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Polyphasic approach to the selection of *Esteya* isolates for the control of the pinewood nematode, *Bursaphelenchus xylophilus*

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ABSTRACT

Pine wilt disease, caused by the pinewood nematode, *Bursaphelenchus xylophilus*, is a major phytosanitary concern to pine forests worldwide. Managing pine wilt disease involves a complex logistical undertaking, with limited effectiveness and significant ecological repercussions. An increasing demand for biosolutions has sparked an interest in microbial antagonists capable of controlling the nematode. *Esteya* spp. are promising fungal biocontrol agents of the pinewood nematode. Here, we carry out an integrative characterization of *Esteya vermicola* and *Esteya floridanum* isolates, through biological, biochemical, and molecular methods, and provide insights into the selection of these isolates for the biological control of the pinewood nematode. Dual culture assays revealed that *Esteya* spp. can compete with ophiostomatoid fungi (*Leptographium terebrantis* and *Ophiostoma ips*) occurring in the pathosystem of pine wilt disease, an often-neglected ecological perspective that could hinder their success as biocontrol agents. Moreover, *E. vermicola* can metabolize more carbon sources than *E. floridanum*, which can have implications on their successful establishment in pine trees. Our experimental approach further shows that both *Esteya* spp. are equally competent in suppressing the pinewood nematode *in vitro*. Overall, our results suggest that a prophylactic application of *Esteya* in pine trees may be preferable for optimal bioprotective effects against the pinewood nematode and fungal pathogens.

1. Introduction

The pinewood nematode (PWN), *Bursaphelenchus xylophilus* (Steiner and Bührer, 1934; Nickle, 1970), is the causal agent of pine wilt disease (PWD), a major phytosanitary concern that have ravaged autochthonous pine forests in Asia and Europe's Iberian Peninsula (Futai, 2013; Mota et al., 1999; Back et al., 2024). The PWN is native to North America and ranks among the most economically and scientifically relevant plant-parasitic nematodes (Tares et al., 1994; Jones et al., 2013; Kantor et al., 2022). The PWN is carried and transmitted by pine sawyer beetles from the *Monochamus* genus upon feeding and/or ovipositioning in pine trees, thus completing the disease triangle (Mamiya 1983; Jones et al., 2008). PWD jeopardizes biodiversity, fragments forest ecosystems, and hinders resilience to climate change (Kiyohara and Bolla 1990; Suzuki 2002). Current management of PWD is a complex logistical undertaking, with limited effectiveness and significant ecological repercussions (Robinet et al., 2020). Regular monitoring of the PWN and its insect vector remain the most common strategies to prevent PWD outbreaks, although introduction and dissemination of the nematode can eventually occur. Beyond the ecological drawbacks, current management practices also have a negative economic impact, as they impede the commercialization of untreated wood (Soliman et al., 2012; Carnegie et al., 2018). Moreover, climate change is expected to foster broader dispersal corridors for the PWN and the spread of PWD to areas previously unaffected (Hirata et al., 2017; de la Fuente et al., 2018; An et al., 2019; Tang et al., 2021). The increasing demand for biosolutions has sparked an interest in antagonists capable of controlling the PWN (Pires et al., 2022). Fungi of the *Esteya* genus have been identified as natural enemies of the PWN, with two currently described species, and some isolates have been patented (Liou et al., 1999; Tzean et al., 2001; Fang et al., 2010; Li et al., 2021). *Esteya vermicola* has shown to suppress the PWN under controlled and natural conditions (Wang et al., 2011, 2018).

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Contrastingly, *Esteya floridanum* is lagging behind, despite encouraging results (Li et al., 2021). Using a polyphasic approach, we carry out a comprehensive characterization of three isolates of *E. vermicola* from the Czech Republic and one of *E. floridanum* from the United States of America. We provide insights into *Esteya* isolate selection for the biological control of the PWN. Lastly, we explore and report for the first time *Esteya*'s ability to compete with naturally occurring ophiostomatoid fungi in the pathosystem of PWD, that could hinder the success of biocontrol strategies.

2. Material and methods

2.1. Maintenance and culturing conditions of fungi and nematodes

The isolates of *E. vermicola* CCF3117, CCF3118 and CCF3131 (Kubátová et al., 2000) were obtained from the Culture Collection of Fungi (CCF) of the Department of Botany at Charles University, Czech Republic, and *E. floridanum* V14639 from the University of Florida, USA. *Leptographium terebrantis* S40.4b and *Ophiostoma ips* S36.1 were previously isolated at the Nematology laboratory of INIAV, Portugal, from PWD-symptomatic *Pinus pinaster* trees (Vicente et al., 2022). *Trichoderma alni* was isolated in the same laboratory from *Monochamus galloprovincialis* (unpublished). The reference isolate of *B. xylophilus* Bx013.003 from the Nematology laboratory of INIAV was used in this study. Nematodes were maintained and multiplied on a non-sporulating strain of *Botrytis cinerea*. All fungi were reared on potato dextrose agar (PDA, BD Difco™) and incubated in the dark at 25 °C until used.

