from inside them.

*B. bassiana* EABps 11/01-Mg spore suspension was prepared by adding 3–4 mycelium plugs in an Erlenmeyer with PDB and stirring at 130–150 rpm for 5 days at room temperature. To achieve the desired concentration ($10^8$ spores $mL^{-1}$), it was necessary to concentrate the spores by centrifuging for 25 min at 4400 rpm and removing the supernatant. Finally, the spores were counted using a Neubauer chamber, and the concentration was adjusted using polysorbate 80 (Tween 80®) 1% as diluent, an emulsifying compound that helps the spores to stick onto the body of the insect. For the control treatments, Tween 80 1% without spores was used.

Inoculation with *B. bassiana* was carried out according to the methodology of Álvarez-Baz et al. 2015. (Fig. 3D). Afterwards, the insect was placed in an individual autoclaved glass jar with a pierced lid and food (Fig. 3E) for 3 days, time waited until the nematodes were extracted. The choice of a right intermediate time, sufficient to ensure the germination of *B. bassiana* spores and the growth of the hyphae inside the insect, but not causing the death of the imago, was indispensable, since the interest was to test the effects of the fungus on nematodes prior to the death of the insect (Liu et al. 2015; Sacristán-Velasco et al. 2018). The extraction (Fig. 3F) and counting of final live nematodes after these 3 days were performed as indicated in Sect. 2.2, in this case dissecting the insect prior to filtration, separating head, thorax, abdomen, and elytra (Fig. 4). In case nematodes were not obtained, extractions were made from the food (that is, from the small wood pieces) to test whether the nematodes were transmitted to the wood by insect feeding during this short period.

### 2.5 Statistical analysis

One-way analysis of variance (ANOVA) and multiple comparison procedures were performed to test the effects of *B. bassiana* and commercial BEA on nematode populations. The ANOVA assumptions (normality and homogeneity of variances) were tested in each analysis by the Shapiro and Bartlett tests. When neither assumption was violated, classical one-way ANOVA and Tukey's HSD tests was applied. When at least one of these assumptions was violated, robust statistical methods were applied (García-Pérez 2010). Heteroscedastic one-way ANOVAs were performed using the generalized Welch procedure and a 0.1 trimmed mean transformation. *T*-tests and Wilcoxon tests (when normality and/or homoscedasticity assumptions were violated) were carried out in order to corroborate the lack of significant differences in the response of the two nematode species to the treatments with *B. bassiana* and BEA commercial. All analyses were carried out using the Wilcox' Robust Statistics (WRS2) package implemented in the R software environment (R Core Team 2024). The primary dataset generated and used for the analysis of this study is available in the Zenodo repository (Sánchez-Gómez 2024).

## 3 Results

### 3.1 In vitro antagonism tests of *B. bassiana* on *B. xylophilus* and *B. mucronatus* populations

The results from in vitro experiments on *B. xylophilus* revealed significant differences among treatments ($F=38.9$; $p<0.001$). Growth of PWN populations was strongly inhibited by the presence of *B. bassiana* ($p<0.01$). The same pattern was followed in the case of in vitro tests on *B. mucronatus*, showing also significant differences between treatments ($F=15.8$; $p=0.02$) and a strong reproductive inhibition of *B. bassiana* on this nematode species ($p=0.04$). It is important to note that, in both cases, treatment with *B. bassiana* not only stopped reproduction but also resulted in high mortality of the initial introduced populations. In the negative growth control (PDA), the nematodes remained alive and even reproduced (final average population of *B. xylophilus*: 2761 ± 480; *B. mucronatus*: 324 ± 81). In contrast, in the positive growth control (*B. cinerea*), a final population exponentially higher than the initial one was garnered (*B. xylophilus*: 165,314 ± 22,217; *B. mucronatus*: 50,333 ± 13,782). Regarding the treatment with *B. bassiana*, the reproduction of the nematodes was totally inhibited (*B. xylophilus*: 30 ± 19; *B. mucronatus*: 4 ± 4) and

