





## Article

# Biocontrol of *Phytophthora xcambivora* on *Castanea sativa*: Selection of Local *Trichoderma* spp. Isolates for the Management of Ink Disease

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**Abstract:** Ink disease is a devastating disease of chestnut (*Castanea sativa*) worldwide, caused by *Phytophthora* species. The only management measures of this disease are chemical and agronomic interventions. This work focuses on the evaluation of the in vitro antagonistic capacity of 20 isolates of *Trichoderma* spp. selected in a diseased chestnut orchard in Tuscan Apennines (San Godenzo, Italy) for the biocontrol of *Phytophthora xcambivora*. Each *Trichoderma* isolate was tested to investigate pathogen inhibition capability by antagonism in dual cultures and antibiosis by secondary metabolites production (diffusible and Volatile Organic Compounds). The six most performing isolates of *Trichoderma* spp. were further assessed for their aptitude to synthesize chitinase, glucanase and cellulase, and to act as mycoparasite. All six selected isolates displayed the capability to control the pathogen in vitro by synergistically coupling antibiosis and mycoparasitism at different levels regardless of the species they belong to, but rather, in relation to specific features of the single genotypes. In particular, *T. hamatum* SG18 and *T. koningiopsis* SG6 displayed the most promising results in pathogen inhibition, thus further investigations are needed to confirm their in vivo efficacy.

**Keywords:** antagonism; antibiosis; secondary metabolites; SEM; enzymatic activity

## 1. Introduction

*Castanea sativa* Mill. is considered a multipurpose tree in Europe, representing an important resource exploited for fruit and timber production as well as for the characterization of mountain landscapes, in addition to its important roles in ecological and biological forest functions [1,2]. Ink disease is one of the most destructive diseases affecting chestnut, which causes root and collar rot of adult trees and of seedlings in nurseries, plantations and forest stands [3,4].

The Oomycetes *Phytophthora xcambivora*, and the more aggressive *P. cinnamomi*, are considered the causal agents of ink disease of *C. sativa* in Europe and the US [2]; although, in very few cases, *P. citricola* and *P. cactorum* are also reported to be associated with the disease in Northern Italy [2]. However, while *P. cinnamomi* is the causal agent of the disease in many European countries, causing severe losses especially in Spain, in Italy *P. xcambivora* is actually considered the main cause [5].

Due to the high economic and environmental value of chestnut in Italy, the most recent outbreak of ink disease has caused considerable concerns in the country [4]. After several decades of regression, the disease re-emerged probably due to climatic changes that occurred in the last 10–15 years, characterized by repeated periods of drought. Summer droughts have weakened root systems, making fine roots more susceptible to infections during the following rainy months, particularly favorable for the pathogen [1]. The symptoms of the disease appear as the necrosis of large and feeder roots that can reach the collar and the base of the trunk. This necrosis causes typical cortical-flame-shaped lesions with characteristic black exudate, which gave the name to the disease. The improper functioning of the roots leads to above ground symptoms, such as chlorosis, microphyllly and wilting, which can be followed by the quick death or progressive decline of the tree, depending on the environmental conditions [2,3].

The management of ink disease is difficult. The main techniques for its containment consist of the removal of infected or dead plants from the field (eradication) or in chemical interventions that could have a negative impact both on soil and inhabiting microbial communities. The distribution of organic soil conditioners (manure, organic fertilizer) around infected plants seems to invigorate the radical system by improving soil structure and stimulating the microbial community to antagonize the pathogens [1]. Chemical fungicide hand-outs, such as potassium and aluminum salts, are only recommended in nursery, as opposed to in forest and orchards. However, these products could have a severe impact on the environment, in addition to their uncertain outcome [1].

The new EU Forest Strategy for 2030, one of the flagship initiatives of the European Green Deal, sets a vision and concrete actions to improve the quantity and quality of EU forests and strengthen their protection, restoration and resilience. In addition, European Directive 2009/128/EC and Regulation CE/1107/2009 established new rules “to achieve a sustainable use of pesticides” and the marketing of plant protection products. This can also promote integrated pest management by the use of biological control agents (BCAs) as an alternative to traditional chemicals [6].

Besides climatic and agronomic aspects, fitness and productivity of chestnuts can be influenced by the interactions established with several microorganisms, among which fungi represent the main group associated with chestnut plants, mostly in soil, that can positively or negatively affect their fitness [7]. Fungi belonging to the *Trichoderma* genus are widely used as beneficial fungi and are among those more frequently isolated from soil and plant root ecosystems [8]. They are marketed as active ingredients of bio-pesticides, biofertilizers, growth enhancers and stimulants of natural resistance [6]. *Trichoderma* spp. exert their beneficial effects by competing with the target pathogen through both exploitation and/or interference strategies. These last consist of: (i) a rapid competition for nutritive resources and (ii) growth inhibition of pathogens by releasing extracellular molecules, such as diffusible and volatile secondary metabolites [9–12], in addition to the production of cell wall degrading enzymes (CWDEs)—such as 1,3- $\beta$ -glucanase and chitinase—allowing the use of pathogens cell-wall components as a nutrient to promote their growth [13,14].

Many studies report the ability of *Trichoderma* to act against a broad spectrum of fungal pathogens [15–21] and, up to now, isolates belonging to four species of *Trichoderma* are those more widely studied and commercialized as BCAs: *T. asperellum*, *T. atroviride*, *T. harzianum* and *T. polysporum* [8,22]. Among a plethora of studies concerning the use of *Trichoderma* in the management of important plant pathogens, few focus on the biological control of *Phytophthora* spp. [23–26], such as those related to the control of *P. ycoparasii* in avocado [27,28]. As far as we know, no information is available about their use to manage *P. xambivora* on *C. sativa*, both at forest and orchard levels. Despite many studies aimed at the elaboration of biocontrol strategies in crops, the same topic is very rarely referred to the forest ecosystem.

In this study, within the framework of the LIFE MycoRestore project (LIFE 18/CCA/ES/001110) focusing on the exploitation of innovative mycological resources to improve the productivity and resilience of Mediterranean forests threatened by climate changes, the

potential beneficial fitness of a pool of locally isolated *Trichoderma* strains has been evaluated against *P. xambivora*. Especially, the biocontrol activity of *Trichoderma* spp. isolates has been evaluated in vitro via inhibition tests and scanning electron microscopy observations. The chitinase, glucanase and cellulase production of selected *Trichoderma* isolates was also evaluated.

## 2. Materials and Methods

### 2.1. Description of the Site and Soil Sampling

Samplings were performed in a mature chestnut orchard for fruit production located in the Tuscan Apennines, Castagno d'Andrea (780 m a.s.l., orientation ENE), municipality of San Godenzo (Florence, Italy), which has been partially affected by ink disease for decades [29]. The soil of the area is classified as typical hapludalfs, fine silty, mixed, mesic (Soil Taxonomy, 2003) and cutanic luvisols (Classification WRB, 1998) based on the pedological map of Tuscany Region, whereas the texture corresponded to loam [29]. Soil samples were collected around 12 asymptomatic plants located close to the core of plants affected by ink disease. Five holes 1–3 m away from the trunk and 15–20 cm deep were made, in two opposite directions, under the canopies of each asymptomatic tree. After removing the superficial litter layer, about 200 g of soil and fine roots were collected from each hole. Soil samples collected under each tree were then pooled together to obtain a single sample per tree.

### 2.2. Isolation of *Trichoderma* spp. and *P. xambivora* Associated with Chestnut

Sampled roots were separated from soil and rinsed with sterile water to isolate *Trichoderma* from the rhizosphere. Washing water, containing rhizospheric soil, was collected and diluted up to  $10^{-3}$ . Each diluted sample was plated in duplicate onto Potato Dextrose Agar (PDA, 39.0 g L<sup>-1</sup>, Difco BD), amended with streptomycin sulphate (0.3 g L<sup>-1</sup>) and hymexazole (0.2 g L<sup>-1</sup>); the latter to reduce *Mucoraceae* development [30]. Therefore, fine roots of less than 1 mm diameter were submerged into a solution of NaClO (1.0% active chloride) in 50.0% ethanol for 1 min, then washed three times in sterile distilled water, and dried on sterile filter paper. Roots were cut into little pieces (about 7 mm length), plated onto PDA + streptomycin sulphate (0.3 g L<sup>-1</sup>) and then incubated at 25 °C for 5 days in the darkness. The colonies, whose morphology was attributable to that of *Trichoderma* (white or green mycelium, shape of phialides, organization of conidiogenic cells), were transferred on PDA plates. Single spore cultures were prepared for each putative *Trichoderma* isolate and the resulting colonies were kept on PDA tubes under mineral oil at 4 °C.

*Phytophthora xambivora* was isolated from soil samples collected around symptomatic plants as described in the soil sampling section, according to Erwin and Ribeiro, 1996 [31]. In detail, 200 g of soil containing chestnut fine roots were placed into transparent plastic boxes. The roots were then accurately crumbled up and 100 mL of distilled water was added to each sample inside the box. After one hour, an additional 400 mL of distilled water was added in each box that was left overnight at room temperature. The ratio sample/water was 1:3 (v/v).