2.2. Total DNA extraction, PCR amplification and sequencing

The mycelium of each *Esteya* isolate was scraped off agar plates with a sterile scalpel and total genomic DNA extracted using the DNeasy Plant Mini Kit (QIAGEN), following manufacturer's instructions. The molecular markers β-tubulin (TUB) and translation elongation factor 1-alpha (TEF) were amplified using the following sets of primers, respectively: Bt2a (5′-GGTAACCAAATCGGTGCTGCTTTC-3′) and Bt2b (5′-ACCCTCAGTGTAGTGACCCTTGGC-3′) (Glass and Donaldson 1995), and EF-728F (5′-CATCGAGAAGTTCGAGAAGG-3′) and EF-2 (5′-GGARGTACCAGTSATCATGTT-3′) (Carbone and Kohn 1999; O'Donnell et al., 1998; Jacobs et al., 2004). The PCR reaction mixture (25 μL) contained ca. 10 ng of genomic DNA, 0.25 μM of each primer, 12.5 μL of 0.5 U Supreme NZYTaq II (NZYTech, Portugal) and 9 μL dH_2O. The PCR conditions for the TUB gene were: 1 cycle of initial denaturation at 95 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 52 °C for 30 s, elongation at 72 °C for 30 s, and a cycle of final elongation at 72 °C for 5 min. For the TEF gene, the PCR conditions were: 1 cycle of initial denaturation at 95 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s for *E. vermicola* isolates and 47 °C for 30 s for *E. floridanum*, elongation at 72 °C for 30 s, and a cycle of final elongation at 72 °C for 5 min. PCR products were cleaned-up with the QIAquick PCR Purification Kit (QIAGEN) prior to sequencing, following manufacturer's instructions. Amplicons were sent to STABVIDA (Caparica, Portugal) for sequencing.

2.3. Phylogenetic analyses

The amplicon sequences were manually trimmed and compared for similarity to other sequences available in GenBank (NCBI) using the BLAST tool. Closely related sequences of TUB and TEF sequences from ophiostomatoid fungi were retrieved from the same database (https://www.ncbi.nlm.nih.gov/genbank/). Multiple sequence alignments were performed in the online version of MAFFT (Katoh et al., 2019) and gaps were cleared with TrimAL (Grüning et al., 2018). Model testing was carried out in CIPRES (Miller et al., 2010) to determine the best nucleotide substitution model for the combined datasets of both genes. Phylogenetic trees were inferred by maximum likelihood (ML) in QIAGEN CLC Main Workbench 21.0.5. TUB and TEF sequences from nematode antagonistic fungus *Trichoderma harzianum* (LT707601.1 and LT707593.1, respectively) were used as outgroups. The best ML generated trees were edited in Adobe Photoshop CC (https://www.adobe.com). The new sequences generated in this study were deposited in GenBank, under the accession numbers: PP480645, PP480646, PP480647, PP480648, PP531526, PP531527, PP531528 and PP531529.

2.4. Growth rate

The growth rate of *E. vermicola* CCF3117, CCF3118 and CCF3131 and *E. floridanum* V14639 was compared on PDA, by placing a mycelial plug from actively growing colonies at the center of agar plates and incubating them in the dark at 25 °C for 15 days. Radial growth was recorded at different time points (1, 3, 7, 10 and 15 days), by measuring two perpendicular radiuses from the periphery of the agar plug to the edge of the colony. The assay was repeated three times, with six replicates per isolate.

2.5. Dual culture assays

To explore fungal compatibility, *Esteya* isolates were paired against one another in dual cultures. Two mycelial plugs from different isolates were excised from the periphery of actively growing colonies, placed 3 cm away from the edge of the plate and 3 cm apart on PDA plates (9 cm ⌀), and incubated in the dark at 25 °C. In positive controls, a single mycelial plug was placed at the center of each plate. The assay ended after 3 weeks of interaction. All *Esteya* pairing combinations were considered, with six replicates per dual culture, and the assay was performed three times independently. To determine *Esteya*'s ability to compete with and dominate fungi occurring in the pathosystem of PWD, a similar assay was carried out with *L. terebrantis* S40.4b, *O. ips* S36.1 and *T. alni*, with slight modifications. A plug of one of four *Esteya* isolates was placed at the center of a PDA plate, and a plug of either *L. terebrantis* S40.4b, *O. ips* S36.1 or *T. alni* was placed at the edge of the plate, due to their faster growth rate. Plates were incubated in the dark at 25 °C, and visually examined after 3 weeks to determine the type of mycelial interaction (Esser and Meinhardt 1984; Mohammad et al., 2011; Pepori et al., 2018). Each assay was performed in triplicate independently, with six replicates per combination.