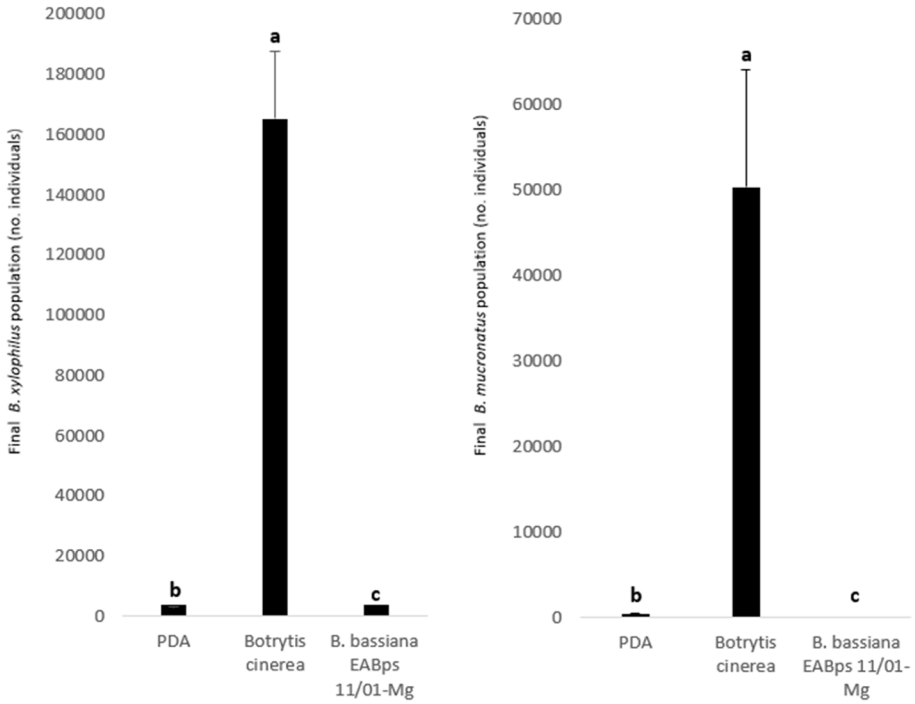


**Fig. 5** *B. xylophilus* and *B. mucronatus* final live populations under negative growth control (PDA), positive growth control (*B. cinerea*) and *B. bassiana* EABps 11/01-Mg. Bars with different letters indicate significantly different means ($\alpha = 0.05$)

a mortality rate close to 100% was obtained (*B. xylophilus*: 89.0%; *B. mucronatus*: 98.5%) (Fig. 5).

### 3.2 Nematicidal effect of commercial BEA on *B. xylophilus* and *B. mucronatus* populations

The results showed a significant nematicidal effect of the BEA commercial 1 mM treatment on both *B. xylophilus* ($F = 58.6$; $p < 0.001$) and *B. mucronatus* populations ($F = 12.7$; $p < 0.01$) (Fig. 6). A comparison of the same results in terms of corrected mortality (and standardized according to the mortality of the controls in each case) shows that the corrected mortality rates of the BEA 1 mM treatments are 44.1% (*B. xylophilus*) and 36.4% (*B. mucronatus*).

It should be also noted that, during the final visual evaluation of individuals undergoing BEA treatment, a brownish "encapsulation" was observed on *B. xylophilus* and *B. mucronatus* (Fig. 7).

All the results described in Sect. 3.1. and Sect. 3.2. lead to a single conclusion: *B. mucronatus* and *B. xylophilus* have very similar response patterns facing the fungi *B. bassiana* and the commercial mycotoxin BEA, at least in terms of reproduction and mortality. In order to statistically support these statements, a comparison of means between each of the analogue treatments of the different trials with both nematode species was carried out. These analyses corroborate that there are not significant differences in the responses of *B. xylophilus* and *B. mucronatus* to *B. bassiana* ($W = 16$; $p = 0.37$). In the case of the commercial mycotoxin BEA, a higher mortality was found in *B. xylophilus* (PWN) ($t = 6.37$; $p < 0.001$). Consequently, the in vivo assays were performed only with the species *B. mucronatus*, assuming that results could eventually be extrapolated to *B. xylophilus*.

### 3.3 In vivo tests to corroborate the nematicidal effect of *B. bassiana* on *B. mucronatus*

Initial results obtained from in vivo tests suggest a potential nematicidal effect of *B. bassiana* on phoretic *B. mucronatus* populations found in *M. galloprovincialis* individuals ($F = 3.8$; $p = 0.08$). As it can be seen in Fig. 8, while the average number of nematodes obtained from the control treatment insects was 691 (per individual), this value only reached 267 in the case of the *B. bassiana* treatment, i.e., the final live population was three times smaller.

