



# Efficacy of *Trichoderma*-based formulat es against Mal secco disease of citrus

Giuseppa Rosaria Leonardi · Alessandro Vitale · Simone Mavica · Vittoria Catara · Giancarlo Polizzi · Dalia Aiello

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**Abstract** The tracheomycotic fungus *Plenodomus tracheiphilus* is a major threat to lemon fruit production in the Mediterranean basin, causing the Mal secco disease. Restrictions on the use of contentious copper-based compounds, due to their negative environmental impact, make the control of this pathogen challenging, especially in organic agriculture. This study evaluated the *in vitro* and *in vivo* antagonistic potential of four commercial *Trichoderma*-based formulations (*T. asperellum* ICC 012 + *T. gamsii* ICC 080, *T. asperellum* T34, *T. atroviride* I-1237, *T. atroviride* SC1) against *P. tracheiphilus* using a stepwise approach and comparing them with a copper compound. Our results showed that all *Trichoderma*-based biocontrol products significantly inhibited mycelial growth through different mechanisms, exhibiting variable efficacy depending on the specific *P. tracheiphilus* isolate. Comprehensively, *Trichoderma* dual culture and volatile organic compounds (VOCs) provided excellent performance in reducing mycelial growth of the pathogen, both higher than those observed for cell-free filtrates. *Trichoderma* colonies provided inhibition values varying from 30 to 71%, whereas VOCs inhibited from 23 to 75% and CFs up to 44%. Under controlled conditions, all *Trichoderma*

products reduced symptoms of leaf vein chlorosis caused by *P. tracheiphilus* on Volkamer lemon seedlings. Specifically, *T. gamsii* ICC 080 + *T. asperellum* ICC 012 averagely showed the best performance since it significantly reduced disease incidence and severity of 50 and 57% at 28 days after inoculation, respectively, thus confirming once again similar performance recorded for the copper-based treatment. The commercial *Trichoderma* mixture was further tested to determine whether the application mode can play a role in mitigating infections. To this regard, the data showed that both soil drenches and foliar sprays effectively managed foliar symptoms caused by *P. tracheiphilus*, reducing disease incidence by 27 and 41% at 28 days, respectively, and symptom severity by 33 and 43%, respectively. Overall, this study confirmed a good efficacy of commercial *Trichoderma* product against *P. tracheiphilus* in controlled conditions using different application modes. Highlights. • *In vitro* and *in vivo* efficacy of some *Trichoderma* bioformulates against *Plenodomus tracheiphilus* (Mal secco) were tested on lemon. • *Trichoderma gamsii* ICC 080 + *T. asperellum* ICC 012 mixture was the most effective bioformulate in managing Mal secco infections. • The performance of *Trichoderma* bioformulate also depends on the application mode. • Although lesser effective than pyraclostrobin, *Trichoderma* bioformulate showed similar efficacy to Cu compound.

G. R. Leonardi · A. Vitale (✉) · S. Mavica · V. Catara · G. Polizzi · D. Aiello  
Department of Agriculture, Food and Environment (Di3A), University of Catania, via S. Sofia 100, 95123 Catania, Italy  
e-mail: alevital@unict.it

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## Introduction

Citrus is a tree crop grown for the appreciated fruit containing health-promoting nutrients and bioactive compounds (Liu et al. 2022), considered to be one of the most economically important fruit crops worldwide, distributed in tropical, subtropical and Mediterranean regions (Vincent et al. 2020; FAOSTAT 2024). Italy is one of the most important producing countries exceeded only by Spain for orange and also by Türkiye for lemon (FAOSTAT 2024). The tracheomycotic fungus *Plenodomus tracheiphilus* (Petri) Gruyter, Aveskamp, and Verkley (syn. *Phoma tracheiphila*) is an economically key pathogen responsible for the Mal secco disease on many citrus species belonging to the genera *Citrus*, *Fortunella*, *Poncirus* and *Severinia* and on their interspecific and intergeneric hybrids (Migheli et al. 2009). Mal secco represents a limiting factor due to its economic impact on the Mediterranean citrus production, where the pathogen is currently spread (EPPO 2025), as well as for its potential introduction in other citrus producing countries (EFSA 2014; Krasnov et al. 2023), given the recent report of *P. tracheiphilus* causing wilt disease on elm trees in Canada (Yang et al. 2024). An increase in disease pressure has been reported in recent years, probably due to a greater diffusion of the most susceptible lemon cultivar ‘Femminello Siracusano 2KR’ and to more frequent hail damage as a result of climate change. Recent studies showed the frequency of extreme climatic events (hail and rainstorms) in the Mediterranean basin has increased significantly over the last two decades, especially in the period 2010–2021 if compared with 1999–2010 (Forestieri et al. 2018). This trend was particularly evident over southern Italy and the Balkans, especially from September to November (Laviola et al. 2022), when the primary infections of the pathogen occur. *P. tracheiphilus* enters through wounds, colonises the xylematic vessels of the plant, and then spreads systemically (Perrotta et al. 1976; Bassi et al. 1980; Nigro et al. 2015). Depending on the primary site of infection, disease symptoms can be different,

but the most common are vein, leaf, and shoot chlorosis, that slowly progress downward from the infected young shoots to twigs and branches causing defoliation and wilting. In severe cases, the pathogen completely infects the vascular tissues of trunk and roots, leading to the death of the plant (Zucker and Catara 1985). Management of Mal secco disease has become challenging since it should include many biological and environmental issues. Agronomic practices are not fully able to guarantee an effective control, and although the use of citrus species with genetic resistance to the pathogen would be the preferred method for controlling the disease, the tolerant lemon accessions are still being evaluated and no resistant varieties with appreciated trade fruit quality have been registered yet (Abbate et al. 2019; Russo et al. 2020; Catalano et al. 2021; Arlotta et al. 2024; Di Guardo et al. 2024). In addition, the use of fungicides is not forward-looking, due to the lack of registered molecules, with Cu compounds being the only authorized for the control of Mal secco (Ministero della Salute 2025). The use of Cu was restricted by the European Union (EU) due to its negative environmental impact (European Chemicals Agency 2018; Triantafyllidis et al. 2020; Dao et al. 2021; Burandt et al. 2024). Consequently, the main control strategy continues to be based on the minimizing of primary inoculum through sanitization practices (Salerno and Cutuli 1982; Migheli et al. 2009). However, an integrated disease management (IDM), based on the combination of agronomic practices and fungicides application using sustainable products could be a suitable strategy to containing the infections. Alternative substances that may represent a substitute to Cu, including biological control agents (BCAs) are included in Annex II of Commission Regulation (EC) 889/2008, which lists the products permitted for plant protection in organic crop production. Biological control agents have been widely investigated in the last decades for the control of Mal secco, especially *Bacillus* and *Pseudomonas* (Coco et al. 2004; Kalai-Grami et al. 2014a; 2014b; Strano et al. 2017; Aiello et al. 2022; Leonardi et al. 2023; Sicilia et al. 2023; Dimaria et al. 2024). *Trichoderma*, also known by its teleomorph *Hypocrea* (Hypocreaceae, Hypocreales), is the most studied and used fungal BCA for the management of phytopathogenic fungi, even applied in pre- and post-harvest (Vu et al. 2021;

Win et al. 2021; Zhou et al. 2021; Khuong et al. 2023). This soil-borne microorganism has the ability to antagonize and mycoparasitize targeted pathogens, to colonize the plant root system and to induce plant defence responses, as well as to promote plant growth and to improve or maintain soil productivity (Hermosa et al. 2012; Woo and Lorito 2022; Woo et al. 2014, 2023). *Trichoderma*-based formulations have been registered worldwide with a total of 144 plant protection products (PPPs) available, containing 11 *Trichoderma* species and 44 strains (Woo et al. 2023). Among these, the commercial product Remedier®, containing a mixture of two *Trichoderma* species (*T. asperellum* ICC 012 and *T. gamsii* ICC 080), is one of the most widely used against several plant pathogens of ornamental, horticultural and fruit crops in various EU (European Commission 2025; Regulation (EU) 2025/99) and non-EU countries (EPA 2025). Among the *Trichoderma* strains registered for commercial use in Italy as plant protection products, Remedier® is currently authorized on citrus for the control of root rot disease caused by *Armillaria mellea* (Ministero della Salute 2025). Due to its non-toxicity and agricultural benefits, this microorganism has only recently gained increasing interest in the control of Mal secco. In a preliminary study, Leonardi et al. (2023) demonstrated the potential biocontrol of Remedier® against *P. tracheiphilus*, which showed a good reduction of foliar symptoms on Volkamer lemon seedlings after foliar application. However, in the above mentioned study the *in vitro* *Trichoderma*-*P. tracheiphilus* interactions, which are important to clarify about action mode throughout *Trichoderma* spp. can act against this pathogen, had not been explored. Moreover, the efficacy in controlling the disease may also depend on the *Trichoderma* species/strains, as they are known to produce several compounds which have different bioactive properties, and on practical factors, including the application mode that can influence its biocontrol activity (Woo and Lorito 2022; Saini et al. 2024). Thus, the objectives of this study were: (i) to evaluate whether different species and strains of *Trichoderma* contained in commercial formulates differ in their efficacy in reducing *P. tracheiphilus* *in vitro* growth and foliar infections in controlled conditions; and (ii) to determine whether the mode of application of *Trichoderma* may influence its efficacy in controlling artificial infections caused by *P. tracheiphilus*.