Impurities were gently removed from the water surface 24 h later, until soil was well settled on the bottom of the box and the water clear. Young leaves of rhododendron, rose, oak, ivy and chestnut were placed with the upside facing upwards on the water surface. Boxes were kept in the laboratory for 5–6 days at 20 °C. Leaf portions showing the typical *Phytophthora* lesions (dark spot/necrosis) were dried on sterile filter paper and plated onto *Phytophthora* selective medium NARPH [32]. Plates were incubated at 20 °C in darkness for 5–7 days. *Phytophthora*-like colonies were then transferred and maintained on PDA plates at 20 °C in darkness. All isolates are maintained at the IPSP-CNR collection of fungal germplasm.

### 2.3. *Trichoderma* and *Phytophthora* Isolates

Each putative *Trichoderma* isolate was transferred onto a 9 mm diameter sterile cellophane disc in plates containing PDA and incubated in the darkness at 25 °C for 5 days. The mycelium was collected from the membrane and lyophilized. DNA was extracted from 20 mg of lyophilized mycelium by using the NucleoSpin® Plant II kit (Macherey Nagel GmbH & Co., Düren, Germany, KG), according to the manufacturer's instruction. Molecular identification was carried out by sequence analysis of TEF-1 $\alpha$  (Translation elongation factor 1 $\alpha$ ). PCR amplification was performed using specific primers EF1-728\_fw/TEF1LLE\_rev [33,34] under the following conditions: initial denaturation at 95 °C for 1 min, 35 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 90 s, and a final elongation step at 72 °C for 7 min. Genomic DNA of *Phytophthora* isolates was extracted as described above and molecular identification was performed by sequence analysis of the rDNA-ITS region with the ITS6/ITS4 primer pair [35]. Thermocycler parameters were 95 °C for 3 min, followed by 35 cycles of 94 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min, and a final elongation at 72 °C for 10 min.

Amplicons were purified with NucleoSpin® Gel and PCR Clean-up kit (Macherey Nagel GmbH & Co., KG), quantified by using Qubit® DNA BR (Broad Range) Assay Kits (Thermo Scientific™, Waltham, MA, USA) by Invitrogen Qubit® 4 Fluorometer (Thermo Scientific™, Waltham, MA, USA) and submitted for Sanger sequencing (eurofins Genomics).

TEF1- $\alpha$  PCR sequences were used to identify *Trichoderma* spp. isolates by using the TrichOKEY v.2.0 database available on the website of the International Subcommittee of *Trichoderma* and *Hypocrea* (ISTH, [www.isth.info](http://www.isth.info), [36] or via GenBank (<http://www.ncbi.nlm.nih.gov>, accessed on 30 April 2022). The obtained sequences were also used for phylogenetic analyses with 13 reference TEF-1 $\alpha$  sequences of *Trichoderma* species available in GenBank (NCBI), and one *Fusarium bostrycooides* selected as outgroup. GenBank accession numbers of the sequences generated in this study are shown in Table 1. The multiple sequence analysis and the construction of the neighbor-joining (NJ) phylogenetic tree were performed using MEGA 11 [37], following the Kimura-two-parameters (K2P) method [38]; 1000 bootstrap replicates were taken in order to examine the reliability of the interior branches and the validity of the trees obtained. The ambiguous nucleotide positions were removed for each sequence pair (pairwise deletion option). ITS sequences of one *P. xcambivora* isolate were blasted in the NCBI database to identify the most similar available sequences.

### 2.4. Temperature Growth Rate and Antagonistic Characterization of *Trichoderma* Isolates

All isolates were grown in triplicate on PDA and the mycelial radial growth was evaluated at 15 °C, 20 °C, 25 °C and 30 °C in the dark for 5 days. Daily, measurements of two perpendicular diameters of the colony were carried out to evaluate their growth rate and to explore the optimum (less stringent) experimental condition for subsequent assays. Additionally, the growth of all isolates was tested at 37 °C to verify their potential ability to live at mammalian temperature (to prevent possible interaction of those fungi with animal life). In order to find the best condition to perform all the further tests, *P. xcambivora* isolate was submitted to the same growth assays as described for *Trichoderma* spp.

#### Inhibition Test

For the evaluation of their biocontrol potential, *Trichoderma* spp. isolates were screened for their ability to reduce the mycelium growth of the locally selected *P. xcambivora* (strain PC003) by three different inhibition tests: (i) dual culture assay of the pathogen and each of the *Trichoderma* spp. isolates onto PDA dishes [39]; (ii) culture filtrate assay of *Trichoderma* isolates (diffusible secondary metabolites) [40]; and (iii) Sandwich Petri test volatile compounds from *Trichoderma* isolates on the growth of *P. xcambivora* [41].

For the dual culture assay, a 6 mm plug of *P. xcambivora* PC003 was cut from the edge of the actively growing colony on PDA, transferred at 1.5 cm from the edge of a 120 mm plate containing 20 mL of PDA and incubated at 25 °C in the dark. After 4 days, a 6 mm plug of each isolate of *Trichoderma* spp. was placed at the opposite side of the plates previously

inoculated with the pathogen (9 cm apart), and incubated under the same conditions. Radial growth of both the pathogen and *Trichoderma* spp. was measured each day, four times per day, until contact [10]. Plates containing two plugs of *P. xcambivora* at opposite sides were prepared as negative controls, while other plates co-cultured with *P. xcambivora* and Radixsoil® (Isagro commercial product based on *Trichoderma asperellum* ICC012 and *Trichoderma gamsii* ICC080) were used as a positive control. For each combination, three independent replicates were performed.

The potential antibiotic activity of secondary metabolites produced by the selected *Trichoderma* spp. isolates against *P. xcambivora* PC003 was investigated. Briefly, a 6 mm PDA plug taken from actively growing colonies of each isolate of *Trichoderma* was inoculated in 100 mL Erlenmeyer flasks containing 20 mL of PDB (Potato Dextrose Broth, 24 g L<sup>-1</sup>) and incubated at 25 °C at 100 rpm in a rotary shaker for two weeks. Inoculated and non-inoculated PDB with Radixsoil®, incubated under the same conditions, were used as positive and negative control, respectively. Cultural filtrates of each isolate were obtained through a micropore disposable membrane (0.22 µm pore size) to remove fungal spores. Two mL of each cultural filtrate were mixed with 18 mL of PDA right before solidification (50 °C) to obtain a final concentration of 10% (v/v) and poured in Petri dishes, further inoculated at the center with 6 mm agar plug of actively growing *P. xcambivora* isolate on PDA and incubated at 25 °C. PDA plates containing uninoculated PDB or PDB added with Radixsoil® were used as negative and positive control, respectively. Radial growth rate (two perpendicular diameters) of *P. xcambivora* isolate was daily recorded in all plates for nine days until the colony reached the edge of the control plates. The test was independently repeated three times.

The production of antibiotic VOCs by each isolate of *Trichoderma* spp. was evaluated through the 'Sandwich test', according to Li et al., 2018 [41]. PDA plates were inoculated in the center with a 6 mm diameter PDA disc made from actively growing colonies of each isolate of *Trichoderma* spp. on PDA and incubated at 25 °C in darkness. After 4 days, the lid of the plates was removed and replaced with a new PDA plate, inoculated upside down in the center, with a 6 mm agar plug of the isolate of *P. xcambivora*. The two plate bases were then fixed with a double layer of parafilm and incubated at 25 °C for 5 days. Negative controls were prepared using the same experimental setup, except that an uninoculated PDA disc or PDA inoculated with a PDA agar plug of *P. xcambivora* isolate were used in place of that inoculated with the *Trichoderma* isolate, while positive control was made by inoculating PDA with Radixsoil®. The experiment was independently repeated three times. Mycelial growth (two perpendicular diameters) of *P. xcambivora* isolate was recorded after 5 days of incubation in all the plates.

On the basis of the results obtained from these tests, *Trichoderma* isolates, representative of each species, which showed the highest antagonistic ability were used for the following analysis.

### 2.5. Enzymatic Activity Assays

The evaluation of the enzymatic activity of *Trichoderma* spp. was conducted to verify the ability of each isolate to produce enzymes that could play a positive role in the interaction with the pathogen. Chitinases and glucanases activities were determined according to Harman et al., 1993 [42] and Lorito et al., 2004 [43], respectively. Since the cell wall of *P. xcambivora* (like all the *Oomycetes*) is made of cellulose and glucane, a cellulases assay was also performed.

Selected *Trichoderma* isolates and positive control (Radixsoil®) were inoculated in 100 mL Erlenmeyer flasks containing 20 mL of either Richard's modified (RM) medium [42] added to 1.0% chitin (chitin suitable for analysis of chitinases, purified powder, Sigma Aldrich, St. Louis, MO, USA), or SMCS (a synthetic medium containing 680 mg KH<sub>2</sub>PO<sub>4</sub>, 870 mg K<sub>2</sub>HPO<sub>4</sub>, 200 mg KCl, 1 g NH<sub>4</sub>NO<sub>3</sub>, 200 mg CaCl<sub>2</sub>, 200 mg MgSO<sub>4</sub>·7H<sub>2</sub>O, 2 mg FeSO<sub>4</sub>, 2 mg ZnSO<sub>4</sub>, 2 mg MnSO<sub>4</sub>, 1.0% Carboxymethylcellulose (CMC, Sigma) and 5 g sucrose in 1 L of distilled water (pH 6.0) [43], respectively. Media were inoculated with an

aqueous spore suspension at the final concentration of  $5 \times 10^6$  conidia  $\text{mL}^{-1}$  and incubated for 4 days in the dark at  $25^\circ\text{C}$ , on a rotary shaker at 100 rpm. Mycelium was then collected from the culture by centrifugation at 5.9 rcf for 15 min, the residual particulates were removed by filtration through a  $0.22 \mu\text{m}$  filter (MF-Millipore MCE Membrane) and the cultural filtrates used for the enzymatic assay.