2.6. Antifungal bioassays

To screen candidate compounds to develop a semi-selective medium tailored for *Esteya* bioprospection from field samples, the minimum inhibitory concentration (MIC) of different antifungals were included in bioassays against *E. vermicola* CCF3117, CCF3118, CCF3131 and *E. floridanum* V14639. The antifungal compounds used were amphotericin B (Sigma–Aldrich), benzoic acid (≥99.5 %; Sigma–Aldrich), cycloheximide (≥90 %; Sigma–Aldrich), dodin (100 %; Sigma–Aldrich), itraconazole (≥98 %; Sigma–Aldrich), miconazole (Sigma–Aldrich), sinefungin (≥95 %; Sigma–Aldrich) and voriconazole (100 %; Sigma–Aldrich). All compounds were dissolved in methanol (MeOH, 99.8 %; Sigma–Aldrich) to prepare stock solutions from initial concentrations of 0.25, 1, 1.5 or 30 mg mL^{-1}. Each compound was then diluted in yeast malt broth (YMB: 10 g L^{-1} malt extract, 4 g L^{-1} D-glucose and 4 g L^{-1} yeast extract), and inoculated with one of four *Esteya* conidial suspensions, previously propagated in YMB and incubated in the dark for 5 days at 25 °C, with constant shaking. The bioassays were performed in 96-well microtiter plates, and all compounds were serially diluted, with MeOH and amphotericin B serving as negative and positive controls, respectively. The 96-well plates were sealed with parafilm and incubated in the dark at 25 °C for 5 days, with constant shaking. Fungal growth was visually assessed, and MIC was recorded as the lowest concentration of each compound inhibiting visible growth of the test

organism. Each treatment was performed in duplicate, and the bioassay repeated twice independently.

Two further experiments were performed on agar plates to determine inhibitory concentrations of cycloheximide (≥90 %; Sigma–Aldrich) and rose bengal (Liofilchem®) on *Esteya* isolates: i) 0.1 g L^{-1}; ii) 0.5 g L^{-1}; and iii) 1 g L^{-1}. *E. vermicola* CCF3117, CCF3118, CCF3131 and *E. floridanum* V14639 were used. Fungi growing on PDA served as positive controls. Plates were observed every other day over 15 days, with ten replicates per treatment, and the assay was conducted three times independently.

2.7. Pinewood nematode mortality bioassays

Freshly grown fungal mats of *E. vermicola* CCF3117, CCF3118 and CCF3131, and *E. floridanum* V14639 were inoculated with 500 mixed developmental stages of *B. xylophilus* per milliliter and incubated in the aforementioned conditions for 7 days. *B. cinerea* served as positive control, as it is routinely used to feed and maintain the PWN under laboratory conditions. Considering our experimental design, a non-treated control was not included, because it could lead to nematode mortality due to starvation, thus preventing us from drawing any conclusion on the suppressive effects of different *Esteya* isolates. Nematodes were extracted using the tray method (Whitehead and Hemming 1965), and the living PWN counted to determine the survival rate (%): (no. nematodes before treatment/no. living nematodes after treatment) × 100. Nematodes were considered dead if not moving or not reacting to physical stimulus. The assay included six replicates per fungus and was repeated three times independently.

2.8. Carbon assimilation profile

The commercial kit API® 50 CH (bioMérieux, Inc.) was used to assess the assimilation profiles of 49 carbon sources by *Esteya* spp., according to Alvarez et al. (2010). To prepare the fungal inoculum of each isolate, a final spore concentration of 5×10^5 CFU mL^{-1} was prepared in 20 mL of yeast nitrogen base (Difco™). Each well of the API® 50 CH strips was then inoculated with 300 µL of the fungal suspension, and the viability of conidia was verified by plating 100 µL of each inoculum onto PDA and incubating at 25 °C for 7 days. Inoculated API® 50 CH strips were incubated in the same conditions, and color change in each well monitored at 24 h intervals. Weak growth of the test organism was still considered a positive result. The API® 50 CH test was repeated two times independently for each isolate.