## Materials and methods

DNA extraction, sequencing and phylogenetic analyses of *Plenodomus tracheiphilus* isolates

*P. tracheiphilus* isolates (PT41, PT54, PT55, PT57, PT77, PT79, PT81, PT90), used to test the efficacy of the *Trichoderma*-based products, were obtained from the fungal collection of the Section of Plant Pathology of the Department of Agriculture, Food and Environment, University of Catania. Selection of these fungal isolates was based on their citrus host and location provenance. The selected pathogen isolates were grown on Potato Dextrose Agar (PDA, Lickson, Vicari, Italy) for 15 days for genomic DNA extraction. The mycelium was scraped off from the colony and processed following manufacturer's instructions of the Wizard Genomic DNA Purification Kit® (Promega Corporation, Madison, WI, USA). The quality and quantity of DNA were measured using a NanoDrop 2000 (Thermo Scientific, Waltham, MA, USA) and samples were stored at 4 °C until use. The complete internally transcribed spacer region (ITS1-5.8S-ITS2) rDNA gene was amplified with primers ITS5 and ITS4 (White et al. 1990). The polymerase chain reaction (PCR) conditions were as follows: initial denaturation step of 30 s at 94 °C, followed by 35 amplification cycles of 30 s at 94 °C, annealing of 1 min at 52 °C; extension of 1 min at 68 °C, and a final elongation step of 5 min at 68 °C. The PCR amplification products were estimated visually by electrophoresis in 1% agarose gels. PCR products were sequenced in both directions by Macrogen Inc. (Seoul, South Korea) and the DNA sequences generated were assembled using Lasergene SeqMan Pro (DNASTAR, Madison, WI, USA) and reference sequences were retrieved from the National Center for Biotechnology Information (NCBI), based on recent and comprehensive phylogenetic studies on the genus *Plenodomus* (Zhao et al. 2021). Fifteen species of *Plenodomus* for which sequences are available were included in the matrix, including type/ex-type strains, and *Leptosphaeria slovacica* CBS 125975 was selected as an outgroup. Sequence alignments for phylogenetic analyses were produced with the server version of MAFFT (<https://mafft.cbrc.jp/alignment/server/>)

and checked and refined using BioEdit Sequence Alignment Editor 7.7.1.0 (Hall 1999). The phylogenetic analyses of the ITS region were performed using Maximum Likelihood (ML) and Maximum Parsimony (MP) methods. ML analyses were performed with RAxML (Stamatakis 2006), as implemented in raxmlGUI 2.0 (Silvestro and Michalak 2012), using the ML + rapid bootstrap setting and the GTRGAMMA substitution model which was selected as the most appropriate model by Modeltest. The matrix was partitioned for the different gene regions, and bootstrap analyses done with 1,000 bootstrap replicates. MP bootstrap analyses were performed with Phylogenetic Analyses Using Parsimony (PAUP) v. 4.0a169 (Swofford 2002). 1,000 bootstrap replicates were implemented using five rounds of heuristic search replicates with random addition of sequences and subsequent TBR branch swapping (MULTREES option in effect, steepest descent option not in effect, COLLAPSE command set to MINBRLEN, and each replicate limited to 1 million rearrangements) during each bootstrap replicate. All molecular characters were unordered and given equal weight, with gaps treated as missing data, and the COLLAPSE command was set to minbrlen. The nucleotide sequence data of our strains were deposited in GenBank (<https://www.ncbi.nlm.nih.gov/>), and the GenBank accession numbers of all accessions included in the phylogenetic analyses are listed in Table 1.

#### *Trichoderma*-based products

All *Trichoderma* species used for these experiments were chosen among the most used for managing crop diseases worldwide, for their plant growth promotion and high suitability for biocontrol ability. The selected *Trichoderma*-based products were Remedier®, containing *T. asperellum* strain ICC 012 + *T. gamsii* strain ICC 080 ( $3 \times 10^7$  spores  $g^{-1}$ ), T34 Biocontrol®, containing *T. asperellum* strain T34 ( $1 \times 10^9$  spores  $g^{-1}$ ), Esquive®, containing *T. atroviride* strain I-1237 ( $1 \times 10^8$  spores  $g^{-1}$ ), and Vintec®, containing *T. atroviride* strain SC1 ( $1 \times 10^{10}$  spores  $g^{-1}$ ). *In vitro* experiments were carried out to evaluate the competitive growth, production of volatile compounds and cell-free filtrates of four *Trichoderma*-based commercial

products against the eight characterised *P. tracheiphilus* isolates (Fig. 1). For the *in vitro* experiments, cultures of *Trichoderma* spp. were prepared from the commercial products, whereas for the experiments commercial formulations were applied according to recommended label rates.

#### Dual culture assay

*In vitro* evaluation of the competitive growth of *Trichoderma* against *P. tracheiphilus* involved the macroscopically observation of antagonistic interaction between cultures on dual inoculated plates according to Bunbury-Blanchette and Walker (2019), with some modifications. A 5-mm mycelial plug of each *P. tracheiphilus* isolate and *Trichoderma*-based product was cut with a sterile corkborer from the edge of 14- and 5-day-old cultures, respectively, and placed opposing one another on the outer edges of a 90-mm diameter Petri plate containing PDA. *P. tracheiphilus* was cultured on the dishes for 5 days prior to inoculation of *Trichoderma* due to its slow mycelium growth rate. Plates containing only a plug of each *Trichoderma* strain or pathogen served as control. The plates were incubated at  $25 \pm 1$  °C in the dark and checked for growth from when the first contact occurred until the pathogen was completely covered. The biocontrol activity was evaluated by measuring two colony radii of the pathogen in the direction of the opposite colony at four and seven days of the coinoculation. The percentage inhibition of mycelial growth (PGI) was also calculated using the following formula:

$$PGI(\%) = \left( \frac{R_c - R_a}{R_c} \right) \times 100$$

where:

$R_c$  and  $R_a$  is the ray of the fungal colony in the control plate and in the presence of the antagonist (cm), respectively. Four replicates, each represented by a Petri plates, were included for each *Trichoderma*-product. Moreover, macroscopic morphological observations indicating an interaction between species, such as development of an inhibition zone, abnormal colour, and overlapping growth were noted as reported by Bunbury-Blanchette and Walker (2019). The experiment was performed twice.

**Table 1** Collection details and GenBank accession numbers of isolates used in the phylogenetic analyses

Species	Strain <sup>a</sup>	Host	Country	GenBank accession number ITS <sup>b</sup>
<i>Leptosphaeria slovacica</i>	CBS 125975	<i>Balota nigra</i>	Netherlands	JF740248
<i>Plenodomus agnitus</i>	CBS 121.89	<i>Eupatorium cannabinum</i>	Netherlands	JF740194
<i>Plenodomus biglobosa</i>	CBS 119951	<i>Brassica rapa</i>	Netherlands	JF740198
<i>Plenodomus collinsoniae</i>	CBS 120227	<i>Vitis coignetiae</i>	Japan	JF740200
<i>Plenodomus confertus</i>	CBS 375.64	<i>Anacyclus radiatus</i>	Spain	AF439459
<i>Plenodomus congestus</i>	CBS 244.64 <sup>T</sup>	<i>Erigeron canadensis</i>	Spain	AF439460
<i>Plenodomus enteroleucus</i>	CBS 142.84 <sup>T</sup>	<i>Catalpa bignonioides</i>	Netherlands	JF740214
<i>Plenodomus fallaciosus</i>	CBS 414.62	<i>Satureia montana</i>	France	JF740222
<i>Plenodomus hendersoniae</i>	CBS 113702	<i>Salix cinerea</i>	Sweden	JF740225
<i>Plenodomus influorescens</i>	CBS 143.84 <sup>T</sup>	<i>Fraxinus excelsior</i>	Netherlands	JF740228
<i>Plenodomus libanotidis</i>	CBS 113795	<i>Seseli libanotis</i>	Sweden	MH862943
<i>Plenodomus lindquistii</i>	CBS 381.67	<i>Helianthus annuus</i>	Canada	JF740233
<i>Plenodomus lingam</i>	CBS 260.94 <sup>T</sup>	<i>Brassica oleracea</i>	Netherlands	MH862462
<i>Plenodomus lupini</i>	CBS 248.92	<i>Lupinus mutabilis</i>	Peru	JF740236
<i>Plenodomus tracheiphilus</i>	MUCL 38481 <sup>T</sup>	<i>Citrus limon</i>	Italy	MW810293
<i>Plenodomus tracheiphilus</i>	ATCC 26007	Mandarin twigs	Italy	MZ049614
<i>Plenodomus tracheiphilus</i>	CBS 551.93	<i>Citrus limonium</i>	Israel	MW810275
<i>Plenodomus tracheiphilus</i>	CBS 127250	<i>Citrus aurantium</i>	Italy	MW810286
<i>Plenodomus tracheiphilus</i>	CBS 539.63	<i>Chrysanthemum</i> sp.	Greece	AB190387
<i>Plenodomus tracheiphilus</i>	<b>PT41</b>	<i>Citrus limon</i> 'Femminello Zagara Bianca'	Italy: Syracuse	<b>PV877297</b>
<i>Plenodomus tracheiphilus</i>	<b>PT54</b>	<i>Citrus limon</i> 'Fino'	Italy: Syracuse	<b>PV877298</b>
<i>Plenodomus tracheiphilus</i>	<b>PT55</b>	<i>Citrus bergamia</i>	Italy: Syracuse	<b>PV877299</b>
<i>Plenodomus tracheiphilus</i>	<b>PT57</b>	<i>Citrus bergamia</i>	Italy: Syracuse	<b>PV877300</b>
<i>Plenodomus tracheiphilus</i>	<b>PT77</b>	<i>Citrus limon</i> 'Femminello 2KR'	Italy: Catania	<b>PV877301</b>
<i>Plenodomus tracheiphilus</i>	<b>PT79</b>	<i>Citrus limon</i> 'Femminello 2KR'	Italy: Catania	<b>PV877302</b>
<i>Plenodomus tracheiphilus</i>	<b>PT81</b>	<i>Citrus limon</i> 'Femminello 2KR'	Italy: Catania	<b>PV877303</b>
<i>Plenodomus tracheiphilus</i>	<b>PT90</b>	<i>Microcitrus australasica</i>	Italy: Catania	<b>PV877304</b>
<i>Plenodomus visci</i>	CBS 122783 <sup>T</sup>	<i>Viscum album</i>	France	JF740256
<i>Plenodomus wasabiae</i>	CBS 120119	<i>Wasabia japonica</i>	Taiwan	JF740257