## 4 Discussion

### 4.1 In vitro antagonism tests of *B. bassiana* on *B. xylophilus* and *B. mucronatus* populations

As noted above, the effects produced by *B. bassiana* on *B. xylophilus* and *B. mucronatus* are uncannily similar: total inhibition of reproduction and mortality close

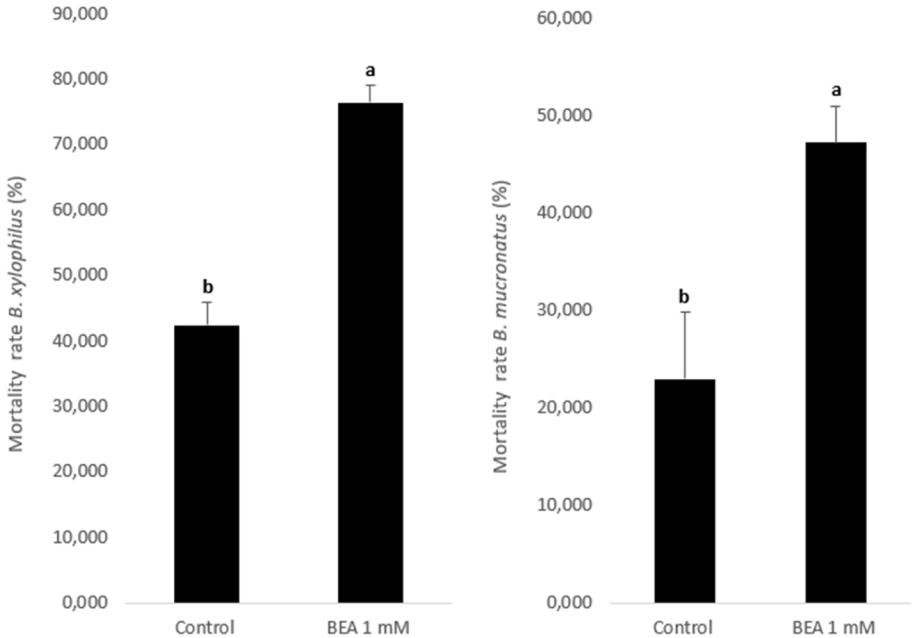


**Fig. 6** Mortality rate of *B. mucronatus* and *B. xylophilus* populations under control treatment (DMSO 5%) and under treatment with commercial BEA. Bars with different letters indicate significantly different means ($\alpha = 0.05$)

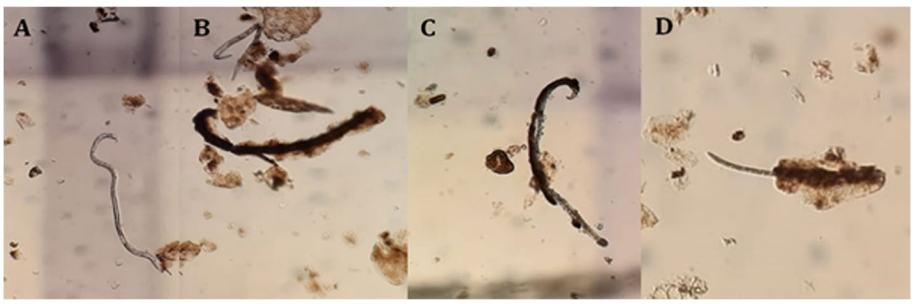


**Fig. 7** *B. mucronatus* individual with a normal appearance (**A**) compared to the "encapsulated" nematodes found in the BEA 1 mM treatments (**B**-**D**)