The chitinase activity of 1,4- $\beta$ -glucosaminidase (EC 3.2.1.30) and chitobiosidase (EC 3.2.1.29) was spectrophotometrically evaluated by determining the acetylglucosaminidase (NAGasi) and chitobiosidase (Biase) activities, measuring the absorbance of *p*-nitrophenol released by *p*-nitrophenyl- $\beta$ -D-N-acetylglucosaminide (Sigma) and *p*-nitrophenyl- $\beta$ -D-N, N'-diacetylchitobiose (Sigma), respectively. Both enzymatic substrates were prepared in a potassium phosphate buffer (50 mM, pH 6.7) at a concentration of  $0.3 \text{ mg mL}^{-1}$ . Thirty  $\mu\text{L}$  of enzymatic substrate and 60  $\mu\text{L}$  of RM cultural filtrate of each isolate and of Radixsoil<sup>®</sup> were added in the wells of an ELISA plate and incubated at  $50^\circ\text{C}$  for 30 min. RM cultural filtrate without inoculum was used as negative control.

The glucanase activity of exo-1,3- $\beta$ -glucanase (EC 3.2.1.58) was evaluated by measuring the absorbance of *p*-nitrophenol released from *p*-nitrophenyl- $\beta$ -D-glucopyranoside (Sigma). The substrate was prepared in potassium phosphate buffer (50 mM, pH 6.7) at a concentration of  $0.3 \text{ mg mL}^{-1}$ . Thirty  $\mu\text{L}$  of enzymatic substrate and 60  $\mu\text{L}$  of SMCS cultural filtrate of each isolate, and of Radixsoil<sup>®</sup>, were added in each well of an ELISA plate and incubated at  $37^\circ\text{C}$  for 30 min. SMCS cultural filtrate without inoculum was used as negative control.

The reactions of these three enzymes were stopped by adding 30  $\mu\text{L}$  of  $\text{Na}_2\text{CO}_3$  0.4 M and the absorbance at 405 nm was measured [42]. Each test was made in triplicate. Enzymatic activity was expressed in nktal (enzyme quantity required to convert one mole of substrate in one second).

The cellulase activity assay was performed with different techniques, using the Congo Red assay [44] and the k-CellG5-2V kit (MEGAZYME, Wicklow, Ireland), following the manufacturer's instructions. The Congo Red assay consists of a qualitative determination of cellulase activity based on the interaction of iodine with cellulose and its components in its degraded form; whereas areas with cellulose hydrolyzed by enzymes results in clear zones or the appearance of a pale halo. The halo was measured for the subsequent calculation of the enzymatic index (EI), a semi-quantitative estimate of the enzyme activities:

$$\text{EI} = \text{Diameter of hydrolysis zone (halo)} / \text{Diameter of colony}$$

Fungal samples were cultured onto water agar added with 1% CMC and incubated at  $25^\circ\text{C}$  in the darkness for 10 days. The agar medium was flooded with an aqueous solution of Congo Red ( $1 \text{ mg mL}^{-1}$ , Sigma) for 15 min. The Congo Red solution was then poured off, and plates further washed by flooding with 1 M NaCl for 15 min. Immediately after, the possible presence of the halo was evident.

The Megazyme test was carried out using cultural filtrates of each sample grown in SMCS. Cultural filtrate of Radixsoil<sup>®</sup> and uninoculated liquid medium were used as positive and negative control, respectively. The kit provides a *Trichoderma* cellulase standard solution ( $3 \text{ U mL}^{-1}$ ) to verify that the assay was correct. One unit of activity (CellG5 Unit) is defined as the amount of enzyme, in presence of thermostable  $\beta$ -glucosidase, required to release one  $\mu\text{mole}$  of 4-nitrophenol from CellG5 reagent solution ( $600 \text{ U mL}^{-1}$  of thermostable  $\beta$ -glucosidase in 10% DMSO plus sodium azide) in one minute under the defined assay conditions. The calculation of CellG5 Unit was realized using the Mega-Calc sheet available on the Megazyme website ([www.megazyme.com](http://www.megazyme.com), accessed on 22 February 2022), filling the required fields with the obtained values of absorbance and the dilution of the samples used for the enzymatic assay. Both assays were carried out in triplicate for each selected isolate of *Trichoderma* spp.

### 2.6. Evaluation of the Mycoparasitic Activity by Light and Scanning Electron Microscopy

The intimate interaction occurring between the above selected isolates of *Trichoderma* spp., and *P. xambivora* PC003 were observed under both light microscope (LM) (Zeiss, Axioskope, Germany) and Scanning Electron Microscope (SEM) (Gaia 3, Tescan s.r.o, Brno, Czech Republic). For observation at LM a sterile cellophane disk was placed on Water Agar (18.0 g L<sup>-1</sup>) plates and inoculated with a 0.5 cm plug from a PDA culture of *P. xambivora* isolate positioned at the edge of each plate and incubated at 25 °C in the darkness. After three days, a 0.5 cm agar plug of each isolate of *Trichoderma* spp. grown on PDA, was placed in each plate, 3 cm apart from the pathogen and incubated at 25 °C. Once the two colonies came into contact with each other, a little piece of the corresponding cellophane disk was cut with a sterile scalpel and placed on a slide for microscopy. Mycelium was stained with a drop of lacto-phenol/cotton blue, covered with a coverslip, and examined at LM at 400× and 630× magnifications. SEM observations were carried out preparing dual culture plates of the selected *Trichoderma* isolates and *P. xambivora* PC003 as described before, but without adding the cellophane layer on the agar. An agar plug made where the two colonies came into contact with each other was cut and used for observations. Samples were fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 6.7). After incubation for at least 24 h at 4 °C, samples were washed two times in 0.1 M phosphate buffer for 10 min each and dehydrated for 20 min in a graded series of ethanol (30, 50, 70, 80, 90 and 100%). Dehydrated samples were then dried to critical point using liquid carbon dioxide. Samples were mounted with double-sided tape on aluminum stubs and coated with silver particles in a coater sputter. Coated samples were observed with scanning electron microscope using an electron beam for SEM imaging at the voltage of 10 kV, operating in high-vacuum mode and with a secondary electron (SE) detector [45,46]. Evidence of mycoparasitic activity (such as *Trichoderma* hyphae coiling around those of the pathogen and/or degradation of the pathogen's cell wall) was observed and photographed.

### 2.7. Statistical Analysis

Data on growth rates obtained from both dual cultures and secondary metabolites were subjected to analysis of variance (ANOVA) of regression by using GraphPad Prism 10 software in order to compare the slopes of *P. xambivora* in control plates with that in presence of *Trichoderma* spp. colonies/cultural filtrates, assuming  $p \leq 0.05$  as a significant value.

A one-way ANOVA test of mycelium growth rate of *P. xambivora* PC003 obtained from both Sandwich Petri and enzymatic activity assay (chitinases, glucanases, cellulases) assays, was performed with STATISTICA 10 using Fisher LSD test for equal N and assuming  $p \leq 0.05$  as significant level.

## 3. Results

### 3.1. TEF-1 $\alpha$ and ITS rDNA Analysis

PCR amplification of TEF-1 $\alpha$  from all the 20 *Trichoderma* isolates from roots and rhizosphere of *C. sativa* (15 endophytes and 5 from rhizosphere respectively) produced DNA fragments between 930 and 1200 bp. Sequencing of these fragments allowed to identify five different species of *Trichoderma*, as indicated in Table 1. In detail, isolates SG1 and SG13 were identified as *T. longipile*, isolate SG4 as *T. asperellum*, isolates SG5 and SG6 as *T. koningiopsis*, isolate SG19 as *T. koningii* while the remaining isolates (SG2, SG3, SG7, SG8, SG9, SG10, SG11, SG12, SG14, SG16, SG17, SG18, SG20 and SG21) belong all to *T. hamatum* species. Phylogenetic distance among isolates and species considered in this work are shown in the neighbor-joining (NJ) phylogenetic tree (Figure 1). Isolates were grouped according to the references TEF-1 $\alpha$  from GenBank and split in two clades: Harzianum and *Trichoderma*. Isolates of *T. hamatum*, *T. koningiopsis*, *T. asperellum* and *T. konigii* clustered into the *Trichoderma* clade, while both isolates of *T. longipile* were grouped within the Harzianum one (Figure 1). By baiting soil samples, one isolate of *P. xambivora* has been isolated. The ITS amplification of the pathogen resulted in an 844 bp sequence, identified as

*Phytophthora xcambivora* (Accession number ON847330) showing a percentage of similarity of 99.67% with the reference found in database.