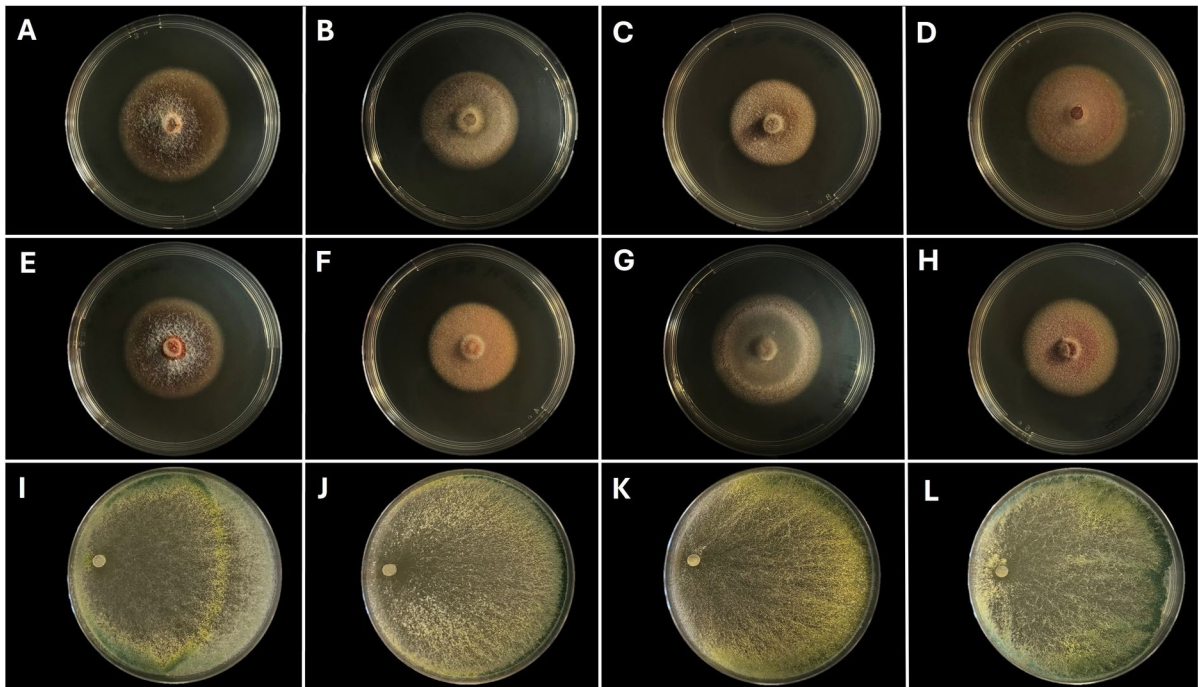
<sup>a</sup>Strains and sequences generated in this study are shown in bold. Ex-type cultures are indicated with a T. ATCC = American Type Culture Collection, USA; CBS = Westerdijk Fungal Biodiversity Institute, Utrecht, the Netherlands; MUCL = Agro-food and Environmental Fungal Collection, Université Catholique de Louvain, Belgium

<sup>b</sup>ITS, internal transcribed spacer

### Volatile antifungal assay

The antifungal activity of volatile organic compounds (VOCs) produced by *Trichoderma* strains against the *P. tracheiphilus* isolates was assessed using the sandwiched Petri plate assays according to Dennis and Webster (1971), with some modifications. A 5-mm mycelial plug from the active growing edge of 14- and 5-day-old PDA culture of *P. tracheiphilus* and *Trichoderma* strains, respectively, were transferred

to the centre of fresh 90 mm PDA plates. The plates inoculated with the antagonists and the pathogen isolates were assembled, removing the lids, inverting the pathogen plate and placing it directly over the bottom plate containing *Trichoderma* plug. The two plates were sealed together with two layers of Parafilm to prevent loss of volatile substances, and incubated at 25 ± 1 °C. This configuration ensured that there was no direct interaction between the BCA and the pathogen. Controls consisted of PDA plates prepared



**Fig. 1** *Plenodomus tracheiphilus* isolates (A: PT41, B: PT54, C: PT55, D: PT57, E: PT77, F: PT79, G: PT81, H: PT90) and *Trichoderma* strains (I: *T. asperellum* ICC 012 + *T. gamsii* ICC 080, J: *T. asperellum* T34; K: *T. atroviride* SC1; L: *T. atroviride* 1-1237) used in *in vitro* assays

similarly but inoculated only with the pathogen and sealed against a sterile PDA plate. After 7 days, the diameter of the fungal colony was measured and the biocontrol activity was evaluated by calculating the PGI (%), as previously described. The experiment was repeated once.

#### Cell-free filtrates antifungal assay

The antifungal activity of cell-free filtrates (CFs) produced by *Trichoderma* on *P. tracheiphilus* isolates was evaluated as reported by Frascella et al. (2022), with some modifications. Eight mycelial plugs were obtained from the actively growing colonies of each of the four *Trichoderma*-based products grown for 10 days on PDA amended with 100 mg L<sup>-1</sup> of streptomycin sulfate. The plugs were transferred into 1 L laboratory bottle containing 100 mL of Potato Dextrose Broth medium (PDB, Vicari, Lickson, Italy) and incubated at 28 °C at 150 rpm in shaking incubator for four days. Spores and mycelia of *Trichoderma* were removed from PDB by filtration with Whatman no.1 filter paper and then using a 0.22 μm

ABLULO® syringe-driven filter (GVS Filter Technology, USA). In total, 10 mL of PDA with 100 μg μL<sup>-1</sup> streptomycin sulfate was mixed with 5 mL of culture filtrate right before solidification (50 °C) to obtain a final concentration of 33% (v/v) and poured in 90 mm Petri dishes. The same amount of PDB liquid medium instead of the CFs was used as the control. A mycelial plug of *P. tracheiphilus* isolate, taken from the actively growing margin of 14-day-old colony, was placed at the centre of the PDA plate and incubated at 25 ± 1 °C. The activity of CFs was determined by measured two perpendicular diameters of *P. tracheiphilus* colony and calculating the PGI (%), as previously described, after 7 days. The experiment was performed with three replications and repeated once.

#### Growth chamber experiments

Two separate *in vivo* experiments were conducted to evaluate: (I) the efficacy of *Trichoderma*-based products (previously used in the *in vitro* assays) in reducing *P. tracheiphilus* foliar infections on young Volkamer lemon seedlings; (II) the application mode (i.e.

root drench and foliar spray) influence on the effectiveness of the best performing *Trichoderma*-based formulate in reducing foliar infections. For the *in vivo* experiments we selected *P. tracheiphilus* PT77, since all isolates tested showed similar growth reductions in dual culture assays. Both the experiments were repeated once.

#### Plant material and growth conditions

Three and seven-month-old Volkamer lemon (*Citrus volkameriana*) plants maintained in plastic trays with a size of 540×280 mm and a depth of 110 mm, and of 800×800 mm and a depth of 850 mm were used in the experiment I and II, respectively. The plant material was obtained by healthy seed sown on a commercial substrate (90% blond peat + 10% perlite) previously fertilized with organic fertilizer (35–40% KNO<sub>3</sub>, 0.3–1% CuSO<sub>4</sub> 5H<sub>2</sub>O, 0.1–0.2% H<sub>3</sub>BO<sub>3</sub>, 0.1–0.2% ZnSO<sub>4</sub>) at 1 kg m<sup>-3</sup> and with micronutrient fertilizer (15% Fe, 2.5% Mn, 0.20% B, 1% Cu, 1% Zn, 0.04% Mo) at 300 g m<sup>-3</sup>. Seedlings were grown in a nursery greenhouse located in Giarre (Catania province, Italy) and two weeks before the experiments, seedling trays were transferred to the growth chamber of the Department of Agriculture, Food and Environment, University of Catania, and maintained at 25 °C, 80% of relative humidity (RH), and in a photoperiod of 16 h of light and 8 h of dark. Seedlings were irrigated at regular intervals of two times per week using a volume of approx. 50 and 70 mL of tap water per plant in the experiment I and II, respectively.

#### Comparison of *Trichoderma*-based formulation treatments

Performances of *Trichoderma*-based formulat es were compared with *T. asperellum* ICC 012+*T. gamsii* ICC 080, previously reported to be effective in reducing *P. tracheiphilus* infections on Volkamer lemon (Leonardi et al. 2023). The experiment also included a standard fungicide [copper hydroxide, Cu(OH)<sub>2</sub>, Kocide Opti®] and an untreated control (seedlings treated with water). The application rates of both *Trichoderma* and Cu formulations were chosen according to label recommendations and reported in Table 2. Seedlings were treated twice

**Table 2** Formul at es selected in the growth chamber experiments

Active ingredient	Trade name	Rates (g or mL 100 L <sup>-1</sup> )
<i>T. asperellum</i> ICC 012+ <i>T. gamsii</i> ICC 080	Remedier®	167
<i>T. atroviride</i> I-1237	Esquive®WG	266
<i>T. atroviride</i> SC1	Vintec®	100
<i>T. asperellum</i> T34	T34-Biocontrol®	100
Copper hydroxide	Kocide Opti®	150

7 days apart before the pathogen inoculation by spraying a volume of 100–150 mL of each formulate onto the leaves with a manual sprayer. Four hours after the second application, young leaves were wounded at two midveins with a sterile needle and inoculated with approximately 0.8 mL of conidial suspension of PT77. The spore suspension of *P. tracheiphilus* PT77, with a concentration of 1×10<sup>5</sup> conidia mL<sup>-1</sup>, was obtained from 21-day-old cultures maintained on PDA medium, as reported in Leonardi et al. (2023). A total of three replicates per treatment were used, each including 15 seedlings, and four young healthy leaves per seedling were chosen to inoculate the pathogen, with a total of 360 inoculation points per treatment. The control consisted of seedlings treated with tap water and inoculated with the pathogen. Seedlings were maintained in the growth chamber at 25±1 °C and 80% of RH. Symptoms were monitored over time, and 14 and 28 days after the pathogen inoculation, disease incidence (DI) and symptom severity (SS) were evaluated. The DI value referred to the assessment of the percentage of positive inoculation points, calculating as follow:

$$DI (\%) = \frac{n}{N} \times 100$$

where: n is the number of symptomatic inoculation points; N is the total number of inoculation point examined.