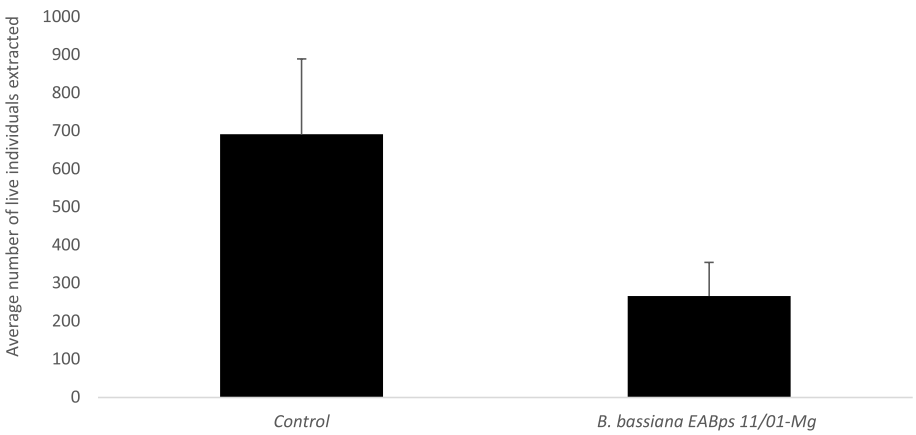


**Fig. 8** Average number of live *B. mucronatus* individual extracted from control insects (Tween 80 1%) and from *B. bassiana* EABps 11/01-Mg treatment (Tween 80 1% + spore suspension $10^8$ sp/mL)

to 100% (Fig. 2). These results were to be expected, given that the nematicidal potential of this fungus has already been tested on the genus *Bursaphelenchus* (Youssef et al. 2020; Ye et al. 2021; Karabörklü et al. 2022; Sánchez-Gómez et al. 2023) and *B. mucronatus and B. xylophilus* are morphological and biologically very similar (Braasch 2001, 2004; Bolla and Wood 2004; Pereira et al. 2013),

Therefore, according to these results, we can state that the effect of this fungal strain under controlled conditions is practically identical on the two nematode species.

It is widely known that both nematode species are mycophagous, at least in one of the stages of their life cycle in the case of *B. xylophilus* (Vicente et al. 2012; Pimentel et al. 2023). This ability to feed on wood-decaying fungi leads to an increase in the development of populations in the presence of *B. cinerea* (positive growth control), while the growth in PDA (negative growth control) was much lower since, in this latter case, the nematodes lack food from the beginning.

There are not many studies of fungal species isolated from or tested as antagonists on *B. mucronatus*. Some of them refer to the genera *Arthrobotrys* (Minghe et al. 2002; Zhang et al. 2021) or *Dactylellina* (Zhang et al. 2021). In this regard, it is worth mentioning the study of Oh et al. (2014), which is the only one (to our knowledge) that studies the nematicidal effect of *B. bassiana* on *B. mucronatus*. This latter study also demonstrated this effect from the fungal species *Auxarthron reticulatum* (Zukal) G.F.Orr and Plunket and *Verticillium saksenae* Kushwaha. Our study has, therefore, an added value in this regard, as it contributes to the scarce knowledge in this area.

### 4.2 Nematicidal effect of commercial BEA on *B. xylophilus* and *B. mucronatus* populations

The disruptive effect of the mycotoxin BEA has already been tested *B. xylophilus* (Shimada et al. 2010; Sánchez-Gómez et al. 2023). Although the nematicidal activity of some plant metabolites, such as chamaechromone 60, umbelliferone 320, and daphnoretin 321, has been tested and corroborated on *B. mucronatus* (Li and Zhang 2023), there are no published works, as far as we know, on the effect of fungal metabolites on this nematode species.

The brownish "encapsulation" mentioned in the Sect. 3 was also observed in BEA assays on *B. xylophilus* (Sánchez-Gómez et al. 2023). The precise mechanism by which BEA affects animal cells remains not fully understood. However, it appears to be associated with its ionophoric activity, promoting heightened ion permeability across biological membranes, thus inducing oxidative stress at the molecular level (Mallebrera et al. 2018). Therefore, the appearance of these tawny "capsules" could potentially derive from the interaction between BEA molecules and nematode epicuticle lipids.

### 4.3 In vivo tests to corroborate the nematicidal effect of *B. bassiana* on *B. mucronatus* under in vivo conditions

The results of the in vivo tests are of particular importance since they show that *B. bassiana* EABps 11/01-Mg strain has a nematicidal effect not only under in vitro conditions but also under simulated natural conditions. This is the same strain used years ago by the research group for the successful field trials to reduce the vector populations (*M. galloprovincialis*). The effective methodology followed for this purpose consists of placing CROSSTRAP® traps, baited with the attractant GALLOPROTECT 2D® (as explained in the introduction, also developed by the group), with a specific collector at the exit of the trap, called an auto-infection or auto-inoculation device. This device consists of a cylindrical collector, with a tube of a specific diameter placed on one side and covered internally with felt. On this felt, the spore powder formulation of *B. bassiana* EABps 11/01-Mg is dispensed so that, when the *Monochamus* escapes from the trap through this tube, the spores remain attached to its body, thus acting as a natural disperser of the entomopathogenic fungus, allowing it to reach and infect other *Monochamus* individuals. Such patented device demonstrated a decrease up to 53% of offsprings in field experiments (Sacristán-Velasco and Martín-García, 2024).