**Table 1.** TEF-1 $\alpha$  and ITS rDNA sequences examined in this study.

Species	Isolate	Source	Geographical Origin	Genbank Accession Number	Reference
<i>T. longipile</i>	SG1	Root endophyte ( <i>C. sativa</i> )	Italy	OM685061	This work
<i>T. hamatum</i>	SG2	Root endophyte ( <i>C. sativa</i> )	Italy	OM906891	This work
<i>T. hamatum</i>	SG3	Root endophyte ( <i>C. sativa</i> )	Italy	OM906892	This work
<i>T. asperellum</i>	SG4	Root endophyte ( <i>C. sativa</i> )	Italy	OM906893	This work
<i>T. koningiopsis</i>	SG5	Root endophyte ( <i>C. sativa</i> )	Italy	OM906894	This work
<i>T. koningiopsis</i>	SG6	Root endophyte ( <i>C. sativa</i> )	Italy	OM906895	This work
<i>T. hamatum</i>	SG7	Root endophyte ( <i>C. sativa</i> )	Italy	OM906896	This work
<i>T. hamatum</i>	SG8	Root endophyte ( <i>C. sativa</i> )	Italy	OM906897	This work
<i>T. hamatum</i>	SG9	Root endophyte ( <i>C. sativa</i> )	Italy	OM906898	This work
<i>T. hamatum</i>	SG10	Root endophyte ( <i>C. sativa</i> )	Italy	OM906899	This work
<i>T. hamatum</i>	SG11	Root endophyte ( <i>C. sativa</i> )	Italy	OM906900	This work
<i>T. hamatum</i>	SG12	Root endophyte ( <i>C. sativa</i> )	Italy	OM906901	This work
<i>T. longipile</i>	SG13	Root endophyte ( <i>C. sativa</i> )	Italy	OM906902	This work
<i>T. hamatum</i>	SG14	Root endophyte ( <i>C. sativa</i> )	Italy	OM906903	This work
<i>T. hamatum</i>	SG16	Root endophyte ( <i>C. sativa</i> )	Italy	OM906904	This work
<i>T. hamatum</i>	SG17	Rhizosphere ( <i>C. sativa</i> )	Italy	OM906905	This work
<i>T. hamatum</i>	SG18	Rhizosphere ( <i>C. sativa</i> )	Italy	OM906906	This work
<i>T. koningii</i>	SG19	Rhizosphere ( <i>C. sativa</i> )	Italy	OM906907	This work
<i>T. hamatum</i>	SG20	Rhizosphere ( <i>C. sativa</i> )	Italy	OM906908	This work
<i>T. hamatum</i>	SG21	Rhizosphere ( <i>C. sativa</i> )	Italy	OM906909	This work
<i>P. xcambivora</i>	PC003	Rhizosphere ( <i>C. sativa</i> )	Italy	ON847330	This work
<i>T. hamatum</i>	Hypo 648	Not specified	France	KJ665514.1	[47]
<i>T. asperellum</i>	T2023	Soil	Malaysia	MG595721.1	[48]
<i>T. gamsii</i>	TW20050	Soil	China	KU523895.1	[49]
<i>T. atroviride</i>	CBS 693.94	Not specified	Poland	KJ786838.1	[50]
<i>T. petersenii</i>	S636	Not specified	Greece	KJ665671.1	[47]
<i>T. koningiopsis</i>	18ASMA001	Leaves ( <i>Smilax aspera</i> )	France	MT671922.1	[51]
<i>T. mediterraneum</i>	S665	Not specified	Spain	KJ665611.1	[47]
<i>T. polysporum</i>	G.J.S. 99-222	Not specified	New Zealand	KJ665741.1	[47]
<i>T. reesei</i>	QM 9414	Not specified	Not specified	Z23012.1	[52]
<i>T. longibrachiatum</i>	CM 1798	Blood culture	Not specified	MH249945.1	[53]
<i>T. atrobrunneum</i>	T17-04	Roots ( <i>Viburnum carlesii</i> )	United Kingdom	MW232522.1	[21]
<i>T. vires</i>	Tvien3	Soil	China	MT081441.1	[54]
<i>T. longipile</i>	120953	Not specified	CBS	FJ860643.1	[55]
<i>F. bostrycooides</i> *	NRRL 52701	Not specified	Colombia	JF740784.1	[56]

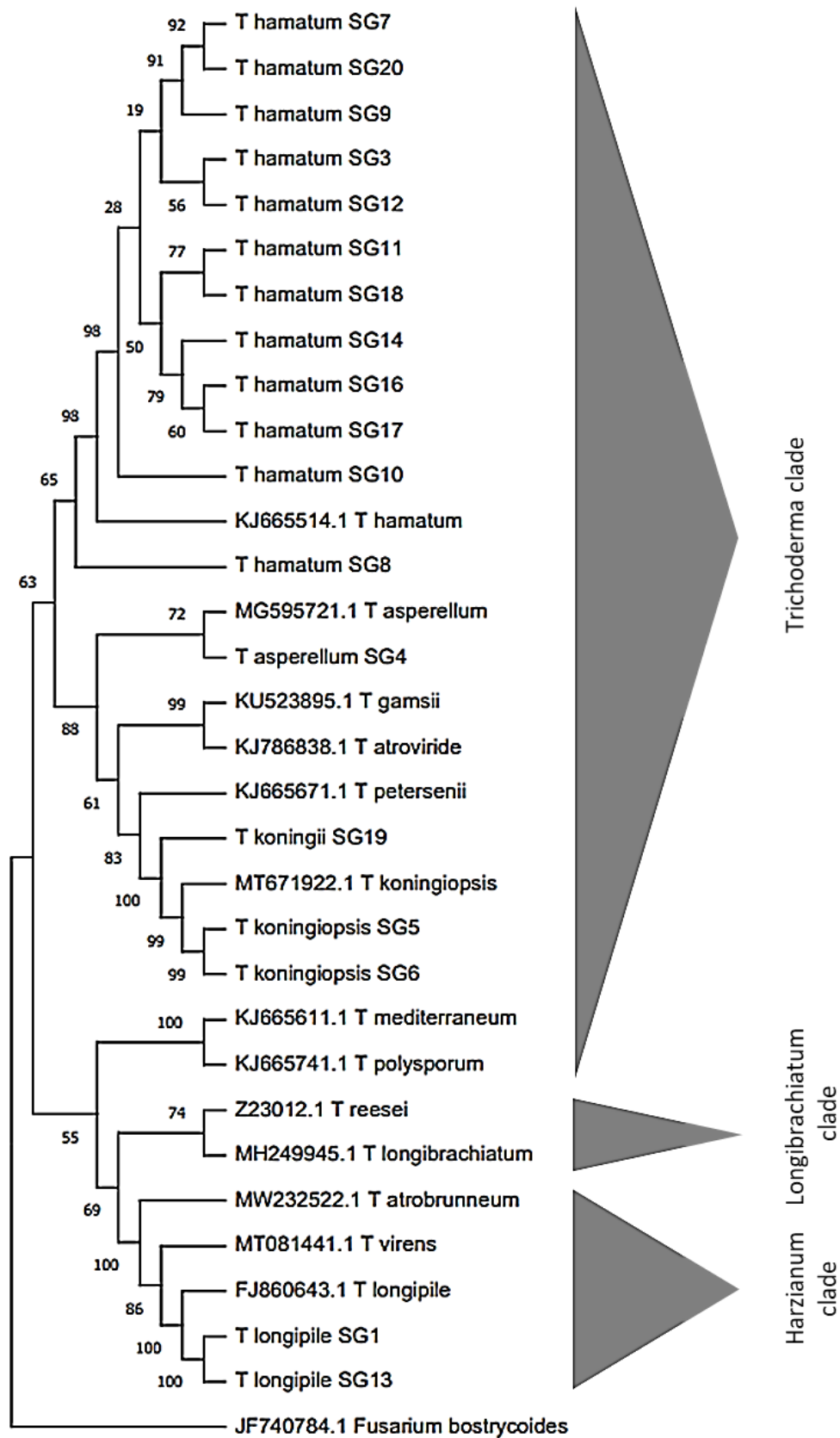
SG Item (included in the white boxes) indicates *Trichoderma* spp. sampled in S. Godenzo (SG) from roots and rhizosphere of *C. sativa*. Within the gray boxes reference isolates, used for phylogenetic analysis, are indicated. \*: *Fusarium bostrycooides* sequence used as outgroup.

### 3.2. Temperature Growth Rate and Antagonistic Characterization of *Trichoderma* Isolates

The growth rate at different temperatures showed no difference among the 20 *Trichoderma* isolates. The optimum temperature of mycelium growth on PDA of all *Trichoderma* isolates was 25 °C, while at 37 °C, no growth was observed.

When submitted for dual culture testing aimed to evaluate the antagonistic ability of *Trichoderma* spp. due to diffusible metabolites, pathogen's growth data was used to create growth curves whose analysis of variance of regression highlighted the differences when the pathogen grew alone and in the presence of each of the 20 potential antagonistic isolates (Table 2). All growth curves (alone and in each combination with the *Trichoderma* isolates) were highly significant as resulting from R<sup>2</sup> and *p* values ( $\geq 0.731$  and  $\leq 0.0001$ , respectively). With respect to the ability of *Trichoderma* spp. isolates to affect *P. cambivora* growth, only in the presence of *T. hamatum* SG2, SG3, SG7, SG10 and SG18, of *T. longipile* SG13 and of *T. koningi* SG19 did the pathogen reduce its growth when compared with control plates. However, in each of these cases, differences in growth rates were not significant ( $P_{\text{slope}} \geq 0.1459$ ). By contrast, *P. xcambivora* paired with *T. hamatum* SG12 and SG16, grew faster compared with the single culture ( $P_{\text{slope}} = 0.0003$  and 0.0121, respectively). The pathogen growing in the presence of the remaining isolates resulted faster than the control, but in each case, differences were not significant.





