



JOSÉ MANOEL FERREIRA DE LIMA CRUZ

**CHARACTERIZATION OF GRAPEVINE ROOT AND TRUNK
ROT DISEASE AND THE USE OF *Trichoderma* TO PROMOTE
PLANTLET ROOTING AND GROWTH**

**LAVRAS – MG
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Thesis presented to the Universidade Federal de Lavras, as part of the requirements of the Post Graduate Program in Agronomy/Phytopathology, area of concentration in Molecular Plant Pathology, for the degree of Doctor in Philosophy.

Ph.D. Jorge Teodoro de Souza
Supervisor

Ph.D. Claudia Rita de Souza
Co-supervisor

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**Ficha Catalográfica elaborada pelo Sistema de Geração
de Ficha Catalográfica da Biblioteca Universitária da UFLA, com dados
informados pelo(a) próprio(a) autor(a).**

Cruz, José Manoel Ferreira de Lima.

Characterization of grapevine root and trunk rot disease and the use of *Trichoderma* to promote plantlet rooting and growth / José Manoel Ferreira de Lima Cruz. - 2025.
106 p. : il.

Orientador: Jorge Teodoro De Souza
Coorientadora: Claudia Rita de Souza

Tese (Doutorado) - Universidade Federal de Lavras, 2025.
Bibliografia.

1. Biostimulant. 2. Diagnosis. 3. *Fusarium*. 4. New species. 5. Pathogenicity.
I. De Souza, Jorge Teodoro. II. Souza, Claudia Rita de. III. Universidade Federal de Lavras. IV. Título.

Elaboração da estrutura de acordo com o AACR2:
Responsável: Tatiana Silva - Bibliotecária-Documentalista (CRB6/1992)

JOSÉ MANOEL FERREIRA DE LIMA CRUZ

**CARACTERIZAÇÃO DAS DOENÇAS DE PODRIDÃO DE RAIZ E TRONCO DA
VIDEIRA E O USO DE *Trichoderma* PARA PROMOVER O ENRAIZAMENTO E O
CRESCIMENTO DE MUDAS**

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APPROVED on April 30th, 2025.

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**LAVRAS – MG
2025**

To my beloved daughter Luiza Antônia Farias Cruz, for all her love and affection.

To my grandfather José Alves de Lima (*in memoriam*), for all his examples and teachings.

I dedicate.

ACKNOWLEDGMENTS

To the honor and glory of the Great Architect of the Universe (Δ), who has always enlightened me with his strength, beauty, and wisdom.

To Federal University of Lavras and Department of Phytopathology for the opportunity.

To Minas Gerais Research Funding Foundation – FAPEMIG for the fellowship provided.

To all technicians and the entire team at the Minas Gerais Agricultural Research Corporation – EPAMIG.

To my parents, Iordam Silva da Cruz and Maria Valéria Ferreira de Lima Cruz, for their endless love, affection, and unwavering support throughout my journey.

To my beloved family, especially my uncles, aunts, and cousins, who are a true example of unity, love, companionship, and affection.

To Ana Karoline Nery Araujo, thank you for your affection, unwavering long-distance support, and heartfelt conversations that brought warmth, comfort, and strength during difficult moments. Your presence, even at a distance, meant more than words can express.

To my advisor, Jorge Teodoro de Souza, for the knowledge shared, the professional growth fostered, and the friendship built during the development of this research.

To my co-advisor, Claudia Rita de Souza, for the valuable discussions, exchange of knowledge, and continued support throughout the project.

To professor Carlos Antonio Inácio, for the friendship, shared laughter, and the valuable mycological knowledge imparted.

To the department technicians, especially Zenóbia de Souza Andrade, Luisa Oliveira Reis, Silvio Antônio Calazans de Freitas, Viviane de Fátima Silva Pessoa, Luciana Andrade Costa, Aline Norberto Ferreira, and Edson Luís Rezende (*in memoriam*), for their personal and professional support during the execution of this research.

To my PhD classmates Mynor Efraín García Munguía, Paul Esteban Pherez Perrony, Janaina Martins de Sousa, Luana Aparecida Gilio, Gabriela Ribeiro Gontijo, Tamires Aparecida Duarte de Souza, Letícia Vaz Molinari, Indiara Carol Lopes Pinheiro, and Thamires Yslanny Oliveira Sousa, thank you for the companionship, shared knowledge, and support throughout this journey.

To the friendships formed in the Molecular Plant Pathology Laboratory—Regiane Alves Belizario, Eduarda Mendes de Carvalho, Alejandra Valencia Rivera, Valter Cruz Magalhães,

Ananda dos Santos Vieira, Pedro Arthur de Sousa Morgado Garcia, Jessica Rembinski, and Larissa Maia de Oliveira—for all the conversations, laughter, and unwavering support.

To all friends who contributed with my academic story. A special thanks to Antonia Thalyta Lopes Silveira, João Vítor da Silva Pereira, Narcya Trindade de Souza, Gabriely Serrão Freire, Larissa Fernanda Andrade Souza, Ana Karoline Vieira dos Santos, Rafael Coelho Silva, Egídio José Aragão da Ponte, Guilherme Chaves de Holanda, Caíke de Sousa Pereira and Luiz Daniel Rodrigues da Silva, whose presence, motivation, and companionship made the challenges more bearable and the achievements even more meaningful. Your support will always be remembered with gratitude.

To my dearest friends Layla Victória da Silva Sousa and Nathalyne Lúcia Moreira Souza, thank you for your unconditional support, heartfelt friendship, and for being by my side with love and kindness through every step of this journey.

To my dear friends from the Raba da Gata house, Sidney Pereira Souza Filho and Brunno Cassiano Lemos Araújo, thank you for the unforgettable moments, constant support, and for sharing not just a home but a true sense of brotherhood throughout this journey.

To the secretary Ariane de Souza Alvarenga from the Graduate Program in Phytopathology, thank you for your dedication, efficiency, and continuous support throughout this academic journey.

To my friends from the Furança Football Club, thank you for the camaraderie, encouragement, and unforgettable moments shared on and off the field.

To the Plant Pathology Study Group – NEFIT, especially to Mário Roberto Nogueira Colares, Felipe Douglas Soares Leal, Islayne Aparecida de Souza Franco, João Vítor Machado de Carvalho, Annelise Cristina de Oliveira e Siqueira, Viviane Bernardes Alves, Lucas Tiago Araújo Américo, Weslei Alan Carvalho Nascimento, Gabriel da Silva Antunes, and Alan Diego do Rosário Chaves, thank you for your friendship and the valuable experiences throughout this academic journey.

I would like to express my heartfelt gratitude to the professors who contributed to my academic journey: Antonia dos Reis Figueira, Claudine Marcia Carvalho, Edson Ampelio Pozza, Eduardo Alves, Flavio Henrique Vasconcelos de Medeiros, Ludwig Heinrich Pfenning, Maria Alves Ferreira, Mario Lucio Vilela de Resende, Ricardo Magela de Souza, Vicente Paulo Campos, and Wagner Bettiol. Each of you, through your teachings, and support, has played a significant role in shaping my knowledge and critical thinking.

“É hoje Mata Limpa”
Iordam Silva da Cruz

RESUMO GERAL

As doenças da videira são uma preocupação para os produtores de uvas devido ao seu impacto econômico. Dentre elas, destaca-se a podridão das raízes e do tronco, uma nova doença observada durante o armazenamento refrigerado de mudas. Essa doença reduz a qualidade das mudas e compromete sua sobrevivência após o transplante ao campo. Neste contexto, o desenvolvimento de novas tecnologias aplicadas à produção de material de plantio de videira surge como uma abordagem promissora para melhorar a qualidade fisiológica do material enxertado. A utilização de ferramentas biotecnológicas visando melhorar o estabelecimento de mudas pode contribuir significativamente para mitigar perdas em viveiros, favorecendo o vigor inicial e o enraizamento. O objetivo geral desta pesquisa foi determinar a etiologia da podridão da raiz e do tronco, bem como testar o potencial de *Trichoderma* na propagação de videira. Observações iniciais indicaram que fungos do gênero *Fusarium* estavam associados à doença. Análises *fingerprinting* por BOX-PCR de 86 isolados de *Fusarium* de plantas doentes revelaram 45 grupos genéticos baseados em 53 bandas polimórficas. O sequenciamento de fragmentos do gene *tefl* de 78 isolados de *Fusarium* (30 de plantas saudáveis e 49 de doentes) revelou 14 espécies dos complexos *F. oxysporum* (FOSC), *F. fujikuroi* (FFSC), *F. tricinctum* (FTSC) e *F. solani* (FSSC). Testes de patogenicidade em campo e casa de vegetação confirmaram que 13 espécies de *Fusarium* (*F. casha*, *F. fredkrugeri*, *F. inflexum*, *F. vaughaniae*, *F. fujikuroi*, *F. annulatum*, *F. foetens*, *F. solani*, *F. landiae*, *F. triseptatum*, *F. guttiforme*, *F. annulatum* e *F. chongqingense*) foram capazes de causar podridão de raízes e tronco em mudas de videira. Análises comparativas de microscopia eletrônica de varredura mostraram que as plântulas doentes apresentavam vasos do xilema obstruídos com tiloses e goma. Espectroscopia de raio-X por energia dispersiva mostrou diferenças na composição mineral das raízes de plântulas saudáveis e doentes. Nas raízes saudáveis, o Ca foi distribuído de forma mais uniforme e concentrando-se na epiderme, enquanto nas mudas doentes, estava mais abundante nos vasos do xilema. O silício não foi detectado em tecidos saudáveis, mas foi distribuído uniformemente em tecidos doentes. Em outra parte do trabalho, o isolado de *Trichoderma* LFM004 foi testado em experimento de campo para promover o enraizamento e o crescimento de mudas de videira. Observou-se que o isolado LFM004 promoveu aumento no pegamento de plântulas de 98% e 55%, bem como no acúmulo de massa seca das raízes em 1,5x e 2,2x, em comparação aos tratamentos com água e hormônio, respectivamente. O isolado LFM004 foi capaz de produzir ácido indol-3-acético e solubilizar fosfato, características que podem estar relacionadas à atividade de promoção do enraizamento e crescimento de mudas. A caracterização polifásica do isolado LFM004, por análises micromorfológicas e moleculares, revelou tratar-se de uma nova espécie, para a qual propusemos o nome *T. harenae* sp. nov. Este estudo contribuiu para a descrição e caracterização de uma nova doença da videira, a podridão da raiz e do tronco, e também para a indicação de uma possível solução biotecnológica para melhorar o crescimento e o estabelecimento das mudas.