Therefore, our in vivo trials demonstrate that the same strain successfully tested in the field to decrease vector populations could also contribute to the reduction of nematode populations while they are in phoretic association with the vector, before the insect dies from the infection itself. However, further in vivo tests should be performed to corroborate these first results.

Regarding the efficiency of our methodology, although it is true that the pathosystem created worked well as a microenvironment to carry out tests, it must be considered that the intermediate losses of nematodes, insects, and even pupation chambers due to contamination are quite high. Of the 2500 nematodes initially introduced into the pupal chamber, not all of them load on the insect. The highest number of live nematodes obtained from an insect-replica was 1666 individuals. Some studies consider that the phoretic affinity between *B. mucronatus* (as well as other species within the genus *Bursaphelenchus*, such as *B. okinawaensis*) and the cerambycid vector insects is not so strongly developed as with *B. xylophilus* (Jikumaru, and Togashi 2003; Kirino et al. 2023). In others, however, it is shown that there are

no significant differences in the phoretic affinity with the insect between *B. mucronatus* and *B. xylophilus* (Vincent et al. 2008). Although there is not yet a clear answer on this matter, in either case, our results suggests that our methodology would work equally well or even better with *B. xylophilus* than with *B. mucronatus.* Aside from the topic of affinity, and as expected, a percentage of the nematodes died during the process, and others remained in the wood of the pupation chamber. Regarding the insects, of the 100 larvae initially extracted and introduced into the pupation chambers, only 26 emerged and were used, 10 for the nematode-insect approach tests (see Sect. 2.4), and 16 for the test itself. The non-emergence of some of the initially introduced insects had already been indicated by Aikawa et al. (2003) where, out of 75 larvae introduced, only 53 managed to emerge.

As already pointed out, there is a serious lack of in vivo trials, probably due to the need of a safety laboratory or greenhouse to put living insects and nematodes together without any environmental risk. Furthermore, the few existing ones (Arakawa and Togashi 2002; Togashi and Arakawa 2003; Aikawa et al. 2003; Ohsawa and Akiba 2014) have not had in any case, to our knowledge, the objective of testing fungi as a feasible biological control method of *B. xylophilus*. While further trials would be needed to refine our methodology and support our results, these first steps contribute as a cornerstone for future in vivo trials in the scope of the biological control of PWN.

## 5 Conclusions

Considering the high risk of spread of *B. xylophilus* and the serious danger that this nematode species presents to European pine forests, it is imperative to fully develop an efficient and enforceable method for managing the PWD. In this regard, our study has demonstrated that *B. bassiana* EABps 11/01-Mg strain presents not only entomopathogenic activity on *M. galloprovincialis* (Álvarez-Baz et al. 2015) but also a nematicidal effect. Thus, it could be implemented in the forest alongside pheromone traps and the previously mentioned self-infection device, thereby creating an integrated management system for PWD. This system would be particularly useful and interesting in those regions or countries where *B. xylophilus* is already established, not to eradicate the disease but to manage it with a biological control strategy.

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**Authors' contributions**
All authors conceived and designed the experiment. T.S-G and P.Z-B performed the experiments. J.M-G analyzed the data. T.S-G. and J.M-G. wrote the paper. All authors contributed to the article and approved the submitted version.

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**Data availability**
The primary dataset generated and analyzed during this study is available in the Zenodo repository (https://doi.org/10.5281/zenodo.13912259).
This dataset can be cited independently as follows:
SANCHEZ-GOMEZ, T. (2024). Dataset_Sanchez-Gomez et al._2024_nematode [Data set]. Zenodo. https://doi.org/10.5281/zenodo.13912259.

## Declarations

**Ethics approval and consent to participate**
Not applicable.

**Consent for publication**
All authors gave their informed consent to this publication and its content.

**Competing interests**
The authors declare no competing interests.

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