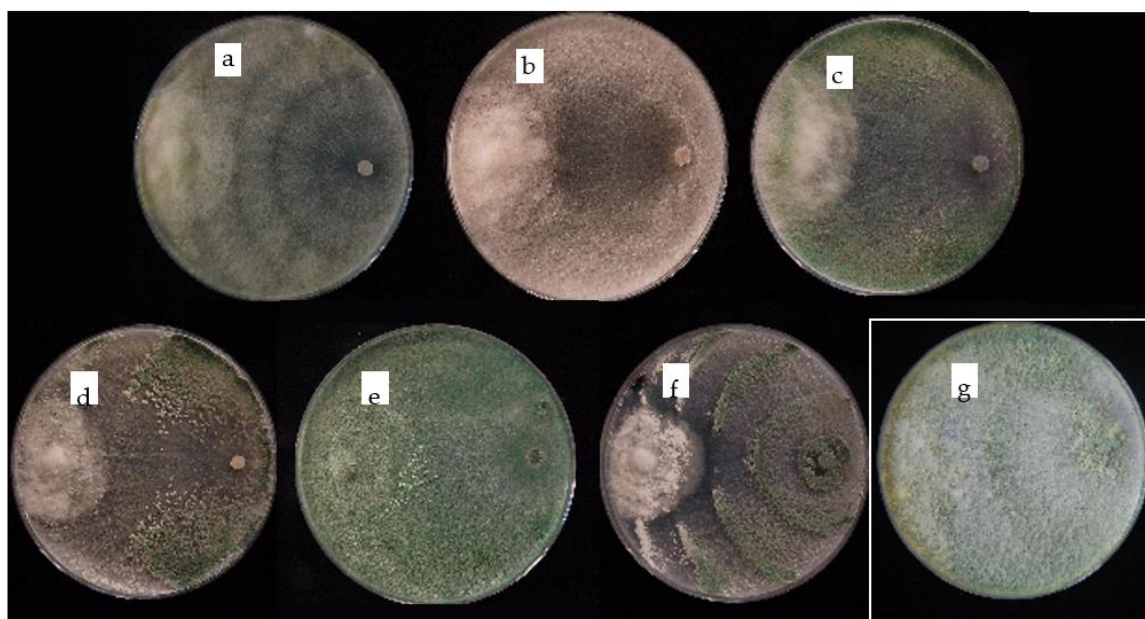
**Figure 1.** Neighbor-joining (NJ) phylogenetic tree of *Trichoderma* s isolates based on TEF-1 $\alpha$  sequence. *Fusarium bostrycoides* was used as outgroup.

**Table 2.** Mycelium growth rate from dual cultures assay between *Trichoderma* isolates and *P. xcambivora*.

Dual Culture	Slope (mm h <sup>-1</sup> )	R <sup>2</sup>	p	P <sub>slope</sub>
<i>P. xcambivora</i> (control)	0.096	0.887	<0.0001	
<i>P. xcambivora</i> vs. <i>T. longipile</i> SG1	0.183	0.734	<0.0001	0.5077
<i>P. xcambivora</i> vs. <i>T. hamatum</i> SG2	0.087	0.877	<0.0001	0.5077
<i>P. xcambivora</i> vs. <i>T. hamatum</i> SG3	0.095	0.731	<0.0001	0.9341
<i>P. xcambivora</i> vs. <i>T. asperellum</i> SG4	0.111	0.864	<0.0001	0.3000
<i>P. xcambivora</i> vs. <i>T. koningiopsis</i> SG5	0.142	0.835	<0.0001	0.0563
<i>P. xcambivora</i> vs. <i>T. koningiopsis</i> SG6	0.135	0.913	<0.0001	0.2043
<i>P. xcambivora</i> vs. <i>T. hamatum</i> SG7	0.088	0.820	<0.0001	0.5330
<i>P. xcambivora</i> vs. <i>T. hamatum</i> SG8	0.107	0.963	<0.0001	0.4981
<i>P. xcambivora</i> vs. <i>T. hamatum</i> SG9	0.115	0.851	<0.0001	0.1337
<i>P. xcambivora</i> vs. <i>T. hamatum</i> SG10	0.071	0.782	<0.0001	0.1459
<i>P. xcambivora</i> vs. <i>T. hamatum</i> SG11	0.117	0.928	<0.0001	0.2180
<i>P. xcambivora</i> vs. <i>T. hamatum</i> SG12	0.173	0.858	<0.0001	0.0003
<i>P. xcambivora</i> vs. <i>T. longipile</i> SG13	0.075	0.937	<0.0001	0.1669
<i>P. xcambivora</i> vs. <i>T. hamatum</i> SG14	0.121	0.787	<0.0001	0.1445
<i>P. xcambivora</i> vs. <i>T. hamatum</i> SG16	0.140	0.953	<0.0001	0.0121
<i>P. xcambivora</i> vs. <i>T. hamatum</i> SG17	0.105	0.916	<0.0001	0.5274
<i>P. xcambivora</i> vs. <i>T. hamatum</i> SG18	0.082	0.779	<0.0001	0.4815
<i>P. xcambivora</i> vs. <i>T. koningii</i> SG19	0.081	0.875	<0.0001	0.2847
<i>P. xcambivora</i> vs. <i>T. hamatum</i> SG20	0.091	0.826	<0.0001	0.7582
<i>P. xcambivora</i> vs. <i>T. hamatum</i> SG21	0.109	0.905	<0.0001	0.4570
<i>P. xcambivora</i> vs. Radixsoil®	0.084	0.743	<0.0001	0.5399

Slope: growth rate (mm h<sup>-1</sup>) of *P. xcambivora*; R<sup>2</sup> and *p*: significance of the growth curve; P<sub>slope</sub>: significance of the difference of growth rate. Data are the average of three independent replicates.

Finally, the presence of Radixsoil® also did not affect the growth of *P. xcambivora* (P<sub>slope</sub> = 0.5399) (Table 2 and Figure 2). The interaction occurring in dual cultures between *Trichoderma* isolates and *P. xcambivora* are shown in Figure 2, where all the beneficial organisms evidenced the capability to overgrow the pathogen and sporulate on it.



**Figure 2.** Dual cultures of *Trichoderma* isolates (right) against *P. xcambivora* PC003 (left) on PDA medium after 9 (a–d,f) and 14 (e,g) days at 25 °C. (a) *T. longipile* SG1; (b) *T. asperellum* SG4; (c) *T. koningiopsis* SG6; (d) *T. hamatum* SG18; (e) *T. koningii* SG19; (f) *T. hamatum* SG20; (g) Radixsoil®.

When the *Trichoderma* isolates were tested for their ability to release secondary metabolites with an inhibiting activity, the growth rate of *P. xcambivora* on PDA was compared with that registered on PDA, added with the cultural filtrates of each isolate of *Trichoderma*. Analysis of variance of regression performed on growth data allowed for the observing of a different behavior of the pathogen depending on the cultural filtrates (Table 3). Filtrate culture assay showed highly significant growth curves as resulting from  $R^2$  and  $p$  values, ( $\geq 0.789$  and  $\leq 0.0001$ , respectively). No mycelial development was observed in *P. cambivora* PC003 grown with culture filtrates of *T. hamatum* SG14 and SG20, as confirmed by the slope values (0.819 and 0.000 mm per day, respectively). A significantly lower growth rate was also detected with *T. longipile* SG1 and SG13, and *T. hamatum* SG8 and SG18, compared to control ( $P_{\text{slope}} \leq 0.001$ ). On the other hand, a relevant increase in growth rate was found in *P. xcambivora* PC003 grown with all other culture filtrates of *Trichoderma* ( $P_{\text{slope}} \leq 0.012$ ), except for *T. hamatum* SG2, SG7 and SG17, and *T. koningiopsis* SG5 which showed no difference (Table 3).

**Table 3.** Mycelium growth rate of *P. xcambivora* PC003 inoculated on PDA added with the cultural filtrates of *Trichoderma* isolates.