Palavras-chave: bioestimulante; diagnose; nova espécie; *Fusarium*; patogenicidade; *Vitis*.

GENERAL ABSTRACT

Grapevine diseases are a concern for grape producers due to their economic impact. Among these, root and trunk rot, a new disease that has been observed during the cold storage of plantlets stands out. The disease reduces the quality of the plantlets and compromises their survival after definitive transplantation to the field. In this context, the development of new technologies applied to the production of grapevine planting material emerges as a promising approach to enhance the physiological quality of the grafted material. The use of biotechnological tools aimed at improving plantlet establishment can significantly contribute to mitigate losses in nurseries, favoring the initial vigor and rooting of the plantlets. The general objective of this research was to determine the etiology of root and trunk rot, as well as to test the potential of *Trichoderma* in the production of grapevine planting material. Initial observations indicated that fungi of the genus *Fusarium* were associated with the disease. *Fingerprinting* analyses by means of BOX-PCR of 86 *Fusarium* isolates from diseased plants revealed 45 genetic groups based on 53 polymorphic bands. Sequencing of *tefl* gene fragments of 78 *Fusarium* isolates (30 from healthy plants and 49 from diseased plants) revealed 14 species belonging to the complexes *F. oxysporum* (FOSC), *F. fujikuroi* (FFSC), *F. tricinctum* (FTSC) and *F. solani* (FSSC). Pathogenicity tests in the field and greenhouse confirmed that 13 *Fusarium* species (*F. casha*, *F. fredkrugeri*, *F. inflexum*, *F. vaughaniae*, *F. fujikuroi*, *F. annulatum*, *F. foetens*, *F. solani*, *F. landiae*, *F. triseptatum*, *F. guttiforme*, *F. annulatum* and *F. chongqingense*) were able to cause root and trunk rot in grapevine plantlets. Comparative analyses of scanning electron microscopy showed that diseased plantlets had xylem vessels obstructed with tyloses and gum. Energy dispersive X-ray spectroscopy analyses showed differences in the mineral composition of roots of healthy and diseased plantlets. In healthy roots, Ca was distributed more uniformly and part of it was concentrated in the epidermis, while in diseased seedlings, it was in the xylem vessels. Silicon was not detected in healthy tissues, but was distributed uniformly in diseased tissues. In another part of the work, the *Trichoderma* isolate LFM004 was tested in a field experiment to promote rooting and growth of grapevine plantlets. It was observed that isolate LFM004 induced an increase in plantlet establishment of 98% and 55%, as well as an increase in root dry mass accumulation of 1.5× and 2.2×, compared to the water and hormone treatments, respectively. The isolate LFM004 was able to produce indoleacetic acid and solubilize phosphate, and these characteristics may be related to the activity of promoting rooting and growth of grapevine plantlets. The polyphasic characterization of isolate LFM004 through micromorphological and molecular analyses revealed that it was a new species, for which we proposed the name *T. harenae* sp. nov. The present study contributed to the description and characterization of a new grapevine disease, root and trunk rot, and also to the indication of a possible biotechnological solution to improve plantlets growth and establishment.

Keywords: biostimulant; diagnosis; *Fusarium*; new species; pathogenicity; *Vitis*.

INDICADORES DE IMPACTOS

A presente pesquisa gerou contribuições tecnológicas, científicas, sociais, econômicas e ambientais no contexto da vitivinicultura e do uso sustentável de recursos biológicos. Essas contribuições estão vinculadas à temática de Meio Ambiente (5) e Tecnologia de Produção (7) da Política Nacional de Extensão, beneficiando pequenos e médios produtores rurais, viveiristas, técnicos agrícolas e instituições de pesquisa no Sul de Minas Gerais. Dentre as contribuições destaca-se três frentes complementares: “Avanço no diagnóstico fitossanitário e manejo de doenças emergentes em viveiros de videira” – A identificação do agente etiológico responsável pela podridão radicular e do tronco de mudas de videira na região Sul de Minas Gerais supre uma lacuna crítica no conhecimento técnico-científico sobre as possíveis causas de falhas no pegamento e mortalidade precoce de mudas enxertadas. O diagnóstico preciso desse patógeno permitirá o desenvolvimento de estratégias de controle direcionadas, prevenindo perdas econômicas, melhorando a sanidade das mudas e aumentando a eficiência da propagação vegetativa em viveiros comerciais. Isso contribui diretamente para o fortalecimento da cadeia produtiva da uva no Brasil, alinhando-se às metas da Agenda 2030 da ONU (ODS 2 – Fome Zero e Agricultura Sustentável; ODS 12 – Consumo e Produção Responsáveis). “Descoberta e descrição de uma nova espécie de *Trichoderma* associada ao bioma Mata Atlântica” – A prospecção e caracterização de uma nova espécie do gênero *Trichoderma*, realizada em área de conservação da Mata Atlântica, destaca a importância da biodiversidade microbiana brasileira como fonte de recursos genéticos com aplicações estratégicas. A descrição formal desse novo táxon contribui para o conhecimento taxonômico do gênero e reforça a relevância da bioprospecção em ambientes naturais pouco explorados, alinhando-se às metas da Agenda 2030 da ONU (ODS 15 – Vida terrestre; ODS 9 – Indústria, Inovação e Infraestrutura). 3. Aplicação biotecnológica do isolado LFM004 como bioinsumo para viticultura sustentável – A demonstração da capacidade bioestimulante do isolado LFM004 como alternativa ao ácido naftalenoacético (ANA) na promoção do enraizamento, brotação e acúmulo de biomassa radicular em mudas de videira reforça o potencial desse microrganismo como biofertilizante natural. O uso desse isolado representa uma alternativa promissora aos reguladores de crescimento sintéticos, contribuindo para a redução de insumos químicos e para o desenvolvimento de práticas agrícolas mais sustentáveis (ODS 12 – Consumo e Produção Responsáveis; ODS 13 – Ação contra a mudança global do clima). Esses resultados não apenas ampliam o entendimento científico sobre os fatores que afetam a produção de mudas de videira, mas também abrem caminhos concretos para o desenvolvimento de novas tecnologias, parcerias público-privado e melhorias sobre a produtividade, sustentabilidade e segurança fitossanitária na viticultura nacional.

IMPACT INDICATORS

This research has generated significant technological, scientific, social, economic, environmental, and cultural contributions in the context of viticulture and the sustainable use of biological resources. These contributions align with the themes of Environment (5) and Technology and Production (7) under the Brazilian National Extension Policy, directly benefiting small and medium-sized farmers, nursery operators, agricultural technicians, and research institutions in southern Minas Gerais. The impacts can be grouped into three complementary areas: “Advancement in phytosanitary diagnostics and management of emerging diseases in grapevine nurseries” – The identification of the etiological agent responsible for root rot in grapevine seedlings in southern Minas Gerais fills a critical technical-scientific gap regarding the causes of poor graft success and early seedling mortality. Accurate diagnosis of this pathogen will enable the development of targeted control strategies, preventing economic losses, improving plant health, and increasing the efficiency of vegetative propagation in commercial nurseries. This directly strengthens the grape production chain in Brazil, aligning with the United Nations 2030 Agenda (SDG 2 – Zero Hunger and Sustainable Agriculture; SDG 12 – Responsible Consumption and Production). “Discovery and description of a new *Trichoderma* species associated with the Atlantic Forest biome” – The prospecting and characterization of a novel *Trichoderma* species, conducted in a protected area of the Atlantic Forest, highlights the importance of Brazilian microbial biodiversity as a source of genetic resources with strategic applications. The formal description of this new taxon contributes to taxonomic knowledge and reinforces the value of bioprospecting in underexplored natural ecosystems, supporting the goals of the United Nations 2030 Agenda (SDG 15 – Life on Land; SDG 9 – Industry, Innovation, and Infrastructure). “Biotechnological application of the LFM004 isolate as a bioinput for sustainable viticulture” – The demonstrated biostimulant capacity of the LFM004 isolate, as an alternative to naphthaleneacetic acid (NAA) in promoting rooting, shoot development, and root biomass accumulation in grapevine seedlings, reinforces the potential of this microorganism as a natural biofertilizer. The use of this isolate represents a promising alternative to synthetic growth regulators, contributing to the reduction of chemical inputs and to the development of more sustainable agricultural practices (SDG 12 – Responsible Consumption and Production; SDG 13 – Climate Action). These results not only expand the scientific understanding of the factors affecting grapevine plantlets production but also open concrete pathways for the development of new technologies, public-private partnerships, and improvements in yield, sustainability, and phytosanitary safety in national viticulture.

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FIRST PART

1 GENERAL INTRODUCTION

Grapevine (*Vitis* spp.) is one of the oldest and most widely cultivated fruit crops worldwide, with a rich history that dates back thousands of years (Guzmán-Ardiles *et al.*, 2023; Margaryan *et al.*, 2021; McGovern *et al.*, 2017; McGovern, 2019). Grape production, intended for both fresh consumption and processing into products such as wine, juice, and other derivatives, plays a key role in the global agricultural economy. It contributes significantly to food security, income generation, and the economic development of numerous regions (Li *et al.*, 2018; Parihar; Sharma, 2021; Silva *et al.*, 2019). The leading grape-producing countries are China, Italy, and France, which together account for approximately 36.3% of global production. Brazil ranks fourteenth, with an output of 1.5 million tons and a harvested area of 74.8 thousand hectares (FAO, 2022).

The Southeast region of Brazil stands out as an important center for the production of wine grapes (*Vitis vinifera* L.) destined for fine wine production (Regina *et al.*, 2006; Regina *et al.*, 2010). An innovative vineyard management technique known as "double pruning," developed and validated by the Agricultural Research Company of Minas Gerais (EPAMIG), has promoted and enabled the cultivation of the 'Syrah' variety in Minas Gerais (Favero *et al.*, 2011). This technique promotes sugar accumulation and the synthesis of phenolic compounds in the berries, thereby improving wine quality (Favero *et al.*, 2011; Mota *et al.*, 2010; Mota *et al.*, 2021; Regina *et al.*, 2011, Souza *et al.*, 2015b). Grafting is another widely adopted practice in vineyards of the region, as *Vitis vinifera* is highly susceptible to phylloxera, an aphid insect that attacks roots and leaves, significantly reducing grapevine productivity. Recent research efforts have focused on combining the desirable traits of rootstocks with wine grape varieties used under the double pruning system, aiming to ensure resistance to biotic and tolerance to abiotic stresses, better soil adaptation, and overall plant vigor (Dias *et al.*, 2017; Souza *et al.*, 2015a; Souza *et al.*, 2020).