The SS value was determined adopting the empirical 0-to-4 rating scale of Luisi et al. (1979), where: 0, no symptom; 1, chlorotic halo around the inoculation point; 2, vein chlorosis close to the inoculation point; 3, extended vein chlorosis to the leaf margin; 4,

extensive vein chlorosis and/or browning close to the inoculation point, and calculated using the following formula:

$$SS = \frac{\sum (C_i \times n)}{N}$$

where: SS is the average index of symptom severity;  $C_i$  is each class detected;  $n$  is the number of inoculation points in each class;  $N$  is the total number of inoculation points examined.

Moreover, the McKinney index (McK-I), which is a measure that include both incidence and severity of the disease, was expressed as the weighted means of the disease as a percentage of the maximum possible level, using the following formula:

$$McK - I(\%) = \frac{\sum (C_i \times n)}{N \times V} \times 100$$

where:  $C_i$  is the number of each class;  $n$  is the number of inoculation points in each class;  $N$  is the total number of inoculation points examined;  $V$  is the maximum value of the severity scale used.

#### *Comparison of foliar spray and root drench treatments of T. asperellum ICC 012 + T. gamsii ICC 080*

The experiment II was carried out using the *T. asperellum* ICC 012 + *T. gamsii* ICC 080 commercial-mixture, which showed the best performance in the previous *in vivo* assay. The experiment also included the standard copper compound, copper hydroxide (Kocide Opti®), and pyraclostrobin (Cabrio® WG, at rate of 37.7 g hL<sup>-1</sup>), a fungicide registered for use on citrus that has never been tested against *P. tracheiphilus*. Control consisted of seedlings treated with water. In this case, *Trichoderma* was applied preventively as foliar and root treatment three times, seven days apart, before inoculation with the pathogen. The frequency of applications was increased compared to the first *in vivo* experiment to promote the effective establishment of the fungal antagonist in the root system, as well as enhancing its persistence. The rate applied was maintained the maximum reported in the label, as in the previously experiment, considering the high inoculum concentration used ( $1 \times 10^5$  conidia

mL<sup>-1</sup>). Specifically, for each application, foliar-treated plants were sprayed using a volume of 100–150 mL of formulation onto the leaves using a manual sprayer and irrigated with 30 mL of sterile distilled water (SDW), while root-treated seedlings were irrigated with 30 mL of the *Trichoderma* commercial mixture and watered by spraying a volume of 100–150 mL of SDW onto the leaves. For chemical products, the treatment was performed four hours prior to the pathogen inoculation. To standardise the amount of water in each treatment, plants were watered twice a week with approximately 70 mL of tap water. Four hours after the third application, young leaves were wounded at two midveins with a sterile needle and inoculated with approximately 0.8 mL of conidial suspension of PT77. A total of three replicates per treatment were used, each including 15 seedlings, and four young leaves in healthy condition per seedling were chosen to inoculate the pathogen, with a total of 360 inoculation points per treatment. The control consisted of seedlings treated and irrigated with tap water as reported above and inoculated with the pathogen. Seedlings were maintained in the growth chamber at  $25 \pm 1$  °C and 80% of RH. Fourteen and 28 days after the pathogen inoculation, disease incidence, severity and McK-I values were evaluated as previously reported.

#### Data analysis

All statistical analyses of *in vitro* and *in vivo* data were performed by using the Statistica package software (version 10; Statsoft Inc., Tulsa, OK, USA). The arithmetic means of mycelial diameter, disease incidence (DI), symptom severity (SS) and McK-I data were calculated averaging the values determined for all replicates of each treatment data and reported in the tables. Percent disease incidence data were previously arcsine ( $\sin^{-1}$  square root  $x$ ) transformed to meet assumptions of homogeneity of variance. In the post-hoc analyses, the main effects of *in vitro* and *in vivo* treatments were evaluated by using the Fisher's least significance difference test at  $\alpha=0.05$  to separate the means of fungal/disease parameters through repeated assays/trials since the ranking of effectiveness was averagely maintained in the repeated assays/trials (Castello et al. 2025).

## Results

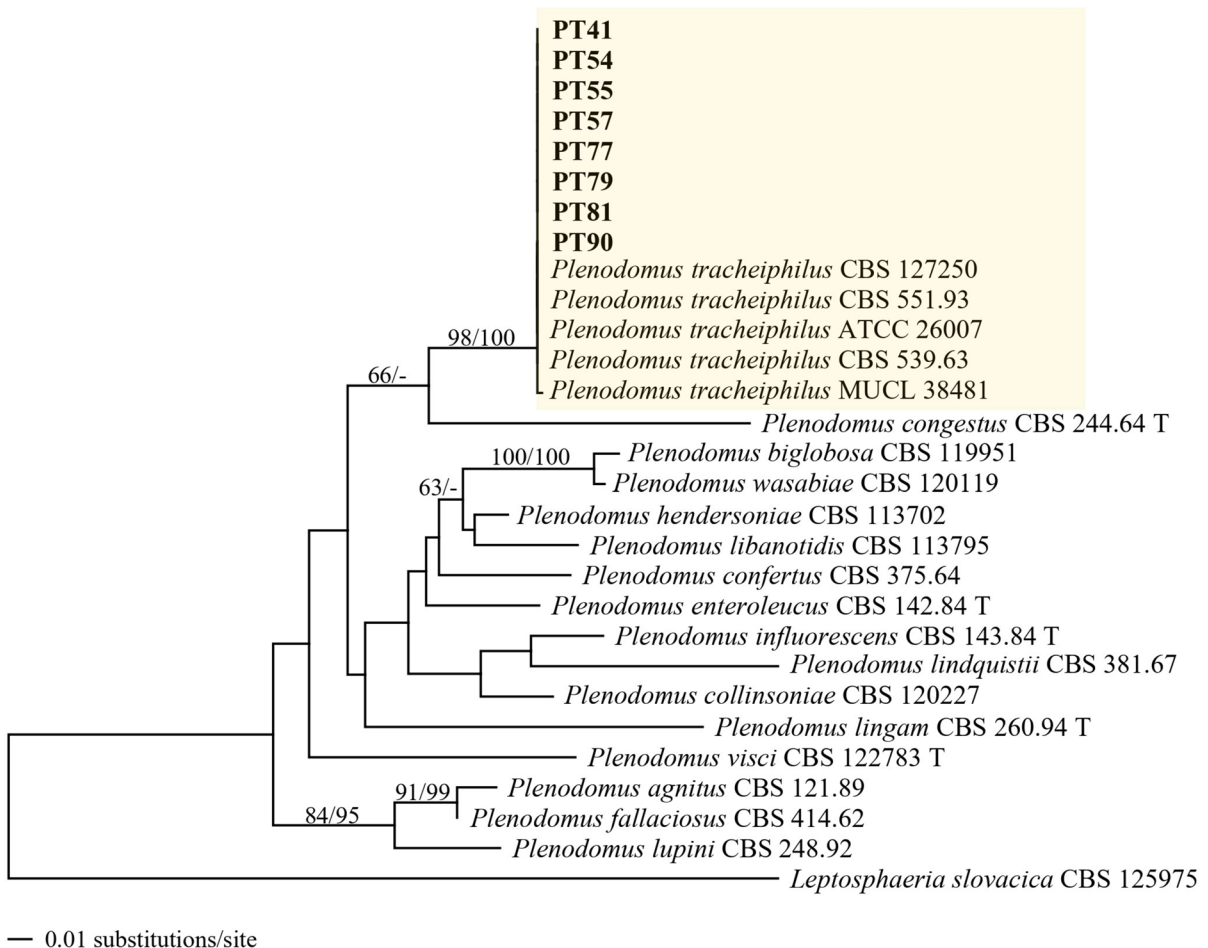
### DNA extraction, sequencing and phylogenetic analyses of *Plenodomus tracheiphilus*

The sequence dataset of the ITS gene was analysed to molecularly identify the *Plenodomus*-like isolates stored in the fungal collection of University of Catania. The dataset consisted of 29 sequences including one outgroup taxon, *Leptosphaeria slovacica* (CBS 125975). A total of 541 characters were included in the phylogenetic analyses, of which 351 were constant, 68 were variable but parsimony-uninformative, and 122 parsimony-informative. The tree topologies

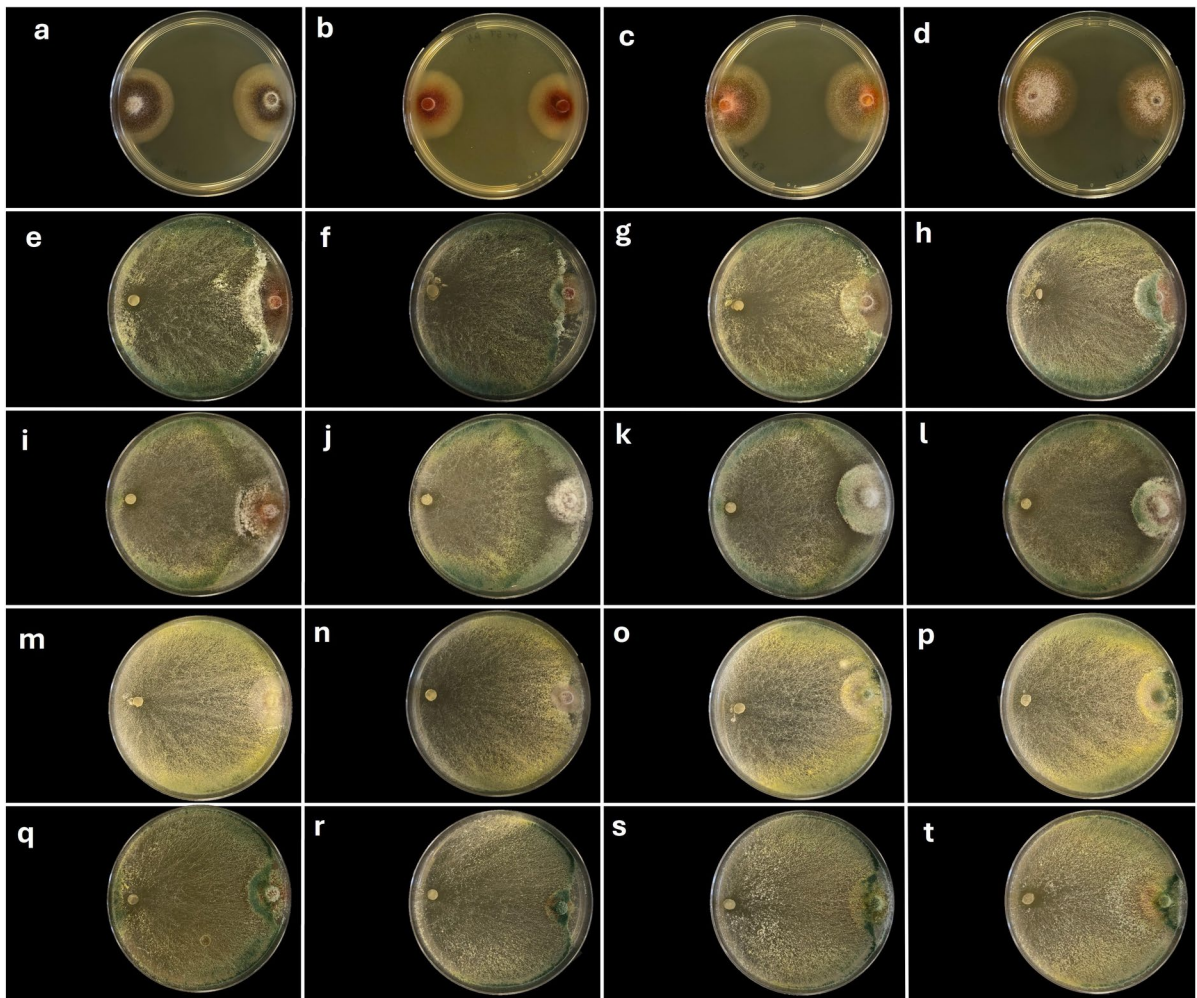
resulting from MP and ML analyses of the dataset were congruent. The ML tree ( $-\ln L = 2915.661158$ ) obtained by RAxML is shown in Fig. 2. The isolates were placed in the clade of *P. tracheiphilus* with high and maximum bootstrap values (98% ML, 100% MP).