2.9. Data analysis

Data analysis was performed using R in Jamovi 2.3 (R Core Team 2020; The Jamovi Project 2023). Normal distribution was checked using the Shapiro–Wilk test. The growth rate of *Esteya* isolates on PDA at different time points was compared by ANOVA. The non-parametric Kruskal–Wallis test was used to infer differences in the survival rate of the PWN interacting with *Esteya* isolates. Multiple mean comparisons were performed using Dwass-Steel-Critchlow-Fligner pairwise comparison ($p < 0.05$).

3. Results

3.1. Molecular characterization of Esteya spp.

The partial sequences of the TUB and TEF genes for all four *Esteya* isolates were determined, and our phylogenetic analyses revealed that both *E. vermicola* and *E. floridanum* are supported in a large monophyletic clade (Fig. 1), closely related to ambrosial fungi of the *Raffaelea sulphurea* complex, recently reclassified as *Dryadomyces* (de Beer et al., 2022), and grouping with other ophiostomatoid fungi. For the TUB gene, *E. vermicola* CCF3117, CCF3131 and CCF3118 clustered with other *E. vermicola* isolates from China, Czech Republic and South Korea, with 100 % bootstrap, while *E. floridanum* V14639 clustered with the same isolate from the United States, also with 100 % bootstrap. As for the TEFgene, *E. vermicola* isolates formed a single sub-clade, clustered with *E. floridanum*, all with good bootstrap support.

3.2. Growth rate

To determine the fastest growing isolates, growth trials were performed on PDA. All *Esteya* isolates had comparable growth rates during the first 3 days ($p > 0.05$), but this trend shifted from the 7th day onward (Fig. 2). By the end of the trials, *E. floridanum* V14639 and *E. vermicola* CCF3118 outgrew other isolates ($p < 0.001$), with colony radiuses reaching 2.2 and 2.05 cm, respectively, and the difference among these two is statistically significant ($p < 0.05$) (Fig. 3). Contrastingly, *E. vermicola* CCF3117 and CCF3131 growth rates were comparable after 15 days ($p > 0.05$) (Fig. 3).

3.3. Dual culture assays

A mycelial barrage along the junction line between opposing *Esteya* isolates was visible in all dual cultures, suggesting incompatibility among isolates (Fig. 3A).

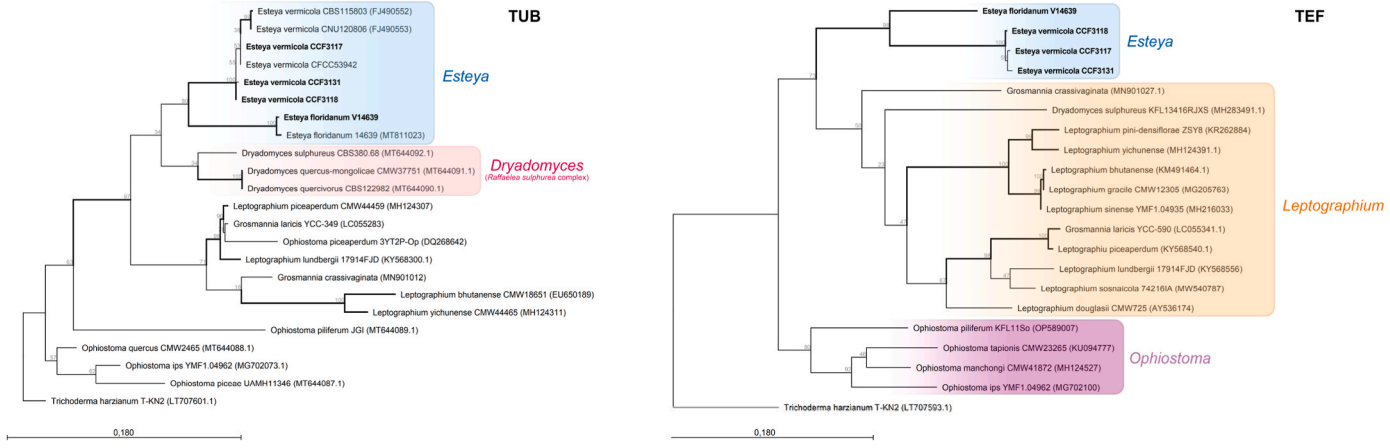

Fig. 1. Maximum likelihood phylogenetic trees of *Esteya* isolates based on β-tubulin (TUB) and elongation factor 1-α (TEF) DNA sequences, with *Trichoderma harzianum* T-KN2 as the outgroup. Novel sequences obtained in this study are presented in bold. Bootstrap values are presented above the respective branch. Bold branches indicate bootstrap values ≥ 0.95 %. Scale bars refer to the phylogenetic distance of the number of substitutions per site.