Recently, the emergence of a new disease characterized by root rot in grapevine plantlets during cold storage has been reported in some nurseries in southern Minas Gerais, Brazil. This issue arises during the refrigeration period (typically at 4°C), which is used to preserve grafted plantlets prior to field planting, and primarily affects hybrid rootstocks such as IAC766, IAC572, IAC313, and 1103P, which are widely adopted in the double-pruning system. The etiological agent responsible for this disease was not previously identified, raising concerns about the potential spread of pathogens and compromising plantlet quality. Among the various pathogens associated with root rot symptoms, species of the genus *Fusarium* stand out, as they

are considered among the ten most important plant pathogens worldwide (Dean *et al.*, 2012; Leslie; Summerell, 2013). *Fusarium* has been reported causing grapevine trunk diseases (GTDs) in several countries, including Afghanistan (Rajput *et al.*, 2020), Brazil (Garrido *et al.*, 2004), Canada (Úrbez-Torres *et al.*, 2023), China (Zhang *et al.*, 2023), Egypt (Ziedan *et al.*, 2011), Spain (Astudillo-Calderón *et al.*, 2019), the United States (Bustamante *et al.*, 2022), and Iran (Chehri, 2017), causing severe damage and reduced yield potential. However, the etiology of these GTDs is distinct from the symptoms observed in the nursery seedlings in southern Minas Gerais, as disease development is closely associated with cold storage conditions. Moreover, although GTDs pathogens can infect plants at the plantlets stage, they typically manifest in more advanced stages of vineyard development, long after field planting (Gramaje; Urbez-Torres; Sosnowski, 2018; Lade *et al.*, 2022; Langa-Lomba *et al.*, 2023). The emergence of such diseases in southern Minas Gerais poses a serious challenge to grape production the economic sustainability of the region (Angelotti *et al.*, 2017; Raftery *et al.*, 2017). These emerging plant diseases, often caused by previously unknown pathogens, can spread rapidly and cause widespread epidemics. Understanding the mechanisms behind their emergence and identifying the causal agents is essential for the development of effective prevention, control, and management strategies (Mwangi *et al.*, 2023; Ristaino *et al.*, 2021; Singh *et al.*, 2023).

In addition to emerging phytosanitary challenges, another critical factor that compromises the efficiency of grapevine plantlets production is the low rooting rate observed after the grafting process (Assunção *et al.*, 2019; ; Boeno; Zuffellato-Ribas, 2023; Goldschmidt, 2014; Wurz; Feldberg; Brighenti, 2022). Although grafting is widely employed in commercial grapevine propagation for its ability to combine productive scion varieties with well-adapted rootstocks (Mudge *et al.*, 2009; Regina, 2002), its effectiveness can be limited by factors such as the vigor and physiological quality of the cuttings, nutrient availability, hormonal imbalance, and susceptibility to pests and pathogens during the early rooting stages (Chen *et al.*, 2024; Gramaje; Armengol, 2011; Pearse, 1943; Vršič; Pulko; Kocsis, 2015; Waite *et al.*, 2015). In this context, vineyard management practices aimed at conserving and restoring soil quality have been extensively explored as sustainable alternatives to improve seedling performance (Jindo *et al.*, 2022; Liu *et al.*, 2024; Visconti, López; Olego, 2024). Among these practices, the use of beneficial microorganisms such as those from the genus *Trichoderma* stands out. This genus has been widely studied for its biostimulant effects and its role in the biological control of phytopathogens (Csótó *et al.*, 2022; Grohs *et al.*, 2017; Zañfano *et al.*, 2024). It acts through multiple mechanisms, including direct antagonism to pathogens, induction of systemic resistance, production of phytohormones such as indole-3-acetic acid (IAA), and enhancement

of nutrient uptake through phosphate solubilization and siderophore production (Tyśkiewicz *et al.*, 2022). Thus, incorporating *Trichoderma* strains into the rooting process represents an effective strategy to overcome propagation challenges in commercial nurseries and reduce reliance on synthetic biostimulants.

2 SCOPE OF THIS THESIS

The general objective of this thesis was to study a new disease of grapevine reported in Minas Gerais and select a potential biological agent to improve plant rooting and growth. This work resulted from a collaboration with EPAMIG (Empresa de Pesquisa Agropecuária de Minas Gerais), specifically with the NUTEV (Núcleo Tecnológico Epamig Uva e Vinho), located in Caldas, MG.

The thesis was structured in three sections: 1) a literature review on the subject of the study; 2) a scientific article already published reporting the ethiology of root and trunk rot disease; and 3) a manuscript that will be submitted on the potential of a novel *Trichoderma* species, *T. harenae* sp. nov., to improve grapevine growth and rooting.

3 THEORETICAL FRAMEWORK

3.1 Origin

The grapevine (*Vitis* spp.) originated in the Northern Hemisphere; however, the exact location of its center of origin remains complex and inconclusive (Guzmán-Ardiles *et al.*, 2023). Potential regions include Asia (Péros *et al.*, 2011; Wan *et al.*, 2008), North America (Liu *et al.*, 2016; Wan *et al.*, 2013), and Eurasia (Bar-Yosef *et al.*, 2011). Although paleobotanical and archaeobotanical evidence indicates the presence of seeds and fruits dating back to the Paleolithic period (Martinoli, 2004; McGovern, 2019), domestication and the beginning of viticulture occurred only in the early Neolithic period, between 6000–5000 BCE, involving [*V. vinifera* L. subsp. *sylvestris* C.C. Gmelin] Hegi for wine production in the Transcaucasia region (Southern Caucasus), corresponding to the present-day republics of Armenia, Georgia, and Azerbaijan (Margaryan *et al.*, 2021; McGovern *et al.*, 2017; Myles *et al.*, 2011).

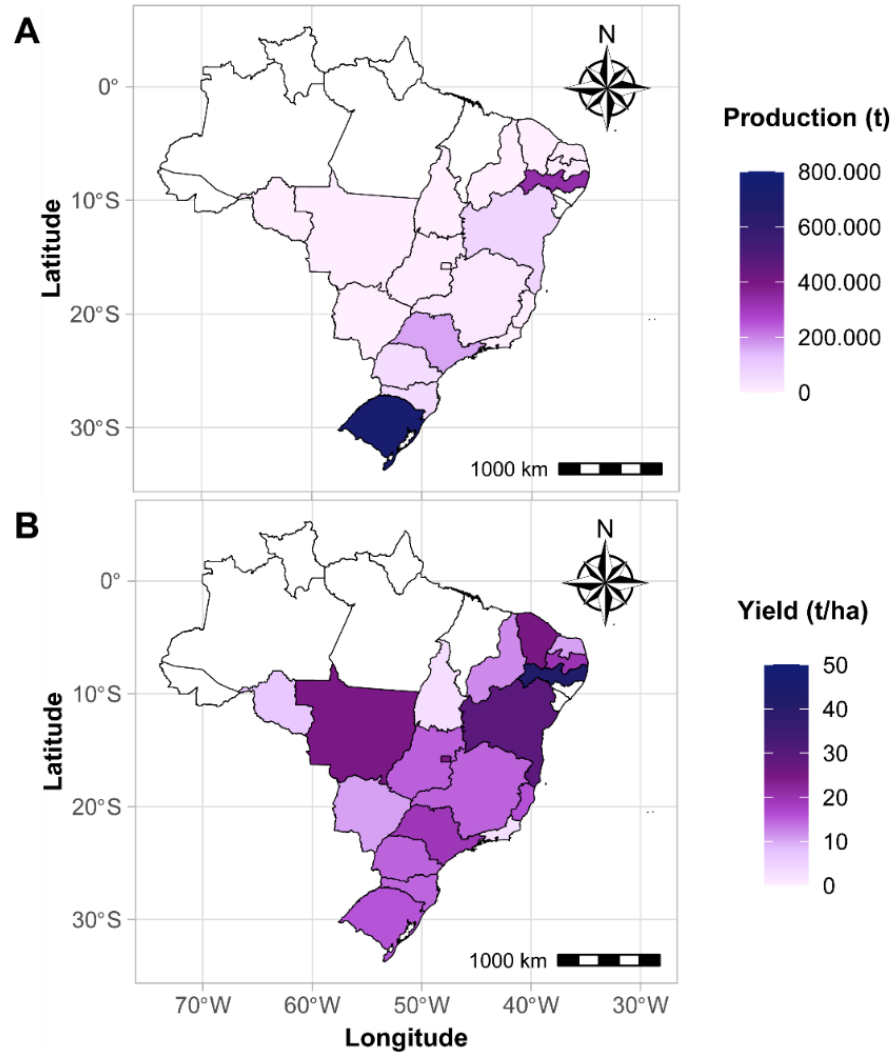
The domestication of the wild grapevine [*V. vinifera* L. subsp. *sylvestris* C.C. Gmelin] Hegi occurred gradually through the selection of desirable traits by early human communities, resulting in biological and architectural changes in the plant that led to the development of *V. vinifera* L. subsp. *vinifera*, the cultivated grapevine. The main traits altered through

domestication used to distinguish the subspecies include seed morphology and the emergence of hermaphroditic flowers, as well as other characteristics such as loss of fruit pigmentation, increased sugar content, firmness, cluster size, and number of grape cluster (Guzmán-Ardiles *et al.*, 2023; Margaryan *et al.*, 2021; Riaz *et al.*, 2018; Terral, 2002; This; Lacombe; Thomas, 2006). Subsequently, grapevine cultivation spread due to the development of the "wine culture," particularly during the Roman Empire, expanding into the Mediterranean, Africa, Central Asia, and Western Europe. In the late 15th century, with the arrival of the Spanish, grapevine cultivation began in the Americas (Forni, 2012; Guzmán-Ardiles *et al.*, 2023; McGovern, 2019; This; Lacombe; Thomas, 2006). In Brazil, grapevine (*V. vinifera* L.) introduction dates back to the Portuguese colonization period, brought by Martim Afonso de Souza to the Captaincy of São Vicente in 1532. However, it was only in the second half of the 19th century that Brazilian viticulture began to gain commercial importance (Leão; Possidio, 2000; Leão, 2010).