Thesis	Slope (mm day <sup>-1</sup> )	R <sup>2</sup>	$p$	P <sub>slope</sub>
<i>P. xcambivora</i> (Control)	6.517	0.921	<0.0001	
<i>P. xcambivora</i> vs. <i>T. longipile</i> SG1	3.479	0.887	<0.0001	<0.0001
<i>P. xcambivora</i> vs. <i>T. hamatum</i> SG2	6.357	0.887	<0.0001	0.080
<i>P. xcambivora</i> vs. <i>T. hamatum</i> SG3	8.571	0.943	<0.0001	0.020
<i>P. xcambivora</i> vs. <i>T. asperellum</i> SG4	9.823	0.952	<0.0001	<0.0001
<i>P. xcambivora</i> vs. <i>T. koningiopsis</i> SG5	7.183	0.935	<0.0001	0.263
<i>P. xcambivora</i> vs. <i>T. koningiopsis</i> SG6	10.100	0.951	<0.0001	<0.0001
<i>P. xcambivora</i> vs. <i>T. hamatum</i> SG7	6.274	0.789	<0.0001	0.772
<i>P. xcambivora</i> vs. <i>T. hamatum</i> SG8	5.071	0.905	<0.0001	0.001
<i>P. xcambivora</i> vs. <i>T. hamatum</i> SG9	8.530	0.953	<0.0001	0.000
<i>P. xcambivora</i> vs. <i>T. hamatum</i> SG10	8.983	0.953	<0.0001	0.000
<i>P. xcambivora</i> vs. <i>T. hamatum</i> SG11	9.177	0.944	<0.0001	0.000
<i>P. xcambivora</i> vs. <i>T. hamatum</i> SG12	9.126	0.952	<0.0001	0.000
<i>P. xcambivora</i> vs. <i>T. longipile</i> SG13	4.579	0.924	<0.0001	0.000
<i>P. xcambivora</i> vs. <i>T. hamatum</i> SG14	0.819	0.185	<0.0001	<0.0001
<i>P. xcambivora</i> vs. <i>T. hamatum</i> SG16	8.923	0.956	<0.0001	0.000
<i>P. xcambivora</i> vs. <i>T. hamatum</i> SG17	7.550	0.914	<0.0001	0.123
<i>P. xcambivora</i> vs. <i>T. hamatum</i> SG18	5.450	0.936	<0.0001	0.044
<i>P. xcambivora</i> vs. <i>T. koningii</i> SG19	8.954	0.944	<0.0001	0.000
<i>P. xcambivora</i> vs. <i>T. hamatum</i> SG20	0.000	1.000	-	<0.0001
<i>P. xcambivora</i> vs. <i>T. hamatum</i> SG21	8.149	0.936	<0.0001	0.012
<i>P. xcambivora</i> vs. Radixsoil®	9.345	0.963	<0.0001	<0.0001

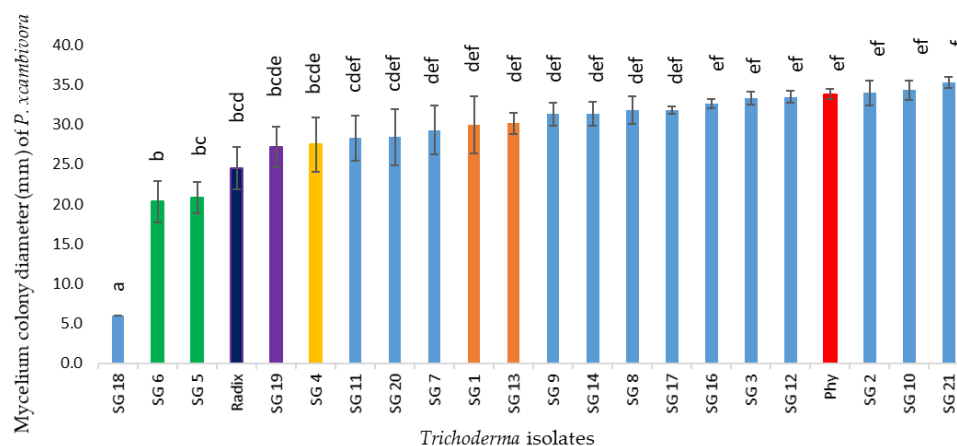
Slope: growth rate (mm day<sup>-1</sup>); R<sup>2</sup> and  $p$ : significance of the growth curve; P<sub>slope</sub>: significance of the difference of growth rate. Data are the average of three independent replicates.

In the Sandwich Petri test, *P. xcambivora* PC003 fungal growth, after 5 days of incubation, showed a significant inhibition in colony growth in combination with *T. koningiopsis* SG5 and SG6, while together with *T. hamatum* SG18 no growth was observed (Table 4 and Figure 3). Radixsoil® significantly reduced pathogen's growth, too. According to all the results described above, the most performing *Trichoderma* isolates for each species, able to contain *P. xcambivora* growth, were selected: *T. longipile* SG1, *T. asperellum* SG4, *T. koningiopsis* SG6, *T. koningii* SG19 and *T. hamatum* SG 18 and SG20, and used for further analysis.

**Table 4.** Enzymatic activity of the selected *Trichoderma* isolates.

Isolate	NAGase (nktal)	BIase (nktal)	Glucanase (nktal)	Cellulase EI
<i>T. asperellum</i> SG4	0.798 a	0.193 a	0.213 a	0.366 a
<i>T. hamatum</i> SG18	0.934 b	0.546 c	0.474 b	0.932 b
<i>T. hamatum</i> SG20	0.915 b	0.394 b	0.808 c	1.029 d
<i>T. koningii</i> SG19	0.987 b	0.496 bc	0.593 b	0.973 c
<i>T. koningiopsis</i> SG6	1.129 c	0.693 d	1.174 d	1.000 c
<i>T. longipile</i> SG1	1.107 c	0.706 d	0.157 a	0.906 b
Radixsoil®	1.103 c	0.698 d	1.127 d	0.910 b

Within each column at different letters correspond values significantly different (ANOVA), assuming  $p \leq 0.05$  as significant level.



**Figure 3.** Effect of volatile metabolites (sandwich test) produced by *Trichoderma* spp. isolates on *P. xambivora* PC003 growth (mm) on PDA after 5 days of incubation. At different letters correspond values significantly different for  $p \leq 0.05$  (One-way ANOVA, Fisher LSD test for equal N). Values are the average of three independent replicates. (Blue) *T. hamatum*; (green) *T. koningiopsis*; (dark blue) Radixsoil®; (purple) *T. koningii*; (yellow) *T. asperellum*; (orange) *T. longipile*; (red) *P. xambivora* (Phy).

### 3.3. Enzymatic Activity Assays

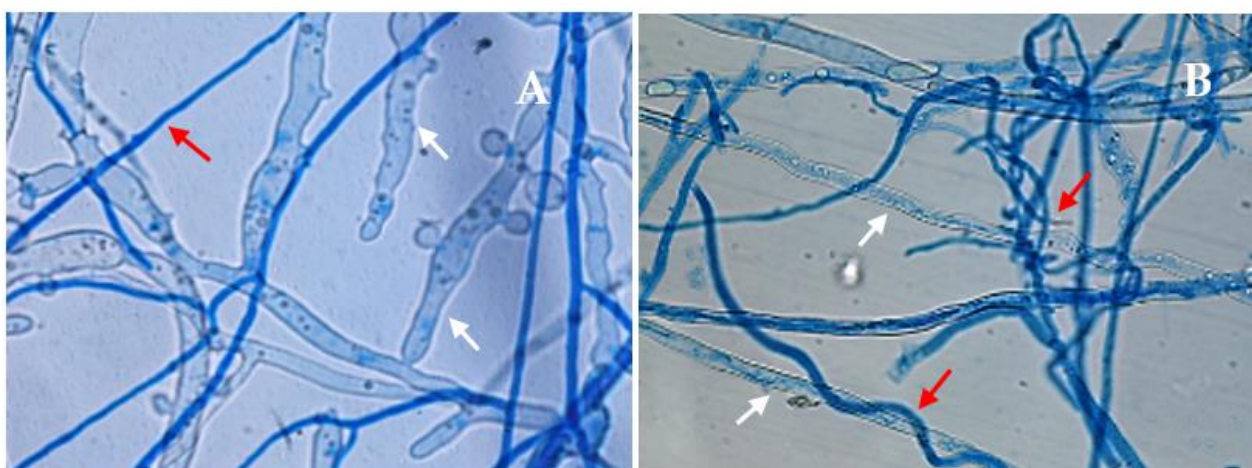
All the selected *Trichoderma* isolates grown in RM liquid medium added with 1% chitin showed a chitinase activity after 4 days of incubation (Table 4). NAGase activity was higher compared to Biase enzymatic activity for all the selected *Trichoderma* isolates. A significant lower level of NAGase activity was detected in *Trichoderma asperellum* (SG4) compared to the control (0.798 nktal), while *T. longipile* SG1 and *T. koningiopsis* SG6 and Radixsoil® showed the higher enzymatic activity (1.129, 1.107 vs. 1.103 nktal, respectively). *Trichoderma koningii* SG19 and both the isolates of *T. hamatum* (SG18 and SG20) showed an intermediate NAGase activity (0.987, 0.934 and 0.915 nktal respectively). Concerning the Biase activity *T. asperellum* SG4 (0.193 nktal), *T. hamatum* SG20, *T. koningii* SG19 and *T. hamatum* SG18, showed a significantly lower activity compared to the control with values of 0.394, 0.496 and 0.546 nktal, respectively. On the other hand, *T. longipile* SG1 and *T. koningiopsis* SG6 showed a Biase activity (0.706 and 0.693 nktal, respectively) similar to that of Radixsoil® (0.698 nktal) (Table 4).

Glucanase activity was detected for all *Trichoderma* isolates grown in SMCS liquid medium supplemented with 1% CMC after 4 days of incubation (Table 4). In detail, the highest production of glucanase was obtained for *T. koningiopsis* SG6 (1.174 nktal), even significantly higher than that of Radixsoil® (0.127 nktal,  $p < 0.01$ ). *T. longipile* SG1 and *T. asperellum* SG4 revealed the lowest enzymatic activity compared to the other isolates and to the positive control, with 0.16 and 0.21 nktal versus 1.13 nktal of Radixsoil®. *T. longipile* SG1 and *T. asperellum* SG4 showed the significantly lowest glucanase activity (0.157 and 0.213 nktal, respectively) followed by *T. hamatum* SG18 and SG20 (0.474 and 0.808 nktal, respectively).