### Dual culture assay

After four days of growth at 25 °C, all *Trichoderma* colonies were in contact with *P. tracheiphilus* isolates, but none of them were able to sporulate, whereas at 7 days they were able to overgrow the pathogen colonies and to sporulate, although slight differences were observed among the *Trichoderma*



**Fig. 2** Phylogram of the best ML tree ( $-\ln L = 2915.661158$ ) revealed by RAxML from an analysis of the ITS gene of *Plenodomus* spp., showing the phylogenetic position of PT isolates (bold), with *Leptosphaeria slovacica* selected as outgroup to root the tree. Maximum Likelihood (ML) and Maximum Parsimony (MP) bootstrap support above 60% are given at first and second position, respectively, above the branches. T = type specimens



**Fig. 3** Dual culture antifungal activity of *Trichoderma*-based formulations against *P. tracheiphilus* isolates (PT) at seven days. **a** to **d**, control plates of PT41, PT57, PT77 and PT79, respectively. **e** to **f**, dual culture of *T. atroviride* I-1237 (Esquive®) and PT41, PT57, PT77 and PT79, respectively. **i** to **l**, dual culture of ICC 012 + *T. gamsii* ICC 080 (Remedier®) and PT41, PT57, PT77 and PT79, respectively. **m** to **p**, dual culture of *T. atroviride* SC1 (Vintec®) and PT41, PT57, PT77 and PT79, respectively. **q** to **t**, dual culture of *T. asperellum* T34 (Biocontrol T34®) and PT41, PT57, PT77 and PT79, respectively

strains (Fig. 3). Specifically, *T. asperellum* ICC 012 + *T. gamsii* ICC 080 and *T. atroviride* SC1 completely colonized the pathogen colony reaching the entire plate after seven days, whereas *T. atroviride* I-1237 and *T. asperellum* T34 did not cover the entire *P. tracheiphilus* colony. The results revealed that the mycelium growth of *P. tracheiphilus* isolates was significantly inhibited by all *Trichoderma* at four days post inoculation (dpi) if compared with the control plates except for PT54 and PT90 in dual culture with *T. atroviride* SC1 and *T. atroviride* I-1237, respectively (Table 3); otherwise at seven

dpi all isolates were significantly inhibited by tested *Trichoderma*-based formulations (Table 4). Overall, the four *Trichoderma* products showed variable performance depending on the target pathogen isolate. The PGI values at seven dpi varied from 30 to 39% for *T. atroviride* I-1237 based formulate, from 34 to 41% for *T. asperellum* ICC 012 + *T. gamsii* ICC 080 commercial mixture, from 33 to 49% for *T. atroviride* SC1 and from 31 to 71% for *T. asperellum* T34, confirming the consistent efficacy of *Trichoderma* over time. For some isolates, *T. asperellum* T34, *T. asperellum* ICC 012 + *T. gamsii* ICC 080 and *T.*

**Table 3** Mean mycelial growth radius (cm) of eight *Plenodomus tracheiphilus* isolates grown in dual culture with *Trichoderma*-based formulat es four days post inoculation (dpi)

Treatment Active ingredient(s)	<i>Plenodomus tracheiphilus</i> isolates <sup>x</sup>								
	PT41	PT54	PT55	PT57	PT77	PT79	PT81	PT90	Mean
Control	1.50±0.01 a	1.27±0.06 a	1.22±0.01 a	1.00±0.04 a	1.30±0.05 a	1.32±0.00 a	1.22±0.05 a	1.14±0.09 a	1.25±0.05
<i>T. atroviride</i> I-1237	1.33±0.03 b	1.04±0.01 c	0.92±0.03 c	0.80±0.18 b	1.07±0.02 b	0.93±0.03 b	0.95±0.08 bc	1.10±0.05 a	1.02±0.05
<i>T. asperellum</i> ICC012+ <i>T. gamsii</i> ICC080	1.02±0.01 d	0.89±0.01 d	0.94±0.06 bc	0.77±0.01 b	0.99±0.02 b	0.96±0.04 b	0.89±0.06 c	0.91±0.16 b	0.92±0.03
<i>T. atroviride</i> SC1	1.09±0.03 cd	1.19±0.06 ab	1.00±0.03 bc	0.64±0.13 c	0.84±0.14 c	0.95±0.03 b	0.96±0.09 bc	0.88±0.03 b	0.94±0.06
<i>T. asperellum</i> T34	1.15±0.03 c	1.16±0.06 b	1.02±0.08 b	0.77±0.21 b	1.00±0.03 b	0.96±0.04 b	1.01±0.03 b	0.81±0.08 b	0.99±0.05

<sup>x</sup>Data derived from four replicates (PDA plates). SEM=standard error of the means. Mean radius values (cm) followed by the same letters within the column are not significantly different according to Fisher’s least significance differences test ( $\alpha=0.05$ ). The last column reports the mean among the mycelial growth values detected among the *P. tracheiphilus* isolates

**Table 4** Mean mycelial growth radius (cm) of eight *Plenodomus tracheiphilus* isolates grown in dual culture with *Trichoderma*-based formulat es seven days post inoculation (dpi)

Treatment Active ingredient(s)	<i>Plenodomus tracheiphilus</i> isolates <sup>x</sup>								
	PT41	PT54	PT55	PT57	PT77	PT79	PT81	PT90	Mean
Control	2.07±0.06 a	2.24±0.26 a	1.89±0.16 a	1.92±0.42 a	1.91±0.09 a	1.71±0.14 a	1.69±0.23 a	1.61±0.13 a	1.88±0.07
<i>T. atroviride</i> I-1237	1.46±0.06 b	1.49±0.33 b	1.23±0.17 b	1.22±0.51 b	1.31±0.20 b	1.14±0.02 b	1.02±0.04 b	1.13±0.06 b	1.25±0.06
<i>T. asperellum</i> ICC012+ <i>T. gamsii</i> ICC080	1.31±0.06 c	1.31±0.16 c	1.22±0.04 b	1.20±0.30 b	1.26±0.02 b	1.14±0.08 b	1.01±0.06 b	1.03±0.14 bc	1.19±0.04
<i>T. atroviride</i> SC1	1.30±0.13 c	1.49±0.18 b	1.18±0.01 b	1.01±0.48 b	0.97±0.01 c	1.01±0.01 c	1.09±0.11 b	0.97±0.01 bc	1.13±0.06
<i>T. asperellum</i> T34	1.37±0.21 bc	1.52±0.23 b	1.19±0.08 b	0.56±0.02 c	1.32±0.16 b	1.17±0.06 b	1.10±0.03 b	0.90±0.03 c	1.14±0.10

<sup>x</sup>Data derived from four replicates (PDA plates). SEM=standard error of the means. Mean radius values (cm) followed by the same letters within the column are not significantly different according to Fisher’s least significance differences test ( $\alpha=0.05$ ). The last column reports the mean among the mycelial growth values detected among the *P. tracheiphilus* isolates

*atroviride* SC1 determined a significant reduction of the colony radii if compared to *T. atroviride* I-1237.