3.2 Economic importance

Grapevine is considered one of the oldest cultivated crops in human history, having played a significant role in the development of human civilization (Li *et al.*, 2018; Martinoli, 2004; McGovern *et al.*, 2017; Trondle *et al.*, 2010). Currently, grapevine ranks among the most economically significant fruit crops globally, with a total output of 74.9 million tons cultivated across approximately 6.7 million hectares. Globally, approximately 47.8% of grapes are used for wine production, 44.2% are consumed fresh as table grapes, and 8% are processed into dried grapes. The main producing countries include China (12.6 million tons), Italy (8.4 million tons), France (6.2 million tons), Spain (5.9 million tons), and the United States (5.4 million tons), which together account for 51.4% of global production. Brazil ranks fourteenth, with a production of 1.5 million tons and a harvested area of 74.8 thousand hectares, surpassing many of the world's leading producers in productivity, with an average yield of 19.4 t/ha (OIV, 2022; FAO, 2022). The Northeast and South regions of Brazil are the major grape-producing areas; especially the states of Pernambuco and Rio Grande do Sul, with productions of 338 and 735 thousand tons and harvested areas of 8 and 46 thousand hectares, respectively (Figure 1A). The highest yields are recorded in the states of Pernambuco (42.4 t/ha), Bahia (29.5 t/ha), and Ceará (25.3 t/ha), all exceeding the national average (Figure 1B) (FAO, 2022; IBGE, 2024).

Figure 1 – Grape (*Vitis* spp.) production (A) and productivity (B) in Brazil during the 2022 agricultural season, according to data from the Brazilian Institute of Geography and Statistics (IBGE).



Source: Cruz, J. M. F. L. (2025)

3.3 Botanical and general aspects

Grapevine (*Vitis* spp.) is botanically classified under the division Magnoliophyta (Angiosperms), class Magnoliopsida (Dicotyledons), order Vitales, and family Vitaceae, which comprises 16 genera and over 950 described species (Christenhusz; Byng, 2016; Wen *et al.*, 2018). The genus *Vitis*, first described by Carolus Linnaeus in 1737, is the most important within the family and includes approximately 70 species. Among these, *V. labrusca* (American grapes) and *V. vinifera* (European grapes) are of the greatest economic significance (Ardenghi *et al.*, 2014; Fortes; Pais, 2016; Schwerz *et al.*, 2023).

In Brazil, American grape varieties predominate due to their excellent adaptation to the country's edaphoclimatic conditions, particularly in regions with high rainfall. These varieties

also exhibit strong vigor, which contributes to lower susceptibility to various diseases and results in high productivity (Camargo *et al.*, 2011; Nascimento-Gavioli *et al.*, 2020; Tecchio *et al.*, 2018). American grapes are mainly cultivated for fresh consumption and juice production (Silva *et al.*, 2019; Toaldo *et al.*, 2015). Although also used to produce table wines, they are not ideal for fine wine production due to their intense foxy flavour, low sugar content, low acidity, and limited alcohol potential during fermentation (Nixdorf; Hermosín-Gutiérrez, 2010; Santiago *et al.*, 2014; Spier *et al.*, 2016). European grape varieties, commonly referred to as vinifera grapes, are the most widely cultivated for the global fine wine and sparkling wine industries. However, they are also used for juice production and fresh or dried fruit consumption, owing to their adaptive characteristics, morphological and genetic diversity, high fruit quality, and oenological potential (Parihar; Sharma, 2021; Petretto *et al.*, 2020; Pulvirenti *et al.*, 2015; Venkitasamy *et al.*, 2019).

According to the International Organisation of Vine and Wine (OIV), wine production in Brazil reached 320 million liters in 2022, marking a 9.3% increase compared to the previous year, with an average consumption of 2.1 liters per person. Brazilian viticulture focused on vinifera grapes is concentrated in the South and Southeast regions of the country, with the main producing states being Rio Grande do Sul, Santa Catarina, São Paulo, and Minas Gerais (Camargo *et al.*, 2011; Gonçalves *et al.*, 2020; Guerra; Pereira *et al.*, 2018; Regina *et al.*, 2011; Sartor *et al.*, 2017; Wurz *et al.*, 2017). Despite increasing wine production in these regions, high rainfall during summer negatively affects vineyards, especially in southern and southeastern Brazil, where excessive humidity during grape maturation promotes disease outbreaks—most notably downy mildew [*Plasmopora viticola* (Berk. & M.A. Curtis) Berl. & De Toni]. This vulnerability is attributed to the high disease susceptibility of European grapevines, likely due to their coevolution with pathogens (Boso *et al.*, 2014; Gong *et al.*, 2019; Gonçalves *et al.*, 2022; Hamada *et al.*, 2008; Regina *et al.*, 2006). Recent studies aiming to ensure effective phytosanitary control have focused on developing resistant varieties and implementing management practices such as the double pruning technique, mainly for southeastern regions. (Almeida Júnior *et al.*, 2019; Favero *et al.*, 2011; Goyal *et al.*, 2021; Oliveira *et al.*, 2020; Wingerter *et al.*, 2021).

3.4 Grapevine propagation

The grafting of grapevine is closely linked to the history of phylloxera (*Daktulosphaira vitifoliae* Fitch), an insect of the order Hemiptera and family Phylloxeridae, which caused

devastating damage to vinifera vineyards worldwide—especially in Europe—during the late 19th and early 20th centuries (Benheim *et al.*, 2012; Tello *et al.*, 2019). Phylloxera is an aphid that feeds on both the roots and leaves of grapevines, causing significant damage and ultimately leading to plant death (Savi *et al.*, 2021; Wilmink *et al.*, 2021). The spread of this pest had a catastrophic impact on global viticulture (Bignon *et al.*, 2017). One of the most important strategies to control phylloxera was the development and use of resistant rootstocks, making grafting an essential practice in the production of grapevine plantlets (Andzejewski *et al.*, 2022; Waite *et al.*, 2015).

Currently, bench or mechanical grafting—an automated technique that utilizes specialized machinery—is the most widely adopted technique for grapevine propagation in large-scale commercial nurseries. This method ensures consistency and quality in the graft union, enhances efficiency and speed of grafting, and reduces operational costs compared to manual methods (Gramaje; Di Marco, 2015; Grohs *et al.*, 2017; Regina *et al.*, 2012). Grapevines are relatively easy to propagate, but the process requires several carefully planned and executed steps to ensure the production of healthy and productive seedlings (Waite *et al.*, 2015). The first step involves the selection of canes with buds from the desired varieties and rootstock cuttings, followed by pruning, sectioning, hydration, fungicide treatment, and cold storage to maintain their viability until grafting. During mechanical grafting, the grafted materials are waxed and stratified in plastic containers with moistened inert substrate, covered with plastic. This stage takes place in a dark, temperature-controlled environment to stimulate callus formation at the graft union. After callusing, the grafts are selected, rewaxed, and treated with rooting hormones at the base before being planted in the nursery. Once the seedlings reach the appropriate stage of development, they are harvested with suitable machinery, sorted, categorized, and packaged for cold storage until final field planting (Regina, 2002; Regina *et al.*, 2012).

For profitable vineyard development, it is crucial that scions of productive varieties are grafted onto rootstocks that offer drought and salinity tolerance, high vigor, excellent sanitary quality, verified varietal purity, and compliance with legal standards (Dias *et al.*, 2017; Fu *et al.*, 2019; Leão, 2023; Souza *et al.*, 2020; Waite *et al.*, 2015; Zhang *et al.*, 2016). Rootstocks are among the main factors influencing vine development and the chemical composition of grape juice and wine (Dias *et al.*, 2017; Blank; Samer; Stoll, 2022; Tecchio *et al.*, 2022). The choice of rootstock during propagation is also closely linked to rooting success in the nursery (Wurz; Feldberg; Brighenti, 2022). The ideal scion/rootstock combination plays a fundamental role in achieving high vigor, disease resistance, and optimal canopy development, as it directly

affects vegetative growth, gas exchange, and water-use efficiency (Faralli *et al.*, 2020; Miele; Rizzon, 2017; Souza *et al.*, 2015a; Waite *et al.*, 2015). Among the rootstocks commonly used in double pruning systems, notable hybrids include IAC766 [(*Vitis riparia* × *Vitis cordifolia*) × *Vitis caribaea*], IAC572 [(*Vitis riparia* × *Vitis rupestris*) × *Vitis caribaea*], IAC313 [(*Vitis riparia* × *Vitis rupestris*) × *Vitis cinerea*], and 1103P (*Vitis berlandieri* × *Vitis rupestris*), known for their adaptability to soil and climate conditions in southern and southeastern Brazil, as well as their high vigor and resistance to both biotic and abiotic stressors (Dias *et al.*, 2017; Leão; Chaves, 2019; Leão, 2023; Souza *et al.*, 2015a; Souza *et al.*, 2020; Tecchio *et al.*, 2013).

3.5 Grapevine trunk diseases

Grapevine Trunk Diseases (GTDs) represent one of the major threats to global viticulture, accounting for significant economic losses, estimated at over USD 260 million per year, due to reduced vineyard yield and vine longevity (Fontaine *et al.*, 2016; Kaplan *et al.*, 2016; Sosnowski; McCarthy, 2017). The term "GTDs" was introduced in the late 1990s to encompass a group of diverse symptoms caused by complexes of necrotrophic fungi that progressively colonize the woody vascular tissues of grapevines (Gramaje; Urbez-Torres; Sosnowski, 2018).

GTDs are highly complex and poorly understood syndromes (Bertsch *et al.*, 2013), caused by fungi belonging to the phyla Ascomycota and Basidiomycota, exhibiting high taxonomic diversity (Brown; Lawrence; Baumgartner, 2020; Travadon *et al.*, 2022). More than 100 fungal species from at least 34 genera are estimated to be associated with GTDs in various wine-growing regions worldwide, leading to internal necrosis of vascular bundles and frequently resulting in the death of mature vines (Gramaje; Urbez-Torres; Sosnowski, 2018). The main diseases included in this group are Eutypa dieback, Esca, Botryosphaeria dieback, Petri disease, Black foot, and Phomopsis dieback, all responsible for vine decline in both young and mature plants (Agustí-Brisach; Armengol, 2013; Bertsch *et al.*, 2013; Cobos *et al.*, 2022; Kenfaoui *et al.*, 2022; Mondello *et al.*, 2018).