Cellulase activity performed by the Congo Red assay showed that, among the tested isolates, *T. hamatum* SG20 displayed the best capability to produce cellulase, followed by *T. koningiopsis* SG6 and *T. koningii* SG19, whose enzymatic index were significantly different from Radixsoil. *Trichoderma asperellum* SG4 resulted in the less efficient isolate in cellulase activity (Table 4). A further cellulase assay implemented by the Megazyme kit resulted as not efficient for all the tested isolates, despite the fact that the assay was successfully carried out since the CellG5 Unit of the standard showed a positive value (12.6 Units/mL).

### 3.4. Evaluation of the Mycoparasitic Activity by Light and Scanning Electron Microscopy

Observations performed with light microscope and SEM, revealed an active interaction between the isolates of *Trichoderma* and that of *P. xambivora* PC003. All *Trichoderma* isolates, except *T. longipile* SG1 grew along *P. xambivora* hyphae, often branching profusely and developing coilings in order to increase the contact with the pathogen mycelium (Figures 4B and 5) [57]. Moreover, hyphae of *P. xambivora* appeared extensively deformed, collapsed or broken (Figures 4 and 5). At last, a profuse sporulation of the beneficial isolates is visible on the pathogen, as for *T. koningii* SG19 (Figure 5D).



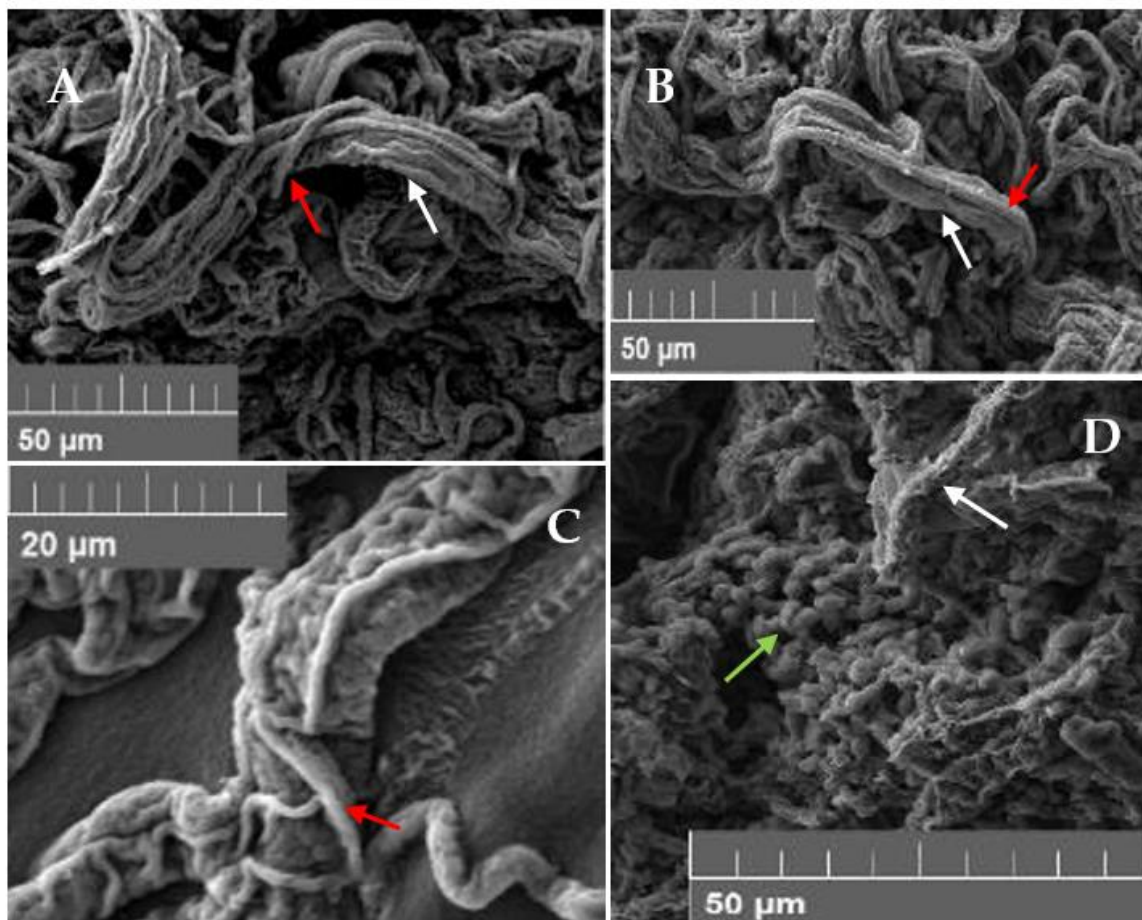
**Figure 4.** Dual culture observation of *Trichoderma koningiopsis* (SG6) vs. *P. xambivora* at light microscope (LM; 630×): (A) hyphae of *T. koningiopsis* (red narrows) and of *P. xambivora* (white narrows); (B) coilings of *T. koningiopsis* (red arrows) choking the hyphae of *P. xambivora* (white arrows).

The aptitude of each selected *Trichoderma* isolate to adopt different antagonistic strategies is summarised in Table 5.

**Table 5.** Schematic representation of the biocontrol strategies adopted by the different *Trichoderma* isolates.

Isolates	Antibiosis				Mycoparasitism
	Dual Culture	Cultural Filtrates (SMs)	Sandwich Test (VOCs)	Enzymatic Activity (CWDEs)	
<i>T. longipile</i> SG1	+	++	/	++	/
<i>T. asperellum</i> SG4	+	/	+	+	+++
<i>T. koningiopsis</i> SG6	+	/	++	+++	+++
<i>T. hamatum</i> SG18	+++	++	+++	++	+++
<i>T. koningii</i> SG19	+++	/	+	++	+++
<i>T. hamatum</i> SG20	+	+++	/	++	+++
Radixsoil®	+	/	++	+++	+++

+++ : strong activity; ++ : moderate activity; + : low activity; / : no activity.



**Figure 5.** Observations of *Trichoderma* vs. *P. xambivora* in dual culture at SEM. Coilings of (A) *T. hamatum* SG18; (B) *T. koningii* SG19; (C) *T. asperellum* SG4 (all indicated with red narrow) around *P. xambivora* hyphae (white narrow). (D) Spore production (green arrows) of *T. koningii* SG19 on *P. xambivora* mycelium (white arrow).

#### 4. Discussion

*Trichoderma* spp. act as biocontrol agents implementing four different strategies to reduce the growth of the pathogen: competition for nutrients in the same growing space, antibiosis by producing secondary metabolites (diffusible and VOCs) toxic for the pathogen, mycoparasitism by the formation of coilings, and synthesis of the cell wall degrading enzyme [58–60]. One of the best strategies to isolate effective beneficial fungi to be used against a specific pathogen is to search/investigate around healthy plants within the outbreaks of a disease or in places where the development of the pathogen is promoted [61,62]. According to this evidence, in this study, 20 *Trichoderma* spp. isolates were collected from rhizosphere and fine roots of asymptomatic chestnut trees growing inside an area affected by ink disease caused by the Oomycetes *P. xambivora* [29]. The collected isolates belong to five different species (*T. asperellum*, *T. hamatum*, *T. koningii*, *T. koningiopsis*, *T. longipile*), which are grouped into two clades (*Trichoderma* and Viride) according to the neighbor-joining tree constructed on TEF-1 $\alpha$  gene marker [63] (Figure 1). These are cosmopolitan species already isolated from soil and as endophytes, with the exception of *T. koningii* and *T. longipile*, whose presence was found in some areas of Europe and North America [40,64,65]. These species, but *T. longipile*, were already isolated in soil from different forest ecosystems (hazelnut orchards, conifer and broadleaf mixed forest), although a higher biodiversity of the *Trichoderma* population was reported by Rees et al., 2021 [21] and Ma et al., 2020 [66] compared to that found in this study. To our knowledge, no information is currently available on the *Trichoderma* community associated with *Castanea sativa*. In the present study, all the isolates

were tested in vitro for their inhibition activity against *P. xambivora*, the causal agent of ink disease, thus assessing the strategy, or the strategies, by which the characterized *Trichoderma* can act as biocontrol agents. Then, the most performing ones were selected to be used, for the first time, in a new possible biological approach for an integrated management of ink disease.

It is well known that *Trichoderma* spp. exhibit a fast up-take of nutrient resources from soil, creating a non-favorable environment for the other rhizosphere microorganisms and leading to a natural management of fungal communities [59,67]. The antagonism activity of the selected *Trichoderma* isolates by diffusible SMs was assessed in this study, evaluating the interaction between the colonies of each *Trichoderma* isolate with that of *P. xambivora* after 14 days of co-culturing (dual culture) on PDA medium. All putative antagonists completely overgrew and sporulated on the colony of the pathogen, particularly some isolates of *T. hamatum* (SG2, SG3, SG7, SG10, SG18), *T. longipile* SG13 and *T. koningiopsis* SG19 that induced a growth slowdown of *P. xambivora* in dual cultures (Table 2). These features represent a very important characteristic to be evaluated in several species of *Trichoderma* used as beneficial fungi against several plant pathogens, in both forest and crop patho-systems. In particular, *T. harzianum*, *T. atroviride*, *T. virens*, *T. koningiopsis*, *T. hamatum* and *T. asperellum* were reported to overgrow and sporulate on the target pathogen in dual cultures, the latter considered as a sign of mycoparasitism [21,27,68–71].