Volatile and cell-free filtrates antifungal assay

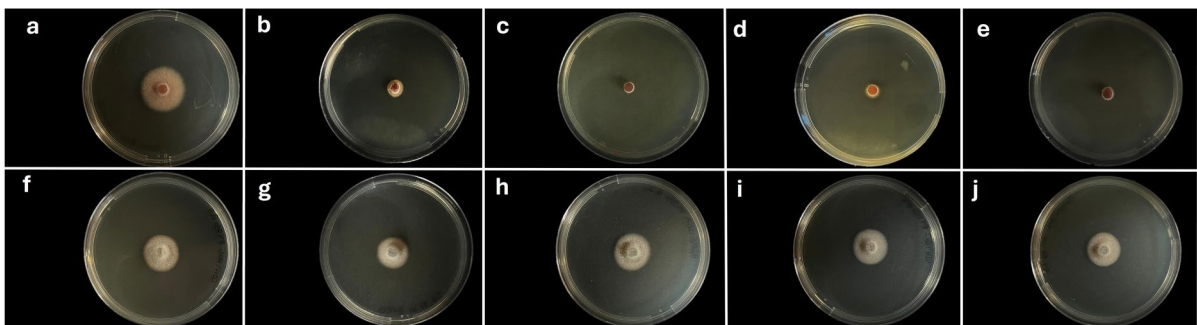
Organic volatiles and CFs produced by *Trichoderma* strains were tested for the antagonistic activity. The results showed that the growth of all *P. tracheiphilus* isolates in the presence of volatiles was strongly reduced (significant data) if compared with the control plates (Table 5, Fig. 4A-E). Percentage reductions of

*P. tracheiphilus* colony growth determined by *Trichoderma* VOCs varied from 23 to 75% among the different isolates. On the contrary, CFs produced by *Trichoderma* strains exhibited a weaker activity compared to VOCs and a variable efficacy depending on the target isolate (Table 6). Of the eight *P. tracheiphilus* isolates, only PT81 growth was significantly reduced by all CFs. However, for the remaining *P. tracheiphilus* isolates, *Trichoderma* CFs did not always provide a significant growth reduction if compared to the relative controls (Fig. 4F-J). Percentage reductions of *P. tracheiphilus*

**Table 5** Mean mycelial growth diameter (cm) of eight *Plenodomus tracheiphilus* grown with volatile organic compounds (VOCs) produced by *Trichoderma*-based formulatates seven days post inoculation (dpi)

Treatment Active ingredient(s)	<i>Plenodomus tracheiphilus</i> isolates <sup>x</sup>								
	PT41	PT54	PT55	PT57	PT77	PT79	PT81	PT90	Mean
Control	3.31±0.31 a	2.71±0.81 a	2.39±0.12 a	2.04±0.04 a	2.13±0.43 a	2.39±0.38 a	2.81±0.24 a	2.57±0.16 a	2.54±0.14
<i>T. atroviride</i> I-1237	1.72±0.44 b	1.06±0.16 b	1.49±0.13 b	1.12±0.08 b	1.15±0.11 b	1.10±0.05 b	1.19±0.19 b	1.18±0.14 b	1.25±0.08
<i>T. asperellum</i> ICC012+ <i>T. gamsii</i> ICC080	1.56±0.43 bc	0.86±0.14 b	1.27±0.44 b	0.92±0.13 c	0.88±0.03 c	1.21±0.16 b	0.78±0.03 c	1.19±0.27 b	1.08±0.09
<i>T. atroviride</i> SC1	1.35±0.28 d	1.04±0.34 b	1.35±0.40 b	0.97±0.09 c	1.16±0.11 b	1.09±0.11 b	0.99±0.06 bc	1.30±0.41 b	1.16±0.05
<i>T. asperellum</i> T34	1.39±0.22 cd	0.87±0.18 b	1.32±0.33 b	1.09±0.14 b	1.04±0.06 bc	1.14±0.16 b	0.89±0.01 c	1.36±0.26 b	1.14±0.07

<sup>x</sup>Data derived from four replicates (PDA plates). SEM= standard error of the means. Mean diameter values (cm) followed by the same letters within the column are not significantly different according to Fisher's least significance differences test ( $\alpha=0.05$ ). The last column reports the mean among the mycelial growth values detected among the *P. tracheiphilus* isolates



**Fig. 4** Antagonist activity of VOCs (a-e) and CFs (f-j) of *Trichoderma*-based formulatates against *P. tracheiphilus* isolates (PT) at seven days of co-incubation. a and f control plates of PT81 and PT55, respectively. b and g, *T. atroviride* I-1237 (Esquivé®) VOCs and filtrates against PT81 and PT55, respectively. c and h, *T. asperellum* ICC 012 + *T. gamsii* ICC 080 (Remedier®) VOCs and filtrates against PT81 and PT55, respectively. d and i, *T. atroviride* SC1 (Vintec®) VOCs and filtrates against PT81 and PT55, respectively. e and j, *T. asperellum* T34 (Biocontrol T34®) VOCs and filtrates against PT81 and PT55, respectively

colony growth determined by *Trichoderma* VOCs varied from 23 to 75% among the different isolates, whereas CFs did not always reduce the colony growth of the pathogen, showing reduction values up to 44%.

#### Comparison of *Trichoderma*-based formulation treatments

In the experiment conducted in growth chamber to evaluate the effectiveness of biological products, containing different *Trichoderma* species and strains, against *P. tracheiphilus* strain PT77, the treatments always had a significant effect on the tested parameters, i.e. DI, SS and McK-I values collected over time ( $P$  value < 0.001)

(Table 7). Otherwise, the treatment × trial interactions were not significant thus indicating a similar ranking of effectiveness between the two trials. Consequently, the two trials were combined and subjected to a post-hoc analysis of the main effects (Table 8).

Based on these data, all *Trichoderma*-based formulatates provided reductions in disease incidence and severity on lemon leaves over time compared to those detected for untreated control (Table 8). Among the *Trichoderma*-based products tested, *T. asperellum* ICC 012 + *T. gamsii* ICC 080 provided the highest reduction in disease incidence and severity over time. Specifically, it reduced by 51 and 62% the disease incidence and symptom severity at 14 dpi, respectively, as well

**Table 6** Mean mycelial growth diameter of eight *Plenodomus tracheiphilus* grown with cell-free filtrates (CFs) produced by *Trichoderma*-based formulates seven days post inoculation (dpi)

Treatment Active ingredient(s)	<i>Plenodomus tracheiphilus</i> isolates <sup>x</sup>									Mean
	PT41	PT54	PT55	PT57	PT77	PT79	PT81	PT90		
Control	2.90±0.00 a	1.87±0.03 a	2.51±0.01 a	1.57±0.02 a	2.46±0.03 a	2.86±0.01 a	3.25±0.02 a	2.90±0.05 a	2.54±0.19	
<i>T. atroviride</i> I-1237	2.88±0.02 a	1.76±0.01 a	2.26±0.03 c	1.56±0.01 a	2.27±0.00 b	1.60±0.00 c	2.86±0.01 b	2.79±0.06 ab	2.25±0.19	
<i>T. asperellum</i> ICC012+ <i>T. gamsii</i> ICC080	2.46±0.01 c	1.49±0.01 b	2.37±0.08 b	1.42±0.01 ab	2.36±0.03 ab	2.52±0.02 b	2.87±0.07 b	2.74±0.01 b	2.28±0.18	
<i>T. atroviride</i> SC1	2.62±0.07 b	1.80±0.00 a	2.27±0.05 c	1.52±0.06 a	2.35±0.05 b	2.54±0.03 b	2.74±0.01 c	2.69±0.08 b	2.32±0.15	
<i>T. asperellum</i> T34	2.49±0.01 bc	1.58±0.02 b	2.28±0.03 bc	1.31±0.03 b	2.16±0.03 c	2.86±0.01 a	2.78±0.07 bc	2.75±0.06 b	2.28±0.20	

<sup>x</sup>Data derived from four replicates (PDA plates). SEM=standard error of the means. Mean diameter values (cm) followed by the same letters within the column are not significantly different according to Fisher’s least significance differences test ( $\alpha=0.05$ ). The last column reports the mean among the mycelial growth values detected among the *P. tracheiphilus* isolates

**Table 7** Analysis of variance for disease incidence (DI), symptom severity (SS) and McKinney index (McK-I) over time (14 and 28 days post inoculation, dpi) and among five treatments in two repeated trials of the *in vivo* experiment I

Factor(s)	df	14 dpi <sup>x</sup>						28 dpi					
		DI (%)		SS (1-to-5)		McK-I		DI (%)		SS (1-to-5)		McK-I	
		F	P value	F	P value	F	P value	F	P value	F	P value	F	P value
Treatment	5	6.164	0.0008	17.006	<0.0001	17.006	<0.0001	9.571	<0.0001	10.694	<0.0001	10.694	<0.0001
Trt×trial	5	0.832	0.540 <sup>ns</sup>	2.312	0.075 <sup>ns</sup>	2.312	0.075 <sup>ns</sup>	0.974	0.453 <sup>ns</sup>	0.833	0.539	0.833	0.539

<sup>x</sup>F test of fixed effects, df=degrees of freedom, and P value associated to F; ns=not significant data

**Table 8** Disease incidence (DI), symptom severity (SS), and McKinney index (McK-I) recorded on leaf inoculated with *Plenodomus tracheiphilus* PT77 after 14 and 28 days on *Citrus volkameriana* seedlings treated with *Trichoderma*-based formulates and standard fungicide

Treatment	14 dpi <sup>x</sup>			28 dpi <sup>x</sup>		
	DI (%)	SS (1-to-5 scale)	McK-I (%)	DI (%)	SS (1-to-5 scale)	McK-I (%)
Control	46.39±0.83 a	0.98±0.04 a	19.53±0.87 a	54.58±1.25 a	1.64±0.11 a	32.87±2.27 a
<i>T. atroviride</i> I-1237 (Esquive®)	32.42±3.81 b	0.55±0.08 b	10.93±1.60 b	35.27±4.43 bc	0.94±0.22 bc	18.77±4.37 bc
<i>T. asperellum</i> ICC012+ <i>T. gamsii</i> ICC080 (Remedier®)	22.50±0.00 c	0.37±0.01 c	7.50±0.03 c	27.08±0.97 c	0.71±0.03 c	14.30±0.63 c
<i>T. atroviride</i> SC1 (Vintec®)	31.55±2.34 bc	0.49±0.07 bc	9.87±1.40 bc	37.50±3.89 b	0.91±0.03 bc	18.23±0.57 bc
<i>T. asperellum</i> T34 (Bio-control®)	32.97±3.97 b	0.52±0.09 bc	10.33±1.73 bc	35.17±3.72 bc	1.05±0.11 b	21.07±2.27 b
Copper hydroxide (Kocide Opti®)	23.75±3.47 bc	0.41±0.11 bc	8.23±2.23 bc	27.92±0.14 c	0.77±0.19 bc	15.33±3.87 bc

<sup>x</sup>Data derived from two trials plus SEM (standard error of the mean). Means for each trial derived from three replicates each formed by 120 inoculation points on 60 leaves belonging to young lemon seedlings. Values followed by different letters within the column are significantly different according to Fisher’s least significance differences test ( $\alpha=0.05$ )

as by 50 and 57% at 28 dpi. However, the reduction of symptom severity recorded at 14 dpi did not significantly differ to that of the other *Trichoderma*-based products, which reductions ranging from 43 to 60%. *Trichoderma atroviride* SC1 exhibited good performance over time, averagely reducing DI and SS by an average of 32 and 47%, respectively. *Trichoderma atroviride* I-1237 and *T. asperellum* T34 were less effective in reducing DI and SS at 14 dpi, achieving a performance similar to that reported for *T. asperellum* ICC 012+*T. gamsii* ICC 080 and copper hydroxide at 28 dpi. In fact, the standard fungicide (copper hydroxide) showed a good reduction of disease parameters at 14 dpi (47% and 56% reduction in DI and SS, respectively), with no significant difference in efficacy compared to *T. asperellum* ICC 012+*T. gamsii* ICC 080 at 28 dpi (49% and 52% reduction in DI and SS, respectively).