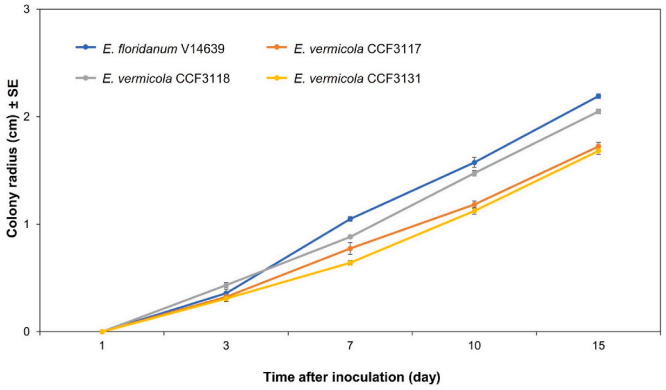

Fig. 2. Colony radius of *Esteya* isolates growing on potato dextrose agar, 15 days after incubation at 25 °C. Values represent the mean ± SE ($F = 3.44$, $df = 19$, $p < 0.001$).

In the second assay, mutual inhibition on contact was apparent when fungi were co-cultured on the same day, with *L. terebrantis* S40.4b and *O. ips* S36.1 growing around *Esteya* in all cases (Fig. 3B). Demarcation zones between opposing colonies were clearer when *Esteya* isolates were paired against *L. terebrantis* S40.4b and *T. alni*, and a more diffuse confrontation zone when paired against *O. ips* S36.1, with a change in pigmentation along the junction line. Surprisingly, *T. alni* seemed to avoid direct contact with *E. vermicola*'s hyphae in some cases, with a tenuous halo around the plug in the interactions *T. alni* × *E. vermicola* CCF3117 and *T. alni* × *E. vermicola* CCF3131 (Fig. 3B–i and Fig. 3B–k). Nevertheless, *T. alni*'s spores were observed spreading over *E. vermicola* CCF3118 and *E. floridanum* V14639 (Fig. 3B–j and Fig. 3B-l). Small mycelial growth was visible in pairings against *T. alni*, but all *Esteya* isolates ceased growing when surrounded by their competitor.

3.4. Pinewood nematode mortality bioassays

The highest PWN mortality rates were recorded in the interaction with *E. vermicola* CCF3117, with a survival rate of 0 % (Table 1). Conversely, the lowest mortality rate was observed in *E. vermicola* CCF3118 after 7 days of interaction, with a survival rate of 18 %. Nevertheless, all *Esteya* isolates significantly suppressed the initial population of the PWN compared to the control ($p < 0.001$).

3.5. Antifungal assays

Among antifungal compounds tested in microplate bioassays, *Esteya* isolates grew in all tested concentrations of CHX and MeOH (Table 2). Likewise, no inhibition was observed for sinefungin at tested concentrations, except for *E. vermicola* CCF3131. All the other compounds inhibited the growth of *Esteya* isolates.

In the case of PDA plates supplemented with antifungal compounds, none of the *Esteya* isolates was inhibited by either CHX or RB, even at the highest concentration (1 g L^{-1}). However, all isolates visibly grew faster at the lowest RB concentration (0.1 g L^{-1}).

3.6. Carbon assimilation profile

The API® 50 CH test revealed that *E. vermicola* CCF3117 and CCF3118 metabolized 46 and 45 carbohydrates 7 days after inoculation,

Table 1
Survival rate of *Bursaphelenchus xylophilus* on fungal mats of *Esteya* spp., 7 days after inoculation. Values represent the mean ± SE. Values in the same column followed by the same letter are not statistically different ($p < 0.001$).

Fungal treatment	No. nematodes before treatment	No. living nematodes after treatment	Survival rate (%)
Esteya vermicola CCF3117	500	1 ± 1^{a}	0 ± 0.15^{a}
E. vermicola CCF3118		88 ± 47^{a}	18 ± 9.31^{a}
E. vermicola CCF3131		10 ± 7^{a}	2 ± 1.47^{a}
E. floridanum V14639		29 ± 24^{a}	6 ± 4.89^{a}
Botrytis cinerea (control)		2189 ± 124^{b}	438 ± 24.7^{b}