Symptoms vary depending on the specific pathogen involved (Bertsch *et al.*, 2013; Gramaje; Urbez-Torres; Sosnowski, 2018; Lawrence *et al.*, 2017). Eutypa dieback, primarily caused by *Eutypa lata*, typically shows asymmetrical foliar symptoms, with small, chlorotic, twisted leaves displaying marginal necrosis. Internally, wedge-shaped necrosis is observed in the woody tissues, leading to progressive xylem death and restricted sap flow (Rolshausen *et al.*, 2014). Esca, associated with a complex of fungi including *Phaeomoniella chlamydospora*,

Phaeoacremonium minimum, and *Fomitiporia mediterranea*, is characterized by interveinal chlorotic spots that evolve into necrotic areas, resulting in the typical “tiger stripe” leaf pattern. In adult vines, sudden dieback of shoots and wood discoloration (white or black) can occur, often accompanied by soft rot caused by basidiomycetes (Cloete *et al.*, 2015; Ye *et al.*, 2021). Botryosphaeria dieback, caused by species within the Botryosphaeriaceae complex such as *Diplodia seriata* and *Neofusicoccum parvum*, is characterized by shoot dieback, cankers, internal necrosis, and gummosis (Reis *et al.*, 2020; Spagnolo *et al.*, 2014). Petri disease, common in young vines, is primarily caused by *P. chlamydospora* and *P. minimum*, leading to chlorosis, stunted growth, and darkening of vascular tissues (Berbegal *et al.*, 2025; Ferreira *et al.*, 2018; Hrycan *et al.*, 2025). Black foot disease caused by fungal genera *Dactylonectria* and *Ilyonectria*, results in black necrotic root lesions, reduced vigor, and death of young vines (Sánchez *et al.*, 2019). Phomopsis dieback, caused by *Diaporthe ampelina*, mainly affects cortical tissues and is characterized by dark lesions on shoots, bark necrosis, and bud mortality (Manawasinghe *et al.*, 2019; González-Domínguez *et al.*, 2021).

The epidemiology of GTDs is notoriously complex, involving multiple infection pathways and long latency periods, which hinder the implementation of effective management strategies (Gramaje; Urbez-Torres; Sosnowski, 2018; Hrycan *et al.*, 2020; Li *et al.*, 2023). The primary infection route is through pruning wounds, which serve as entry points for airborne fungal conidia (Díaz; Latorre, 2022; Rosace *et al.*, 2023). Once inside the woody tissue, pathogens can remain latent for extended periods before symptom expression, complicating early detection (Hrycan *et al.*, 2020). Furthermore, environmental factors such as temperature and humidity play a key role in disease dynamics, with warm and humid conditions favoring infection and symptom development (Sosnowski *et al.*, 2023).

GTDs management requires an integrated approach combining cultural practices, chemical treatments, and biological control methods (Mondello *et al.*, 2018). Preventive measures, such as protecting pruning wounds (Ayres *et al.*, 2017; Leal *et al.*, 2024) and using healthy, resistant plant material, are essential to reduce infection (Carbone *et al.*, 2022; Khattab *et al.*, 2021). Although fungicides have been employed, their efficacy is variable, and concerns persist regarding environmental impact and pathogen resistance (Sosnowski, 2008; Sosnowski *et al.*, 2013). Biological control agents, particularly *Trichoderma* species, have shown promising results in suppressing GTD pathogens through mechanisms such as competition, mycoparasitism, and induced systemic resistance (Martínez-Diz *et al.*, 2021; Mesguida *et al.*, 2023; Kenfaoui *et al.*, 2023).

3.6 New disease in grapevine plantlets

The emergence of new plant diseases is intrinsically linked to the presence of a virulent pathogen, a susceptible host, and a favorable environment (Agrios, 2005). As such, emerging plant diseases pose an increasing threat to global food security, affecting agricultural production and, consequently, food availability. These diseases, often caused by previously unknown or underestimated pathogens, may arise suddenly and spread rapidly, causing significant damage to crops and food supply chains (Anderson *et al.*, 2004; Mwangi *et al.*, 2023; Ristaino *et al.*, 2021). Global climate change in recent decades has created more favorable conditions for the proliferation, dissemination, and emergence of various pathogens in different regions, as well as alterations in host susceptibility (Angelotti *et al.*, 2017; Fones; Gurr, 2017; Raftery *et al.*, 2017; Santos *et al.*, 2020; Singh *et al.*, 2023). In addition, avirulent pathogens may become pathogenic through several co-evolutionary mechanisms during pathogen-host interaction over time, such as mutation, gene recombination, horizontal gene transfer, selective pressure from agrochemicals, and host adaptation (Arie *et al.*, 2019; Burgess; Wingfield, 2017; Covo, 2020; Li *et al.*, 2020; Zhang *et al.*, 2021). Other contributing factors include the sharing of latent pathogens among plant species (Crous *et al.*, 2018), the introduction of pathogens from other geographic regions (Yorinori *et al.*, 2002), and certain agricultural practices, such as monoculture and specific pruning strategies (Almeida Júnior *et al.*, 2019; Zhou *et al.*, 2023). Early identification and monitoring of emerging pathogens are critical to prevent epidemic development and to devise effective control strategies (Buja *et al.*, 2021; Venbrux; Crauwels; Rediers, 2023).

In the southern region of Minas Gerais, commercial grapevine propagation is carried out through mechanical grafting, and this process involves the refrigeration of grafted plants at 4°C after removal from the nursery. This cold storage period lasts between 30 and 90 days, aiming to preserve the seedlings until final field planting (Regina *et al.*, 2012). During this cold storage stage, the EPAMIG Grape and Wine Technology Center (NUTEV) has observed the occurrence of root rot in plantlets, resulting in plant death after transplanting to the field. Preliminary observations by NUTEV technicians indicated the potential involvement of unidentified fungal pathogens as causal agents of the disease. Furthermore, reports suggest that root rot is more prevalent in hybrid rootstocks (IAC 313, IAC572, IAC766, and 1103P), which are widely used in traditional and double pruning systems due to their high grafting success rates, high vigor and resistance to phylloxera (Regina *et al.*, 2012; Dias *et al.*, 2017; Leão *et al.*, 2020; Souza *et al.*, 2020).

Root rot is a disease that causes considerable agricultural losses globally. The extent of the impact varies depending on the causal agent, host susceptibility, and environmental conditions, and in severe cases, it can lead to total crop failure (Bodah, 2017; Williamson-Benavides; Dhingra, 2021). Among the pathogens associated with root rot symptoms, *Fusarium* species stand out. This genus is considered one of the top ten most important plant pathogens worldwide (Dean *et al.*, 2012; Leslie; Summerell, 2013). *Fusarium* is a monophyletic genus known for its complex taxonomy, encompassing over 300 phylogenetically distinct species grouped into 23 evolutionary lineages called "species complexes," based on morphological, biological, and phylogenetic species concepts (Aoki *et al.*, 2014; Al-Hatmi *et al.*, 2020; Geiser *et al.*, 2021). *Fusarium* species have been commonly reported as causal agents of grapevine diseases (*Vitis* spp.) worldwide, including: *F. annulatum*, *F. nirenbergiae*, *F. oxysporum*, *F. solani*, *F. keratoplasticum*, *F. brachygibbosum*, *F. culmorum*, *F. clavum*, *F. avenaceum*, *F. brachygibbosum*, *F. falciforme*, *F. fredkrugeri*, *F. glycines*, *F. nanum*, *F. nematophilum*, *F. nirenbergiae*, *F. curvatum*, *F. proliferatum*, *F. ramigenum*, *F. commune*, *F. equiseti*, *F. decemcellulare*, and *F. delphinoides* (Andrade, 1993; Astudillo-Calderón *et al.*, 2019; Akgül *et al.*, 2024; Chehri *et al.*, 2017; Bustamante *et al.*, 2022; Bustamante *et al.*, 2024; Li *et al.*, 2023; Rajput *et al.*, 2020; Úrbez-Torres *et al.*, 2023; Yurchenko *et al.*, 2020; Zhang *et al.*, 2023). However, the symptomatology and etiology associated with these species are linked to grapevine trunk diseases (GTDs), which differ entirely from the root rot observed in seedlings from the southern region of Minas Gerais. Investigations in these propagation nurseries are essential to anticipate potential epidemics that could arise from the spread of the pathogen to unaffected areas. Moreover, accurate identification of the causal agent is crucial for developing effective control methods, including the use of biological control strategies to promote sustainable viticulture.

3.7 Biotechnological potential of *Trichoderma*

The genus *Trichoderma* is a filamentous fungus widely distributed across various ecosystems, especially in soil and the rhizosphere of plants. This genus has been widely studied for its role as a biocontrol agent against phytopathogens and for its beneficial effects on plant growth (Woo *et al.*, 2023). According to the current classification in MycoBank, the *Trichoderma* genus belongs to the domain *Eukaryota*, kingdom *Fungi*, phylum *Ascomycota*, subphylum *Pezizomycotina*, class *Sordariomycetes*, order *Hypocreales*, and family *Hypocreaceae*. It currently includes over 382 species characterized based on molecular and

morphological traits (Cai *et al.*, 2022). The success of *Trichoderma* species is attributed to their rapid growth on diverse substrates and tolerance to a range of toxic chemicals, including fungicides such as azoxystrobin, chlorothalonil, cyproconazole, and trifloxystrobin. Studies have shown that different *Trichoderma* isolates can tolerate and grow in the presence of these fungicides, although sensitivity may vary among species and strains. For instance, isolates of *T. rifaai* and *T. atroviride* have demonstrated substantial growth even in the presence of azoxystrobin and trifloxystrobin (Escudero-Leyva *et al.*, 2022). Furthermore, *Trichoderma* colonizes a wide range of habitats by employing a combination of strategies that enable it to compete for resources, withstand environmental stress, repair cellular damage, and modify ecological conditions to its advantage (Woo *et al.*, 2023). One such strategy includes acidification of the environment, as observed in *T. atroviride*, which can reduce the pH of its growth medium (Pelagio-Flores *et al.*, 2017).

The biocontrol activity of *Trichoderma* against phytopathogens involves multiple mechanisms that may act independently or synergistically (Alfiky; Weisskopf, 2021). These include antibiosis, characterized by the production of secondary metabolites with antimicrobial properties that inhibit spore germination, hyphal development, and/or cause cell death (Gualtieri *et al.*, 2022; Jiménez-Bremont, *et al.*, 2024; LI; LI; Zhang, 2019). Other mechanisms include parasitism, hyperparasitism, and mycoparasitism (Kubicek *et al.*, 2011; Mukherjee *et al.*, 2022), as well as competition for space and nutrients—aided by rapid mycelial growth, high sporulation rate, and strong colonization ability—which provides *Trichoderma* with a competitive advantage (Benítez *et al.*, 2004). *Trichoderma* is also known to induce systemic resistance in host plants by activating defense-related signaling pathways involving jasmonic acid, ethylene, and salicylic acid (Kumhar *et al.*, 2022; Nawrocka; Malopepsza, 2013; Yuan *et al.*, 2019).

Beyond biocontrol, various *Trichoderma* species exhibit biostimulant properties that contribute to plant development by enhancing root growth, nutrient uptake, and tolerance to abiotic stresses (Carvalho *et al.*, 2011). One such effect is the stimulation of root development, essential for water and nutrient absorption. This effect is associated with the production of phytohormones such as indole-3-acetic acid (IAA). In addition to IAA, recent studies have shown that secretes anthranilic acid, a compound that stimulates lateral root growth through auxin signaling and remodeling of the endodermal cell wall, promoting secondary root formation (Chen *et al.*, 2024). These substances stimulate cell division and elongation, enhancing both primary and lateral root development. Moreover, *Trichoderma* promotes plant growth by improving the uptake of essential nutrients such as phosphorus (P) and iron (Fe).