#### Comparison of foliar spray and root drench treatments of *Trichoderma asperellum* ICC 012+*Trichoderma gamsii* ICC 080 formulate

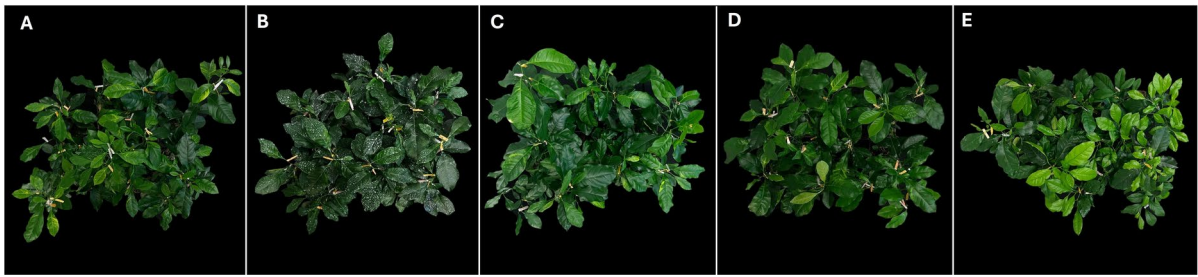
Since the ranking of effectiveness between the two trials in the experiment II was very similar, both relative data were combined and subjected to a post-hoc analysis of the main effects (Table 9).

In detail, the treatments always had a significant effect on DI, SS and McK-I values collected over time (14 and 28 dpi). Foliar symptoms caused by *P. tracheiphilus* appeared approx. six to eight days after inoculation. From the first onset of symptoms, a slower disease progression was clearly recorded over time in tested treatments if compared with the relative untreated controls (Table 9). At 14 dpi, the incidence of leaf vein chlorosis was reduced by 24, 42, 41 and 80% by the root drench and foliar spray treatments with *T. asperellum* ICC 012+*T. gamsii* ICC 080, copper hydroxide and pyraclostrobin, respectively, and the McK-I was reduced by 14, 48, 50 and 85%, respectively. At 28 dpi reductions of 27, 41, 42 and 78% of DI were observed for root drench and leaf spray applications of *T. asperellum* ICC 012+*T. gamsii* ICC 080, copper hydroxide and pyraclostrobin, respectively. The McK-I was also reduced by 33, 43, 48 and 81%, respectively. The reduction at 28 dpi in the McK-I for the root drenching application of *T. asperellum* ICC 012+*T. gamsii* ICC 080 did not significantly differ from that obtained for leaf spray application. Pyraclostrobin was the most effective, being able to significantly reduce both the DI and McK-I compared with the remaining treatments and untreated control (Fig. 5).

**Table 9** Disease incidence (DI), symptom severity (SS), and McKinney index (McK-I) recorded on leaf inoculated with *Plenodomus tracheiphilus* PT77 after 14 and 28 days on *Citrus volkameriana* seedlings treated with *T. asperellum*+*T. gamsii* bioformulate and fungicides

Treatment	14 dpi <sup>x</sup>			28 dpi <sup>x</sup>		
	DI (%)	SS (0-to-4 scale)	McK-I (%)	DI (%)	SS (0-to-4 scale)	McK-I (%)
Control	46.72 ± 1.06 a	0.86 ± 0.07 a	17.27 ± 1.4 a	57.11 ± 3.44 a	1.53 ± 0.00 a	30.60 ± 0.00 a
<i>T. asperellum</i> ICC012+ <i>T. gamsii</i> ICC080 (Remedier®)—root drench	35.42 ± 3.75 b	0.75 ± 0.02 b	14.90 ± 0.3 b	41.90 ± 7.33 b	1.03 ± 0.08 b	20.50 ± 1.63 b
<i>T. asperellum</i> ICC012+ <i>T. gamsii</i> ICC080 (Remedier®)—leaf spray	27.00 ± 4.67 c	0.45 ± 0.07 c	8.90 ± 1.4 c	33.72 ± 5.72 c	0.87 ± 0.18 bc	17.33 ± 3.67 bc
Copper hydroxide (Kocide Opti®)—leaf spray	27.61 ± 6.28 c	0.43 ± 0.13 c	8.60 ± 2.6 c	32.89 ± 4.89 c	0.79 ± 0.22 c	15.87 ± 4.44 c
Pyraclostrobin (Cabrio® WG)—leaf spray	9.31 ± 0.70 d	0.13 ± 0.01 d	2.63 ± 0.2 d	12.81 ± 2.53 d	0.29 ± 0.06 d	5.70 ± 1.17 d

<sup>x</sup>Data derived from two trials plus SEM (standard error of the mean). Means for each trial derived from three replicates each formed by 120 inoculation points on 60 leaves belonging to young lemon seedlings. Values followed by different letters within the column are significantly different according to Fisher's least significance differences test ( $\alpha=0.05$ )



**Fig. 5** Foliar symptoms on *Citrus volkameriana* seedlings 28 days post-inoculation of *Plenodomus tracheiphilus* PT77. **a**, untreated control. **b** and **c**, leaf spray and root drench treatments with *Trichoderma asperellum* ICC 012 + *Trichoderma gamsii* ICC 080, respectively. **d** and **e**, leaf spray treatments with copper hydroxide and pyraclostrobin, respectively

## Discussion

Beneficial microorganisms are one of the main tools available to control plant diseases. Although the activity of some BCAs has already been preliminary investigated for the management of Mal secco (Coco et al. 2004; Kalai-Grami et al. 2014a; 2014b; Aiello et al. 2022; Leonardi et al. 2023; Sicilia et al. 2023; Dimaria et al. 2024), the effectiveness of these means still poses challenges in the comprehensive management of the disease. *Trichoderma* is one of the largest groups of fungi used as biological control agents due to the broad spectrum of pathogens, included in the genera *Fusarium*, *Rhizoctonia*, *Botrytis*, *Sclerotinia*, *Phytophthora* and *Pythium*, amongst many others on which its properties have been demonstrated (Benitez et al. 2004; Harman et al. 2004; Vinale et al. 2008a; Aleandri et al. 2015). Different mechanisms of actions are involved in the *Trichoderma* control of plant diseases. These include direct actions on phytopathogens mainly due to the ability to mycoparasite, to compete for space and nutrient and to produce secondary metabolites with antimicrobial activity, and indirect mechanisms through the induction of local and systemic defences in plants (Woo et al. 2023). In a preliminary study, it was obtained an important result on the ability of *T. asperellum* ICC 012 and *T. gamsii* ICC 080, preventively applied on wounded lemon leaves, to reduce foliar symptoms caused by *P. tracheiphilus* under growth chamber conditions (Leonardi et al. 2023). Since several studies have shown that not all *Trichoderma* species and strains have the same ability to control plant pathogens (Tucci et al. 2011; Carrero-Carrón et al. 2018; Bazghaleh et al. 2020), herein we compared the performances of three

*Trichoderma*-based formulates with the previously tested by Leonardi et al. (2023), in inhibiting the *in vitro* mycelium growth of *P. tracheiphilus*, as well as in minimizing symptoms on young lemon seedlings. Among commercial products authorized in Italy, we selected those belonging to the Section *Trichoderma* (*sensu Viride* clade) (i.e. *T. atroviride*, *T. gamsii*, *T. asperellum*) which contains important species and strains for biological control (Cai and Druzhinina 2021). According to our results, all *Trichoderma* species and strains exhibited a considerable antagonistic activity in the dual culture assays, inhibiting the mycelial growth of all tested *P. tracheiphilus* isolates. Specifically, inhibition observed after four days of the co-inoculation was mediated by contact of all the *Trichoderma* strains, which after seven days were able to overgrow and sporulate on the pathogen colony. Several studies reported that *Trichoderma* acts using different types of interaction, including the ability to compete for space and nutrients and to produce lytic enzymes (e.g. chitinases and  $\beta$ -1,3-glucanases) (Vinale et al. 2008a, 2008b; Woo and Lorito 2022). As reported by these studies, our findings of *in vitro* assays showed that *T. atroviride*, *T. gamsii* and *T. asperellum* strains are able to compete for space and nutrients due to their fast growth rate, even though *P. tracheiphilus* was inoculated five days earlier. Interestingly, a recent study about the enzymatic activity of *T. asperellum* ICC 012 and *T. gamsii* ICC 080 (a.i. of Radix Soil®), clearly showed that these strains exhibited a higher competitive enzymatic profile (chitinase, glucanase, and cellulase activity) than those of other *Trichoderma* soil- and rhizosphere-inhabitants of chestnut (i.e. *T. asperellum*, *T. hamatum*, *T. koningii*, *T. koningiopsis* and *T. longipile* strains). These

enzymes have reported to hydrolyze the cell-wall of many fungi and oomycetes, allowing the colonization of the pathogen mycelium (Frascella et al. 2022). Although the evaluation of the mycoparasitic activity of *Trichoderma* on *P. tracheiphilus* was not herein microscopically observed, the ability of *Trichoderma* strains to overgrowth and sporulate on the pathogen surface is reported as a sign of mycoparasitism (Andrade-Hoyos et al. 2020; Rees et al. 2021). The ability of *Trichoderma* to inhibit the pathogen growth by antibiosis was also assessed by evaluating the production of volatile and non-volatile metabolites. Our findings indicate that the effects of VOCs on the mycelial growth of *P. tracheiphilus* colonies are higher than those observed for the cell-free filtrates. Several studies reported that *Trichoderma* spp. produce VOCs such as ketones, terpenes, lactones, and alcoholic substances which have a crucial role in the interaction with plants as well as in the inhibitory effect on other fungi (Vinale et al. 2008b; Korpi et al. 2009; Siddiquee et al. 2012; Kong et al. 2022). In this paper, there was a variable effect of cell-free filtrates produced by *Trichoderma*-based products based on *P. tracheiphilus* isolates, for which sometimes they did not show efficacy in reducing the mycelial growth of the pathogen. However, the lack of effect observed could be attributed to cultural conditions given that *Trichoderma* usually secretes volatile and non-volatile compounds in response to the presence of the pathogen (Stracquadanio et al. 2020). Indeed, the production of metabolites is generally reported to be more common when a complex net of microorganisms living together (Guo et al. 2019).