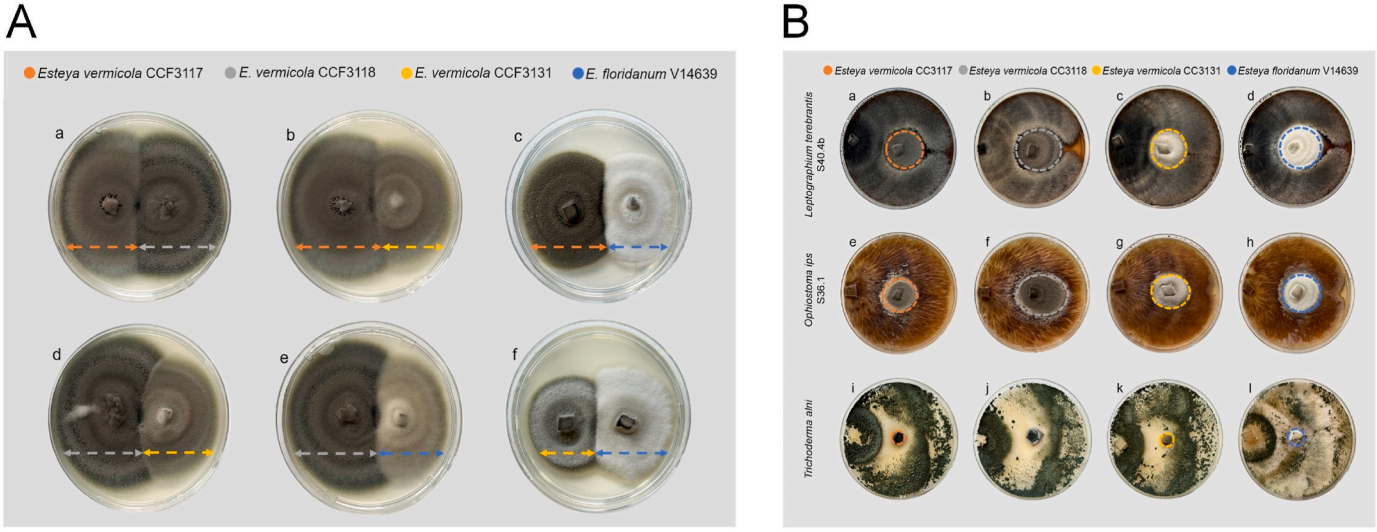

Fig. 3. Fungal co-culture assays on PDA, three weeks after inoculation: A – interactions among *Esteya* isolates: a) *E. vermicola* CCF3117 × CCF3118; b) *E. vermicola* CCF3117 × CCF3131; c) *E. vermicola* CCF3117 × *E. floridanum* V14639; d) *E. vermicola* CCF3118 × CCF3131; e) *E. vermicola* CCF3118 × *E. floridanum* V14639; f) *E. vermicola* CCF3131 × *E. floridanum* V14639; B – interactions between *Esteya* isolates (inoculated in the middle of the plates) and three potential competitors (*Leptographium terebrantis* S40.4b, *Ophiostoma ips* S36.1 and *Trichoderma alni*, inoculated on the left side and at the edge of the plates): a) *E. vermicola* CCF3117 × *L. terebrantis*; b) *E. vermicola* CCF3118 × *L. terebrantis*; c) *E. vermicola* CCF3131 × *L. terebrantis*; d) *E. floridanum* V14639 × *L. terebrantis*; e) *E. vermicola* CCF3117 × *O. ips*; f) *E. vermicola* CCF3118 × *O. ips*; g) *E. vermicola* CCF3131 × *O. ips*; h) *E. floridanum* V14639 × *O. ips*; i) *E. vermicola* CCF3117 × *T. alni*; j) *E. vermicola* CCF3118 × *T. alni*; k) *E. vermicola* CCF3131 × *T. alni*; l) *E. floridanum* V14639 × *T. alni*.

Table 2
Minimum inhibitory concentration of antifungal compounds on different isolates of *Esteya*, after 5 days of incubation at 25 °C.

Fungal treatment	Minimum inhibitory concentration (μg mL^{-1}), 5 days after inoculation								
	SIN	MIZ	DOD	CHX	ITZ	VRZ	BA	AmB	MeOH
E. vermicola CCF3117	NI	<0.52	16.65	NI	<0.52	3.125	500	0.26	NI
E. vermicola CCF3118	NI	<0.52	16.65	NI	<0.52	6.25	500	0.26	NI
E. vermicola CCF3131	66.6	<0.52	8.325	NI	<0.52	6.25	250	0.26	NI
E. floridanum V14639	NI	<0.52	4.1625	NI	<0.52	3.125	250	0.26	NI

Sinefungin (SIN), miconazole (MIZ), dodine (DOD), cycloheximide (CHX), itraconazole (ITZ), voriconazole (VRZ), benzoic acid (BA), amphotericin B (AmB) and methanol (MeOH). NI = no inhibition.

respectively, whereas *E. vermicola* CCF3131 and *E. floridanum* V14639 only assimilated 6 and 5 carbon sources, respectively (Fig. 4; Supplementary Table 1). Indeed, all isolates assimilated D-glucose, esculin, D-maltose, glycogen, and potassium 5-keto-gluconate. Contrastingly, none of the tested isolates was able to metabolize amygdalin, potassium gluconate or potassium 2-keto-gluconate as a sole carbon source.