Phosphorus availability, often limited in tropical soils, is enhanced by the production of organic acids that chelate cations bound to phosphate, releasing bioavailable forms of the nutrient. Additionally, acid and alkaline phosphatases secreted by *Trichoderma* mineralize organic compounds, releasing inorganic phosphate. For iron, *Trichoderma* produces siderophores—chelating molecules that bind Fe^{3+} and increase its bioavailability in the rhizosphere. These fungi also positively influence the expression of iron transporter genes in plants and contribute to soil pH modulation, creating favorable conditions for nutrient uptake (Altomare *et al.*, 1999; Harman *et al.*, 2004; Pelagio-Flores *et al.*, 2017; Vinale *et al.*, 2008; Yedidia *et al.*, 2001). Another notable effect of *Trichoderma* is the mitigation of abiotic stress. Studies show that *Trichoderma* inoculation enhances net photosynthetic rate, stomatal conductance, proline content, and water use efficiency, in addition to modulating hormonal balance under drought and saline stress conditions (Oljira *et al.*, 2020). These physiological responses significantly contribute to plant growth under adverse environmental conditions. The dual roles of *Trichoderma* as a biocontrol agent and plant growth promoter explain its wide application in sustainable agriculture (Tyśkiewicz *et al.*, 2022).

According to Carvalho *et al.* (2015), Brazilian soils host a diverse range of *Trichoderma* species, making them a valuable source for the biotechnological exploration of promising isolates. In this context, research focusing on the characterization of these microorganisms has proven fundamental for the discovery of strains with applications in plant pathogen control and growth promotion (Bader *et al.*, 2020; Batistello *et al.*, 2022; Bononi *et al.*, 2020; Marcuzzo; Carvalho, 2018). Considering the challenges associated with grapevine seedling production—such as the significant increase in demand for high-quality propagative material and the pursuit of sustainable technologies—the prospecting and use of new *Trichoderma* strains emerge as a strategic solution. Inoculation with selected isolates that possess bio-stimulant capacity, in addition to their well-established role in the biological control of phytopathogens, may contribute to enhanced rooting efficiency, faster seedling establishment, and reduced reliance on synthetic inputs, establishing *Trichoderma* as a key tool for the advancement of sustainable viticulture in Brazil.

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SECOND PART – PUBLICATION/MANUSCRIPT

Article 1 – Written according to the journal's guidelines – Published version	
Article title:	A New Root and Trunk Rot Disease of Grapevine Plantlets Caused by <i>Fusarium</i> in Four Species Complexes
Authors:	José Manoel Ferreira de Lima Cruz Otília Ricardo de Farias Brunno Cassiano Lemos Araújo Alejandra Valencia Rivera Cláudia Rita de Souza Jorge Teodoro de Souza
Journal:	Journal of Fungi
ISSN	2309-608X
DOI	https://doi.org/10.3390/jof11030230

Article

A New Root and Trunk Rot Disease of Grapevine Plantlets Caused by *Fusarium* in Four Species Complexes

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Abstract: Grapevines are propagated by grafting, but the rootstocks used in commercial plantations are susceptible to several diseases. In this study, we focused on a novel root and trunk rot disease of grapevine plantlets that show symptoms during cold storage, before field establishment. Our objectives were to study the aetiology, symptomatology, plant resistance responses, and mode of action of the pathogen that was initially identified as *Fusarium*. The characterisation of this pathosystem was performed by isolation, pathogenicity assays, genetic diversity studies with BOX-PCR, and identification by sequencing a fragment of the *tef1* gene. Scanning electron microscopy and X-ray spectroscopy were used to study the mode of action and plant resistance responses. The results showed that 12 species of *Fusarium*, initially isolated from both healthy and diseased plantlets, and classified into 4 species complexes, were pathogenic to grapevines. Comparative analyses between diseased and healthy roots showed typical resistance responses in diseased plantlets, including tyloses formation, translocation of Ca, and accumulation of Si. Field experiments confirmed that 100% of the diseased plantlets died within 90 days of transplantation. This study contributes to a better understanding of root and trunk rot disease under cold storage and provides insights for the development of management strategies.

Keywords: aetiology; endophytic colonisation; genetic diversity; grapes; pathogenicity; *Vitis* spp.



Academic Editor: Baoshan Chen

Received: 10 February 2025

Revised: 11 March 2025

Accepted: 13 March 2025

Published: 17 March 2025

Citation: Cruz, J.M.F.d.L.; Farias, O.R.d.; Araújo, B.C.L.; Rivera, A.V.; de Souza, C.R.; de Souza, J.T. A New Root and Trunk Rot Disease of Grapevine Plantlets Caused by *Fusarium* in Four Species Complexes. *J. Fungi* **2025**, *11*, 230. <https://doi.org/10.3390/jof11030230>

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1. Introduction

Grapevines (*Vitis vinifera*) are economically and socially important as they offer a wide range of products, including fresh and dried fruits, juices, liquors, and wines [1]. In Brazil, the production of grapes for fine wines is concentrated in the southern and southeastern regions, and the south of Minas Gerais stands out for both the expansion of cultivation and the high quality of the grapes and wines produced [2,3]. This performance is directly related to the favourable climate conditions allied with vineyard management practices, such as the production of high-quality plantlets (planting material) and the double pruning system, with a direct impact on the quality [2–7].

Production of grapevine plantlets is a long process that takes more than a year in Minas Gerais state, Brazil. The production of grafted grapevine plantlets is carried out

using the table grafting system, in which the rootstock and scion of commercial cultivars are mechanically grafted. These unrooted plantlets undergo a stratification for the formation of callus at the junction between the scion and rootstock for a period of approximately 20 days at 20–29 °C and 90–95% humidity. After this period, the unrooted plantlets are transferred to a nursery installed in the field, where they remain for one year for root development. After root development, the rooted plantlets are removed from the soil, cleaned, have their roots trimmed, and are stored in a cold chamber at 4 °C for periods that may go up to one year before commercialisation [8].

The Agricultural Research Agency of Minas Gerais State (EPAMIG) is one of the institutions responsible for producing grapevine plantlets to expand grape cultivation in Minas Gerais. Plantlets with rotten roots and trunks were first observed in 2016 at the plantlet producing facility coordinated by EPAMIG in the Caldas municipality, Minas Gerais. Preliminary observations suggested that *Fusarium* was associated with these symptoms. This root and trunk rot disease was more prevalent in plantlets with the hybrid rootstocks IAC 313, IAC 572, IAC 766, and 1103P, widely used due to their high vigour and resistance to phylloxera aphids [6,9,10]. Initial observations indicated that root and trunk rot symptoms only developed under cold storage. An informal quantification of the incidence of root and trunk rot indicated that up to 50% of the susceptible plantlets could develop symptoms (Souza, C.R., personal observation). Since its discovery, little research has been undertaken to understand the aetiology and control of this disease.

It is well known that grapevines are susceptible to several diseases, including grapevine trunk decline (GTD), certainly the most complex and one of the most destructive diseases of this crop. GTD shows symptoms in the field, typically after 5 years of cultivation, but varies according to a great number of factors [11,12]. The disease may cause between 30 and 94% of losses and involves a great number of fungal pathogens in its aetiology [13]. The symptoms involve root rot, trunk rot, and general decline, which may vary according to the causative agent. More than 140 fungal species were listed as causative agents, including *Neofusicoccum*, *Phaeomoniella*, *Diplodia*, *Lasiodiplodia*, *Botryosphaeria*, *Phaeoacremonium*, *Pleurostoma*, *Cadophora*, *Campylocarpon*, *Cylindrocladiella*, *Dactylonectria*, *Ilyonectria*, *Neonectria*, and *Thelonectria* [11,12,14–20]. *Fusarium* is among the agents of GTD, but it is always in association with other pathogens [21]. *Fusarium* is a pathogen with a wide host range, frequently acting as an opportunist, infecting plants under stressful conditions [22,23]. *Fusarium* was recently reported as the causative agent of trunk rot in plantlets in Turkey [22].

In this study, we report a new disease caused by *Fusarium* that we named root and trunk rot of grafted grapevine plantlets under cold storage, and it is clearly different from GTD. In this initial characterisation of the disease, we described the symptoms, identified the *Fusarium* species associated with healthy and diseased plantlets, and studied the reactions of the plant to the pathogens. The factors that are thought to influence disease development and possible control strategies are discussed.

2. Materials and Methods

2.1. Plant Material and Fungal Isolation

Healthy and diseased grapevine plantlets of cv. Syrah (*Vitis vinifera*) grafted onto IAC766 rootstock [(*Vitis riparia* × *Vitis cordifolia*) × *Vitis caribea*] were obtained from the plantlet production areas of the EPAMIG research station, in Caldas, Minas Gerais, Brazil. The samples were placed in plastic bags and stored at 4 °C until fungal isolation. A total of 25 healthy and 25 diseased plantlets of grafted cv. Syrah on cv. IAC766 were used in these isolations. In the laboratory, tissue fragments (diam = 5 mm) from the trunk and roots of healthy and diseased plantlets were surface sterilised by immersion in 70% ethanol for 30 s and 1% NaClO for 60 s, washed three times with sterile distilled water, and transferred to

Petri dishes containing potato dextrose agar (PDA) medium with streptomycin (250 mg/L) for 7 days at 25 °C, with a 12 h photoperiod. Subsequently, the fungal structures that developed on the fragments were purified and preserved in sterile distilled water [24] and glycerol (40%) at −80 °C. Fungal strains were identified at the genus level based on the morphology of the reproductive structures observed under a microscope and comparison with descriptions in the specialised literature [25,26].

2.2. Clustering and Identification

Strains of *Fusarium*, selected for being the most prevalent genus in both healthy and diseased plantlets, were subjected to total genomic DNA extraction following the procedures described by [27]. The purity of the extracted DNA was estimated, and its concentration was quantified using a spectrophotometer (Nanodrop Lite UV-Vis; Thermo Fisher Scientific, San Francisco, CA, USA). The DNA was then stored at −20 °C for further analyses.