In our study, the activity of the *Trichoderma*-based products was also confirmed on lemon seedlings inoculated with *P. tracheiphilus* under controlled condition. Artificial inoculations of *P. tracheiphilus* on leaf veins of *C. volkameriana* were carried out as reported in other studies (Coco et al. 2004; Kalai-Grami et al. 2014a, 2014b; Aiello et al. 2022; Oliveri et al. 2022; Leonardi et al. 2023) in order to reproduce the main infection way of the pathogen, which typically enters the plant through leaf and twigs wounds (Recupero et al. 2006; Uzun et al. 2012; Ziadi et al. 2012; Abbate et al. 2019; Russo et al. 2020). This inoculation method enables a more rapid and reliable assessment of the BCAs performance in reducing disease amount, in according to other studies (Kalai-Grami et al. 2014a; 2014b; Aiello et al. 2022; Leonardi et al.

2023; Dimaria et al. 2024). It is well known, in fact, that pathogen causes a very rapid and systemic plant death ('Mal fulminante' syndrome) when it enters through the roots, infecting the outermost woody rings of large roots, or a slow-progressing internal black discolouration ('Mal nero' syndrome) when it enters through the rootlets, remaining segregated inside the plants for many years, independent of plant age (Nigro et al. 2015). However, root inoculation may not allow the assessment of disease amount, given that a designated ordinal scoring system is crucial to evaluate carefully symptom severity and detect slight differences between treatments (Bock et al. 2024). The first *in vivo* experiments revealed that all *Trichoderma*-based products are able to reduce Mal secco infections. *T. asperellum* ICC 012 + *T. gamsii* ICC 080 resulted the most promising since they gave similar performance of those reported for the standard fungicide. These data are consistent with those obtained in the previous study in which we compared the efficacy of the same *Trichoderma* strains with that of the oomycete *Pythium oligandrum* and Cu-compound on *P. tracheiphilus* PT41 infections (Leonardi et al. 2023). The efficacy of *Trichoderma* in reducing the disease parameters may be related to their ability to rapidly colonize wound sites and prevent the pathogen entry. Previous authors highlighted the importance of applying BCAs as preventative treatment to optimize their biological activity, allowing them to colonize the plant sites and provide the time required to develop systemic plant resistance (Amira et al. 2017; Ferreira et al. 2020; Bian et al. 2021; Díaz-Gutiérrez et al. 2021; Woo and Lorito 2022). Furthermore, this interesting finding could be due to the synergistic role of the two mixed *Trichoderma* species. Further studies recently showed that the combination of more than one species or strain of *Trichoderma* can increase the protection level against plant pathogens, as well as enhancing the effect on the plant growth, and mitigation of environmental stresses (Thangavelu and Gopi 2015; Musa et al. 2018; Anshu et al. 2022). To clarify the role of each *Trichoderma* species in reducing *P. tracheiphilus* infections, further studies should test each *Trichoderma* species separately.

Since the application mode could affect the performance of BCAs (Saini et al. 2024), our study also investigated on performance variability depending on leaf spray or root drench application of *T. asperellum* ICC 012 and *T. gamsii* ICC 080.

To this regard, our data showed that commercial *Trichoderma* mixture was always effective in reducing Mal secco infections although the leaf sprays provided the best performance. Thus, the paper confirms the hypothesis according to which the direct contact with the target pathogen could have enhanced the biocontrol efficacy (Wu et al. 2017; Di Marco et al. 2022), as preliminary demonstrated in laboratory assays. Moreover, our data suggest that soil drench of *T. asperellum* ICC 012 and *T. gamsii* ICC 080 could be a promising approach against soil-borne phase of *P. tracheiphilus*, which can persist in the infected soil debris (Mosca et al. 2024). The reduction of the potential soil inoculum represents a key step in disease management, given the ability of *Trichoderma* to well grow in this environment (Woo et al. 2023). To the light of these results, *Trichoderma*-based product by root application may act indirectly by priming plant immunity of the aboveground organs from pathogen infection. The induction of systemic resistance (ISR) by *Trichoderma* that contribute to prevent the infections was demonstrated by several authors (Yedidia et al. 2003; Djonovic et al. 2007; Korolev et al. 2008; Ilham et al. 2019; Yuan et al. 2019; Galletti et al. 2020; Xu et al. 2020). Although the ISR is triggered by root colonization with beneficial microorganisms, a study conducted on transgenic lemon plants supported the hypothesis that expression of the *chit42* gene from *T. harzianum* mimics some of the molecular responses typically associated with ISR, in particular it was correlated with increased transcript levels of ISR-marker genes (PAL, HPL, AOS), enhanced Reactive Oxygen Species (ROS) production and reduced symptoms caused by *P. tracheiphilus* and *Botrytis cinerea* (Gentile et al. 2007; Distefano et al. 2008). Since the increased in systemic resistance was observed to be strain specific and no molecular studies have been conducted in citrus so far, further studies need to establish how *T. asperellum* ICC 012 + *T. gamsii* ICC 080 may reduce foliar infections caused by *P. tracheiphilus* after preventive root applications. Although *Trichoderma*-based product showed a good efficacy similar to that observed for copper hydroxide, in this experiment pyraclostrobin was the most effective in reducing foliar infections. This active ingredient, belonging to the quinone outside inhibitors (Q<sub>o</sub>I), is currently the only fungicide along with Cu-based

compounds, fludioxonil and dodine authorized in Italy for the management of citrus fungal diseases (Ministero della Salute 2025). Its field application as aerial spray is common during the favourable season for infections of main citrus pathogens, *Alternaria* and *Colletotrichum* (Aiello et al. 2015; 2020), by which has proven to be effective (Miles et al. 2005; Colturato et al. 2009; Piccirillo et al. 2018). Unfortunately, although no studies have been conducted on fungicides sensitivity of *P. tracheiphilus* so far, the major issue of using pyraclostrobin on a field scale is the risk of resistance development in target pathogen populations (FRAC 2025). To this regard, some tolerant and resistant strains of the citrus pathogen *A. alternata* (Fr.) Keissl. have been reported recently in Italy and other citrus-growing countries, respectively (Mondal et al. 2005; Chitolina et al. 2019; Camiletti et al. 2022; Leonardi et al. 2024). Moreover, chemicals may affect non-target organisms, including BCAs, or cause problems for human health (Friis et al. 2004; Mackie et al. 2013; Van Gestel et al. 2019; Vitale 2023; Álvarez et al. 2025). Several studies, in fact, have shown that pyraclostrobin and copper hydroxide can negatively affect the *in vitro* growth of *T. asperellum* at the recommended label rate (Dethoup et al. 2022), although the sensitivity to these chemicals may vary depending on the specific *Trichoderma* strain (Juniors et al. 2020; Küpper et al. 2022). For this reason, further studies are needed to understand how different copper compounds could affect the growth and survival of the *Trichoderma* strains tested in this study against *P. tracheiphilus*, in order to explore the possibility of integrating low doses of Cu and *Trichoderma* in the field. Taking into account the shorter shelf-life and higher cost of biological products than those of chemicals, integrating *Trichoderma* and Cu could be an effective strategy during a transitional phase to ensure the economic sustainability of disease management. Overall, these results highlight the current differences in efficacy between chemical and biological strategies, as a consequence of the complexity of BCAs which performance is typically highly dependent on multiple environmental and biological factors (Fedele et al. 2020), while chemical fungicides tend to maintain their efficacy more consistently under varying environmental conditions (Fenibo et al. 2022). Based on these results and considerations,

*T. asperellum* ICC 012 and *T. gamsii* ICC 080 are promising BCAs for the integrated management of Mal secco. Considering this commercial product is currently registered in Italy already for *A. mellea*, the target spectrum could also include *P. tracheiphilus*. *Trichoderma* performance depends on different factors, including competition with other organisms and environmental conditions. It is well known, in fact, that the phyllosphere is more subjected to environmental factors, such as radiation and pollution (Kadivar and Stapleton 2003; Suda et al. 2009; Yuthammo et al. 2010), thus considered a temporary environment compared to the rhizosphere (Vorholt 2012). Therefore, given that soil is the most suitable habitat for the long-term persistence of *Trichoderma* (Woo et al. 2023), this mode of application could be a more sustainable and efficient long-term strategy for reducing foliar symptoms caused by *P. tracheiphilus*. Nevertheless, the good efficacy of *Trichoderma* product strongly encourages its further evaluation in field under different agronomic, phytosanitary and environmental conditions, as well as assessing their ability to colonize and persist in plant system, in order to fully define their potential in different application modes. This research represents a milestone for developing future sustainable and eco-friendly management approach against Mal secco to replace and/or reducing the use of Cu compounds in integrated and organic citriculture in the near future.

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**Author Contribution** Giuseppa Rosaria Leonardi: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing – original draft, Writing – review & editing. Alessandro Vitale: Conceptualization, Data curation, Formal analysis, Methodology, Resources, Supervision, Validation, Visualization, Writing – review & editing. Simone Mavica: Investigation. Vittoria Catara: Writing – review & editing. Funding acquisition. Giancarlo Polizzi: Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Validation, Visualization, Writing – review & editing. Dalia Aiello: Conceptualization,

Methodology, Resources, Supervision, Validation, Visualization, Writing – review & editing.

**Data Availability** No datasets were generated or analysed during the current study.

## Declarations

**Competing interest** The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper. The authors declare no competing interests.

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