4. Discussion

The economic value of biological control is not always easy to estimate, but it potentially provides one of the highest returns on investment within the framework of integrated pest management (Naranjo et al., 2015; van Wilgen et al., 2020). Biopesticides are increasingly gaining attention worldwide and the bioprotection market is anticipated to soar within the coming decade (Desaeger et al., 2020). The use of single microorganisms in bioformulations has been widely studied but does not necessarily warrant consistent results over time in natural conditions, and single microorganisms can be outperformed by indigenous microbes in the system they are intended for, which are major constraints for their wider adoption (Trivedi et al., 2017, 2020; Mitter et al., 2019). Furthermore, loss of virulence under laboratory conditions has been known for a long time and it is another downside of microbial biocontrol agents (LaRue 1925; Zuckerman et al., 1989; Krokene and Solheim 2001; Butt et al., 2006). Nevertheless, microbial consortia can improve efficiency and consistency by enhancing the suppressiveness of biocontrol agents through synergistic effects, by targeting alternative metabolic pathways or employing distinct control strategies (Faust 2019; Mitter et al., 2019). For fungal biocontrol agents to thrive in their environment, a successful and rapid colonization ability, coupled with a high sporulation rate, are desirable fungal traits (Garbelotto et al., 2015). However, such characteristics are rarely found in a single isolate. In this sense, exploring compatibility among microbial agents is essential (Ram et al., 2022; Nunes et al., 2024).

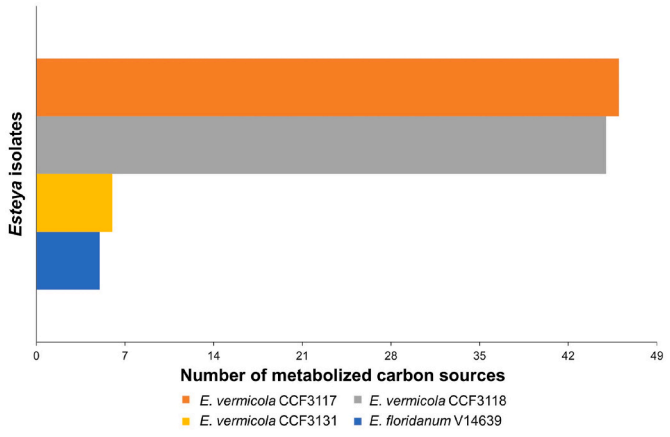

Fig. 4. Total carbon sources assimilated by *Esteya* isolates (CCF3117, CCF3118, CCF3131 and V14639), assessed through color development 7 days after inoculation, using the API® 50 CH test.

Here, a comprehensive approach to assess the compatibility of four *Esteya* isolates and their ability to compete with and dominate other fungi in the pathosystem of PWD was explored for the first time. This work revealed an incompatibility among *Esteya* isolates, even within the same species, under controlled conditions. Our results further showed that while they were outgrown by their competitors *in vitro*, *Esteya* isolates were markedly antagonistic to *L. terebrantis* S40.4b and *O. ips* S36.1, with both fungi avoiding *Esteya*'s hyphae. More importantly, *Esteya* isolates were observed to spread over the mycelium of *L. terebrantis* S40.4b in our experiments. Vicente et al. (2022) also noted that *Leptographium* sp. T3.6 inhibited the growth of both *L. terebrantis* S40.4b and *O. ips* S36.1 on artificial substrate, revealing fungal antagonism within the same order. *T. alni* appeared to avoid direct contact with *Esteya*'s hyphae in some cases, but ended up colonizing the entire plate over the course of the experiment. Whether the suppression of *Esteya*'s growth when interacting with *T. alni* was due to competitive exclusion, antibiosis or mycoparasitism is unclear. Nevertheless, *Trichoderma* is known to be an aggressive colonizer, either by producing antagonistic compounds such as chitin and glucan degrading enzymes, by direct competition for space and nutrients, or both (Dutta et al., 2023). Since *Esteya* spp. are slow-growing fungi, *T. alni* quickly colonized the culture medium and most likely depleted resources, thereby preventing *Esteya* from growing. Although it was not investigated in this work, another plausible cause is that secondary metabolites may have been produced, as none of the *Esteya* isolates was able to grow in the presence of *T. alni* (Oszust et al., 2020). Indeed, coexisting microorganisms often dispute access to resources in order to survive, and catabolite repression is a crucial strategy to accommodate changes in resource-depleted environments, or to outcompete others under nutrient-limiting conditions (Nair and Sarma 2021; Guzmán-Guzmán et al., 2023), an aspect of microbial biocontrol agent ecology that is often neglected.