BOX-PCR, a reproducible whole-genome fingerprinting technique used here to study the genetic diversity of the *Fusarium* strains, was performed in 25 µL reactions with the primer BOX-A1R (5′-CTA CGG CAA GGC GAC GCT GAC G-3′) [28]. Cluster analysis was performed using the unweighted pair group method using arithmetic averages (UPGMA) hierarchical method in R statistical software version 4.4.2 [29]. Genetic similarity was assessed based on Jaccard's distance [30] and calculated from a binary matrix indicating the presence (1) or absence (0) of amplified products (bps).

Fusarium strains obtained from healthy plantlets, as well as representative strains of each BOX group identified through cluster analysis, were selected for sequencing a fragment of the *tef1* gene (translation elongation factor 1- α). Polymerase chain reaction (PCR) amplification of the gene was performed using the primers EF-1 (5′-ATG GGT AAG GAR GAC AAG AC-3′) and EF-2 (5′-GGA RGT ACC AGT SAT CAT GTT-3′) following the recommendations of [31]. Amplified fragments were purified using the commercial Biospin PCR kit and sequenced in a private facility (ACTgene Molecular Analysis, Porto Alegre, RS, Brazil). Consensus sequences were obtained by assembling and editing in Sequencher v. 5.4.6 (Gene Codes Corporation, Ann Harbor, MI, USA) and compared with the most closely related sequences of type material deposited in public databases [32] using the BLASTn programme (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>, accessed on 20 November 2024) [33]. Phylogenetic analyses using the *tef1* sequences obtained and sequences from the closest type material deposited in databases (Table S1) were performed to identify the strains at the species level. Alignments were conducted using MAFFT (multiple alignment using fast Fourier transform) v. 7 [34,35] and refined using GBlocks v. 0.91b [36], eliminating low-quality regions and preserving only the conserved blocks. Evolutionary relationships were inferred in RAxML-HCP2 v. 7.0.4 and MrBayes on Access (3.2.7a) [37], implemented in the CIPRES platform [38], using maximum likelihood (ML) and Bayesian inference (BI) methods separately, with 1000 bootstrap replications. Final phylogenetic trees were edited using FigTree v. 1.4.3 [39] and *tef1* sequences were deposited with GenBank.

2.3. Pathogenicity and Virulence

Three inoculation methods were performed in parallel using *Fusarium* strains on cv. IAC766 grafted with cv. Syrah, (i) trunk inoculation, (ii) inoculation of rooted plantlets, and (iii) inoculation of the base of rootless plantlets [22,40].

For method (i), mycelium discs (3 mm diameter) from each strain, previously grown on PDA plates, were used. The area between the base and the first node of the plantlets was punctured with a sterile cork borer to insert the inoculum. PDA discs without mycelium were used as negative controls. To prevent water loss, sterile cotton soaked in distilled water was placed on the opposite side of the lesion. Pathogenicity is defined here as the

capacity of a pathogen to cause disease and virulence as the amount (incidence in this case) of disease, which was quantified with a descriptive scale (Table 1). The pathogenicity of representative strains from each BOX-PCR cluster was tested following method (i). Plants were examined under a stereomicroscope to visualise disease symptoms and pathogen signs at 7 days after the inoculations. The virulence was determined on the basis of the number of plantlets with trunk rot incidence using the scale described in Table 1. Nine strains were chosen to complete Koch's postulates by performing re-isolations on PDA. The assays with method (i) were conducted in a completely randomised design with 15 plantlets per treatment.

Table 1. Descriptive scale for evaluating the virulence of trunk rot in grapevine grafted plantlets infected with *Fusarium* in the pathogenicity assays with method (i). All strains listed in this study were tested with this method.

N° Diseased Plantlets	Pathogenicity/Virulence
0	-
<5	+
6–10	++
>11	+++

Methods (ii) and (iii) consisted of inoculations with conidial suspensions prepared by growing the fungi in liquid malt extract medium for 7–10 days at 25 °C, subsequently filtering the culture through a double layer of gauze and adjusting the concentration to 10^7 conidia/mL in a Neubauer chamber. For method (ii), rooted plantlets that were produced in field nurseries were immersed in the suspensions of each *Fusarium* strain for 24 h and stored in a cold chamber for 3 months. For method (iii), rootless plantlets (without roots) were inoculated as described above, planted in the nursery field for 12 months, and subsequently, the plantlets were removed from the soil, cleaned, and stored in a cold chamber (4 °C) for 3 months, following standardised methods described in [8]. The experiments with methods (ii) and (iii) were installed in a randomised block design with four replicates of 25 plantlets per replicate, totalling 100 plants per treatment. The fungicide treatment consisted of immersing the plantlets for 24 h in a solution containing 200 mL/100 L of water of a commercial product composed of 50% of the active ingredient Iprodione. The survival rate of the plantlets and disease incidence were evaluated after 3 months of storage in the cold chamber. Later on, 100 healthy and 100 diseased plantlets, without discriminating the *Fusarium* strains in diseased plantlets, were transplanted to the field to assess their survival rate. Mortality, shoot length, and chlorophyll a, b, and total chlorophyll content were measured using ClorofiLOG model CFL 1030 (Falker, Porto Alegre, RS, Brazil). These measurements were performed on 100 healthy and 100 diseased plantlets growing in the field by recording chlorophyll measurements in three leaves per plant. The trials were conducted with the five most virulent strains among those that fulfilled Koch's postulates performed with method (i). All analyses were performed using the R software v.4.3.3 [29], with the data subjected to residual normality tests using Shapiro–Wilk and the variance homogeneity test of Bartlett. Differences between the means of healthy and diseased plantlets were analysed using the *t*-test 5 and 1% probability.

2.4. Pathogenicity and Colonisation of Other Plant Species by *Fusarium*

In order to evaluate whether *Fusarium* strains from diseased plantlets were capable of colonising and causing root and/or trunk rot in other crops, conidial suspensions containing 10^7 conidia/mL were used to inoculate common bean cv. Carioca and tomato cv. Santa Cruz seedlings, and grapevine rooted plantlets of cv. Syrah grafted onto cv.

IAC766. Grapevine plantlets were rooted in a mixture (1:1) of sterile sand and a substrate (Tropstrato, Campinas, SP, Brazil) in a greenhouse for approximately 1 month before the inoculations. The roots of 25 plants of each species were surface sterilised as described above, immersed in conidial suspensions of each strain that had fulfilled Koch's postulates, and incubated for 7 days in Petri dishes covered with filter paper moistened with sterile distilled water to maintain the humidity. After the incubation period, disease incidence was visually assessed and roots were cut in 0.5 cm-long fragments, surface sterilised as described in Section 2.1, and then 100 fragments per plant species per *Fusarium* strain were plated on PDA to quantify root colonisation. The fungal colonies obtained were visually compared with the originally inoculated strains.

2.5. Comparative Analysis of Diseased vs Healthy Tissues

Scanning electron microscopy (SEM) and X-ray spectroscopy were used to assess the interaction between pathogenic *Fusarium* strains and grapevine plantlets. The study included a structural analysis and the determination of the chemical composition of both healthy and diseased plantlets.

Fragments from roots and trunks of healthy and diseased plantlets were fixed in a modified Karnovsky solution (2.5% glutaraldehyde, 2.5% paraformaldehyde, 0.05 M sodium cacodylate buffer, pH 7.2, and 0.001 M CaCl₂), following the protocol described in [41]. The samples remained in this solution for 24 h, then were washed with cacodylate buffer (0.05 M) and subjected to sequential dehydration in acetone at concentrations of 25%, 50%, 70%, and 90% for 10 min each, followed by 100% acetone three times for 10 min each. After dehydration, the samples were dried at the critical point using CO₂ (Baltec, model CPD 030; Electron Microscopy Sciences, Hatfield, PA, USA). The specimens were mounted on aluminium stubs (covered with aluminium foil) using double-sided carbon tape and coated with gold (Balzers SCD 050, Oerlikon Balzers, Balzers, Liechtenstein). The prepared samples were stored in a desiccator with silica gel until SEM analysis (model Leo Evo 40 XVP, Carl Zeiss, Jena, Germany).

For chemical composition analysis, the fragments were directly fixed on aluminium stubs for 24 h in a desiccator containing silica gel for dehydration. The samples were then coated with carbon in a sputtering machine (MED 010, Oerlikon Balzers, Balzers, Liechtenstein). Mineral element mapping was performed using energy-dispersive X-ray spectroscopy (EDS), integrated with the SEM, using Bruker Espirit software (version 1.9). Images were generated and digitally captured under the working conditions of 20 kV, at 9.0 mm working distance, $\pm 180\times$ magnification, and Kcps values ranging between 3 and 4. The nutrients that showed variations in distribution between diseased and healthy plantlets were selected for graphical representation. The images from the morphological analysis and the chemical mapping results were processed and edited using CorelDraw and CorelPhoto-Paint v. 22.0.

3. Results

3.1. Symptoms of Root and Trunk Rot of Grapevine Plantlets Under Cold Storage

The symptoms of root and trunk rot in grapevine plantlets only appear during storage in a cold chamber at 4 °C. Root and trunk rot under cold storage was first detected in cultivar Syrah grafted onto IAC766 rootstock and since 2016 its occurrence has been noticed in the plantlet-producing facility of EPAMIG in Caldas, Minas Gerais state. However, we were not able to quantify its incidence precisely as it varies greatly from year to year, but it currently affects roughly around 5–10% of the plantlets produced annually (C.R. Souza, personal observation). Diseased plantlets showed reduced vigour when they are grown in a greenhouse, and yellowing and leaf fall is quickly followed by plant death (Figure 1A,B).

The disease progresses from the rotten roots (Figure 1C), reaches the crown and trunk, and finally the callus formed at the union between the scion and the rootstock (Figure 1D–F). The internal tissues of the trunk show darkening of the xylem vessels accompanied by areas of rot that appear as dark longitudinal bands (Figure 1E,F).