*P. xambivora* growth stimulation, here observed when in presence of *T. longipile* SG1 and two isolates of *T. hamatum* (SG12 and SG16) (Table 2), could be considered as a reaction to the presence of the antagonist sharing the same plate, i.e., as a response of the pathogen to an adverse environment. This behavior has already been observed in other pathogen-*Trichoderma* dual cultures (*F. graminearum* vs. *T. gamsii*) where faster growth of the pathogen was explained by Zapparata et al., 2021 [11] as probably due to the lack of nutrients (which can stimulate an initial overgrowth to escape the stressful situation) or the sensing of secondary metabolites synthesized by *Trichoderma*, which stimulate ‘the prey’ to accelerate its growth.

Direct antagonistic activity exerted by *Trichoderma* also implies a cross-talk between the antagonist and the pathogen during which the sensing of secondary metabolites (SMs) diffused across the media, or released in the shared headspace, interferes with fungal cellular activities [58]. As well as for all microorganisms, *Trichoderma* SMs play pivotal roles in chemical defense and communication, and some of them are largely studied, showing antibiotic abilities to be of importance in biocontrol applications (peptaibols, polyketides, pyrones, terpenes and diketopiperazine-like compounds) [72]. Among these compounds, 6-pentyl- $\alpha$ -pyrone (6-PP) is the most studied for its antifungal property and is responsible for the “coconut aroma” associated with some species of *Trichoderma*, such as *T. harzianum*, *T. virens*, *T. asperellum* [59,73–76], and that in our study was observed in all the tested *T. hamatum* isolates, and weakly in *T. asperellum* SG4. In this study, the capability of *Trichoderma* to act through antibiosis strategy was assessed by also evaluating the eventual release of diffusible and volatile secondary metabolites, as well as their inhibitory activity towards the growth of *P. xambivora* in vitro. Each tested *Trichoderma* showed a different inhibition degree of *P. xambivora* growth due to SMs (both diffusible and volatile), which appeared unrelated to the species they belong to, but rather to specific features of the single genotypes. For the first time, the effectiveness of *T. longipile* SG1 and *T. hamatum* SG20 diffusible SMs in the inhibition of *P. xambivora* in vitro was documented here, showing a strong (SG1) or total (SG20) inhibition of mycelium growth of the pathogen (Table 3). In recent studies, the crude extract from *T. harzianum*, *T. viride*, *T. reesei*, *T. asperellum* and *T. asperelloides* is reported to inhibit the development of *P. palmivora* lesions on palm oil [77–79], and a treatment with *T. atroviride* and *T. petersenii* extract inhibited *Phytophthora* growth, inducing defense-related genes [80].

Noteworthy results were obtained through *T. koningiopsis* (SG5, SG6) and *T. hamatum* SG18 VOCs emission, which strongly or totally prevented mycelial growth of *P. xambivora* in vitro (Figure 3). Similar results were obtained by Elsherbiny et al., 2020 [81], revealing

that VOCs emitted by two strains of *T. atroviride* induced 80% inhibition of mycelial growth of the pathogen. Given the promising results of this study, further investigations will be devoted to the characterization of the pool of both diffusible and volatile SMs in the selected *Trichoderma* isolates by GC-MS/SPME (VOCs) and HPLC (diffusible compounds).

In addition to SMs, a set of lytic enzymes are also produced by *Trichoderma* enhancing the host cell wall lysis, whose components can then be used as nutritional resources. Chitinases and  $\beta$ -1,3-glucanases are the best studied groups of CWDEs within the genus *Trichoderma*—being strictly associated with the mycoparasitic lifestyle—to the point that their number has been documented to be higher than in other filamentous fungi [82]. In particular, *T. atroviride*, *T. virens* and *T. reesei* are reported to produce higher chitinases and glucanases activity in comparison to other Ascomycetes [82,83]. *T. reesei* and *T. koningii* are the best-known producers of cellulolytic enzymes [84], mainly studied for the improvement of industrial cellulases production processes and more recently in biofuel applications [85]. In the present study, chitinases (NAGase and Biase) and glucanases activities of the cultural filtrates of the six selected *Trichoderma* isolates were determined. Moreover, cellulases activity was investigated by biochemical and colorimetric assays since cellulose is the main component of the cell wall of the fungal-like *P. xambivora* [86]. Chitinases and glucanases activities appear to be isolate-specific: *T. koningiopsis* SG6 and *T. hamatum* SG20 displayed the best performances, followed by *T. hamatum* SG18 (Table 4). Similar results were obtained for *T. asperellum*, *T. harzianum*, *T. viride*, *T. hamatum* and *T. koningiopsis* in response to phytopathogenic fungi in biocontrol studies [70,87,88]. The synergic action of SMs and lytic enzymes offers a more advanced level of antagonism than the performance of either antibiotics or enzymes acting alone [61]. We can likely infer that, isolates displaying the best performances in both SMs and lytic enzymes tests, develop the best aptitude to act against the pathogen by exerting an antibiosis strategy, as observed for *T. koningiopsis* SG6 and *T. hamatum* SG 18 and SG20. The lack or very low cellulases activity showed by the tested *Trichoderma*—including the positive control Radixsoil<sup>®</sup>—using the commercial “megazyme assay” could depend on cultivation conditions (pH) and, more presumably, on the high carbon and nitrogen sources present in the growing media. In fact, it is observed that the use of sugars as a carbon source leads to a repression of cellulases encoding genes transcription [89]. A different result was obtained testing the same enzymatic activity in water agar added with 1% CMC as carbon source. All isolates showed a different behavior in terms of cellulases biosynthesis, regardless of the species they belong to, demonstrating again that each isolate displays specific features related to its genotype. *T. hamatum* SG20 showed the higher cellulases activity, followed by *T. koningiopsis* SG6 and *T. koningii* SG19 (Table 4) in contrast with what demonstrated by Rabinal et al., 2020 [90], which showed a very low cellulases activity by the latter two species. In the same work, a very weak cellulases activity was reported for *T. longipile*, similar to results obtained in this study [90]. The synthesis of lytic enzymes generally occurs right before and during the physical contact between the antagonist and the recognized prey, followed by the formation of twists of *Trichoderma* around the pathogen’s hyphae, thus penetrating them by developing appressoria [7,90,91]. This is a mycoparasitic mechanism implemented by *Trichoderma* against a target pathogen and represents an important condition to be observed for an effective use of these beneficial fungi [92]. This strategy is very well documented in several species of the genus and is considered as one of the major mechanisms involved in the antagonistic activity. The hyperparasitism of *T. harzianum* against *F. solani* by coilings formation was already reported by Kumar et al., 1998 [93] and more recently described also for *T. koningii*, *T. viride*, *T. hamatum* and *T. asperellum* with regards to several fungal pathogens and Oomycetes [94–96]. Coilings formation of the tested isolates in this study was evaluated by both a light and scanning electron microscope, revealing that all the selected *Trichoderma* isolates showed this aptitude but *T. longipile* SG1, which was still able to overgrow *P. xambivora* in dual cultures (Figures 4 and 5). Since no literature is currently available regarding the mycoparasitic strategy implemented by *T. longipile*, it could be proposed that such isolate exerts its beneficial effect through the mechanism of antibiosis,



mainly producing diffusible secondary metabolites with a strong inhibitory effect on the growth of *P. xambivora*.

The in vitro selection process of putative BCAs is crucial to understand the features of the studied isolates before deeply exploring their efficacy in vivo. Indeed, the beneficial behavior of *Trichoderma* is a complex trait to be predicted, since a given strain can synchronously use several strategies or even act in different ways against different pathogens. This is the case of *T. asperellum* tested in this study, the results of which are less efficient in the inhibition of *P. xambivora* in vitro, compared to the other examined isolates; although, it is well recognized in the literature as one of the most performing *Trichoderma*-based biocontrol agents in the management of several plant diseases [97–99].

To our knowledge, this is the first time that *Trichoderma* isolates belonging to *hamatum*, *koningii*, *koningiopsis* and *longipile* species have been tested for the specific management of *P. xambivora*. All the selected isolates displayed the capability to control the pathogen in vitro acting by coupling synergistically antibiosis and mycoparasitism at different levels. In detail, *T. hamatum* SG18 and SG20, *T. koningiopsis* SG6 and *T. koningii* SG19 isolates positively replied to all the tests herein performed, *T. longipile* mainly used antibiosis and *T. asperellum* mainly acted as mycoparasite as demonstrated by the coilings differentiated around the pathogen's mycelium. Particularly relevant results were shown by *T. hamatum* SG18 and *T. koningiopsis* SG6, raising a strong interest for their capability to highly inhibit the growth of the pathogen. They could be promising isolates for an innovative and environmentally friendly approach for ink disease management; thus, further investigations are needed to also confirm their efficacy in vivo. Given the hybrid nature of *P. xambivora*, future investigations should take into consideration several strains of the pathogen, and their respecting mating types, to evaluate the efficacy of the selected beneficial organism on the representative pathogen population diversity.

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