Carbon and nitrogen sources were shown to have positive effects on conidiogenesis and pathogenicity in *E. vermicola* (Wang et al., 2011, 2016; Zhu et al., 2021; Chen et al., 2023). In this work, we investigated the nutritional demands of four isolates of *Esteya* to identify a potential niche overlap for specific carbon substrates. Our results show that isolates from the same geographic origin have contrasting nutritional requirements, which may have implications for their successful establishment in pine tree tissues and, consequently, their ability to suppress the PWN under natural conditions. Indeed, two Czech isolates of *E. vermicola* (CCF3117 and CCF3118) were able to metabolize more carbohydrates than the rest. As significant differences were observed among these two isolates in terms of growth rate but not pathogenicity to the PWN, we hypothesize that carbon metabolism is more correlated with biomass production than with pathogenicity in *Esteya*. Contrastingly, Vieira Dos Santos et al. (2019) found that isolate original substrate, pathogenicity, and rhizosphere colonization ability were significantly correlated with carbon metabolism in *Pochonia chlamydosporia*, irrespective of geographical origin. Frascella et al. (2023) reported a contrasting range of monosaccharide assimilation in cosmopolitan fungi from the *Trichoderma* genus, regardless of species or forest soil they were isolated from.

Prospecting new *Esteya* isolates from environmental samples is difficult due to their slow growing nature, but it is paramount that we expand our biological arsenal against the PWN. A semi-selective medium tailored for *Esteya* would make this task easier. Here, we screened several antifungal compounds to maximize the probability of detection of *Esteya* from environmental substrates. Taking into consideration cost-benefit of all tested compounds, cycloheximide and rose bengal have proven the most promising, and can be included in a semi-selective medium with PDA at the concentration of 0.1 g L^{-1}. The high tolerance to cycloheximide among ophiostomatoid fungi was first reported by Fergus (1956), later expanded upon by Harrington (1981), and the molecular basis behind it was clarified by Wingfield et al. (2022).

Previous phylogenetic data suggests that *Esteya* diverged independently from many ophiostomatoid fungi, with the Ophiostomatales order having undergone considerable revisions in recent years and the status of *Esteya* remaining somewhat unresolved (Lehenberger et al., 2021; de Beer et al., 2022). Our phylogenetic analyses based on the TUB and TEF genes revealed that both *E. vermicola* and *E. floridanum* are supported in a large monophyletic clade, closely related to ambrosial fungi of the *R. sulphurea* complex, reclassified as *Dryadomyces* (de Beer et al., 2022), and grouping with other ophiostomatoid fungi, thus corroborating previous studies (Li et al., 2021; Araújo et al., 2022; de Beer et al., 2022).

Overall, our results suggest that a prophylactic application of *Esteya* to asymptomatic pine trees in intervention zones may be preferable for optimal bioprotective effects against the PWN and fungal pathogens. Furthermore, the carbohydrate metabolic profiles of *Esteya* isolates are asymmetric. In this sense, *E. vermicola* CCF3117 and CCF3118 could be the best suited candidates for biocontrol of the PWN, given their capacity to assimilate more carbon sources in their environment, their comparable antagonistic nature towards the PWN and their complementary growth rates. Further experiments are needed to verify this assumption *in vivo* and in a broader ecological context. Moreover, these results should be compared to *Esteya*'s ability to colonize pine tissues (Supplementary Fig. 1) in order to identify potential correlations. This knowledge could prove useful in the future to speed up screening isolate colonization ability and predict their success as biocontrol agents, based on their metabolic profiles. Lastly, although carbon catabolite repression was not studied here, it should also be the object of future research to unravel its role in the infective cycle of *Esteya*.

CRediT authorship contribution statement

David Pires: Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Cláudia S.L. Vicente:** Writing – review & editing, Supervision, Funding acquisition, Formal analysis, Conceptualization. **Manuel Mota:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization. **Maria L. Inácio:** Writing – review & editing, Supervision, Project administration, Funding acquisition.

Ethics approval

This article does not contain any studies with human participants or animals, other than nematodes, performed by any of the authors.

Data availability

The datasets generated during the current study are available from the corresponding author on reasonable request.

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Declaration of competing interest

The authors have no financial or non-financial interests to disclose.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.funbio.2024.10.001.

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