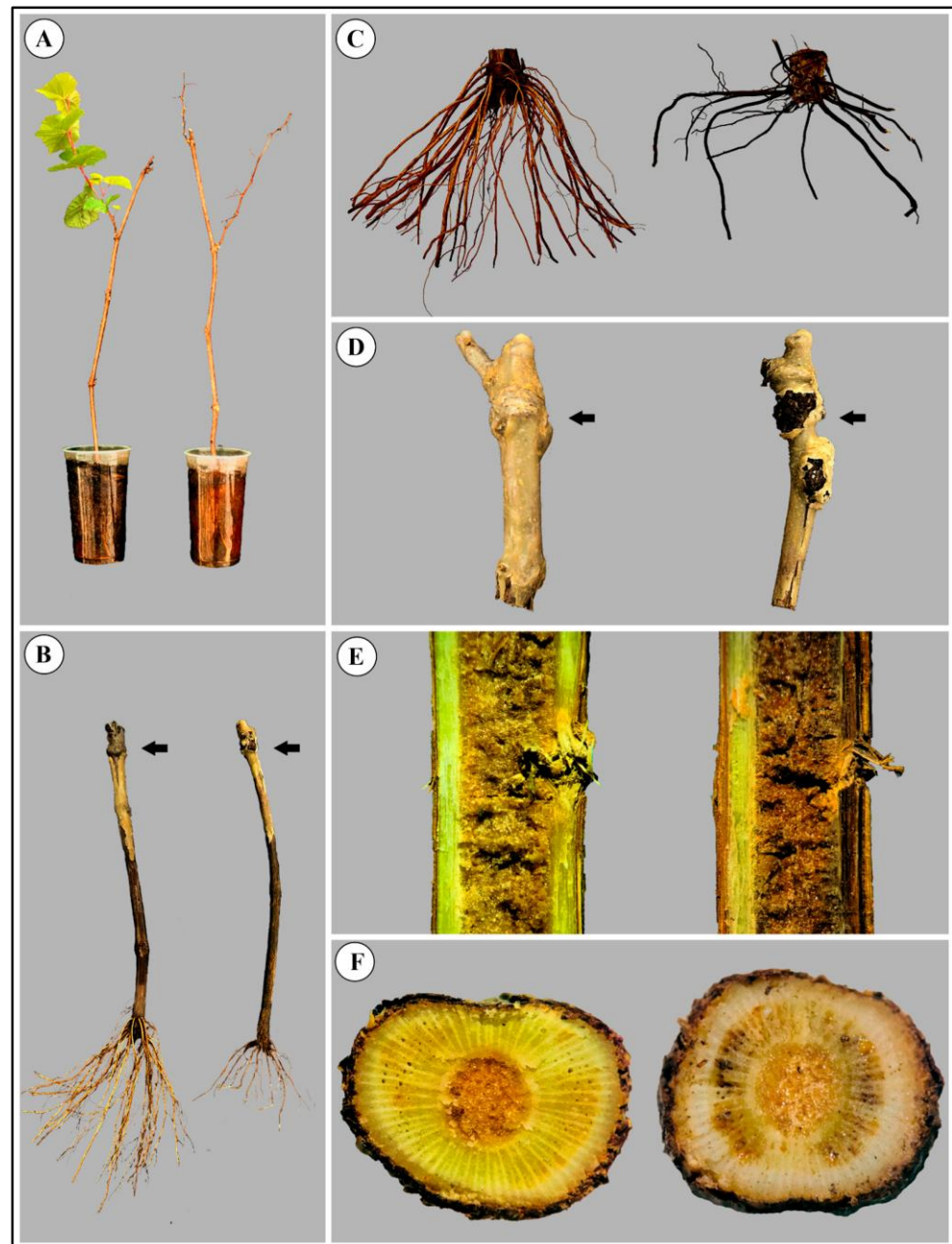


Figure 1. Symptoms of root and trunk rot in grapevine plantlets cv. Syrah grafted onto IAC766 under cold storage. Healthy plant material is always shown on the right side and diseased plant material on the left side. (A) Whole plantlet showing branching and diseased plantlet without leaves. (B) Normal roots on a healthy plantlet and rotten roots on a diseased plantlet. (C) Healthy and diseased roots. (D) Callus formed in the junction between the scion and the rootstock in healthy and diseased plantlets. (E) Longitudinal section in the vascular tissue of healthy and diseased plantlets showing darkening. (F) Cross sections in healthy and diseased vascular bundles of the trunk showing darkening due to the disease. Black arrows indicate the junction between scion and rootstock.

Diseased plantlets sprouted when transferred to the field and produced shorter shoots, but 100% of them (n = 100) died in a period of 90 days after field transplantation, whereas

no death of healthy plantlets was observed in this period. Healthy plantlets produced significantly longer and more vigorous shoots that were on average 21 cm long, in contrast to diseased plantlets that produced shoots 8.2 cm long on average (Figure 2A–C). Healthy plantlets showed significantly higher levels of chlorophyll than diseased plantlets. These values were approximately 1.8, 2.8, and 2× higher in healthy than in diseased plantlets for chlorophyll A, chlorophyll B, and total chlorophyll, respectively (Figure 2D). Furthermore, healthy plantlets exhibited more developed branches and leaves with an intense and uniform green colour (Figure 2B,C,E,F).

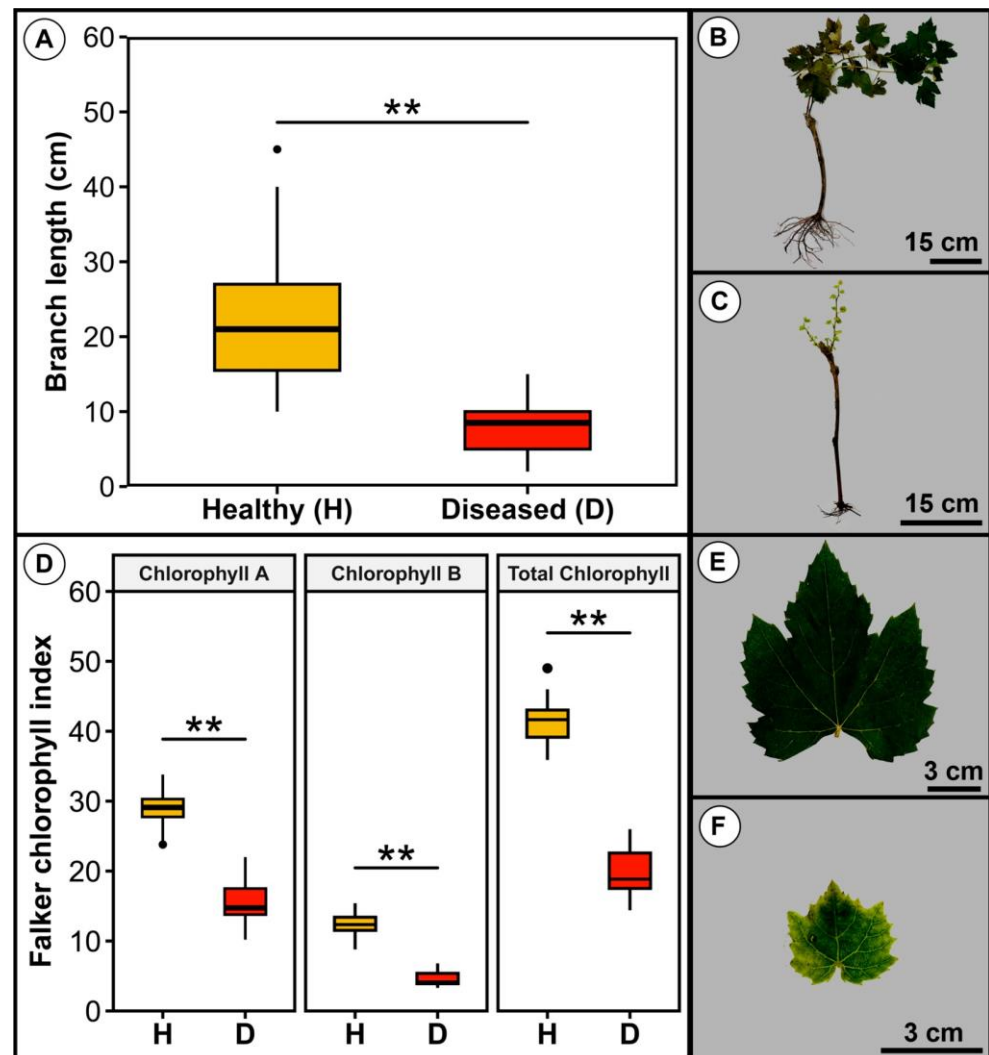


Figure 2. Sprouting and chlorophyll content in healthy—H and diseased—D plantlets of cv. Syrah grafted onto IAC766 rootstocks. (A) Branch length in 100 healthy and 100 diseased plantlets. (B) Visual aspect of a sprout in a healthy plantlet. (C) Sprouts in a diseased plantlet. (D) Chlorophyll content in 100 healthy and 100 diseased plantlets, with the measurement of 3 leaves per plantlet. (E) Aspect of a leaf in a healthy plantlet. (F) Leaf on a diseased plantlet. Asterisks indicate significant differences according to the *t*-test at 1% probability. Scale bars are shown at the lower right corner of figures in the right panel.

3.2. *Fusarium* Was the Most Prevalent Genus in Both Healthy and Diseased Plantlets

In order to identify the pathogens associated with root and trunk rot of grapevine, healthy and diseased plantlets of cv. Syrah grafted onto rootstock IAC766 were subjected to the isolation of microorganisms in culture media. The genera *Fusarium* and *Epicoccum* were the most commonly isolated genera from the roots of both diseased and healthy plantlets,

as identified by morphological analysis (Figure 3). Bacteria were also isolated, but their numbers were considered low and, therefore, our attention turned to the fungi.

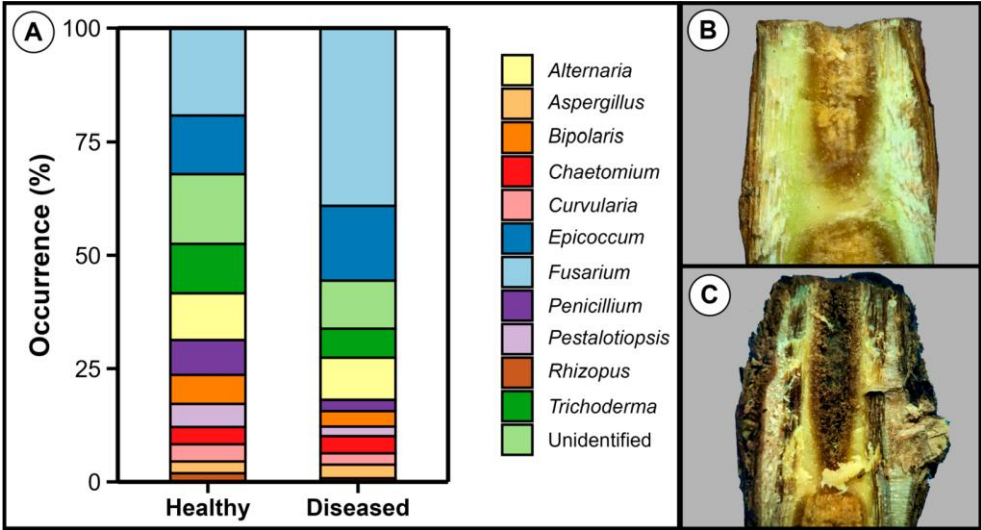


Figure 3. Microorganisms associated with healthy and diseased grapevine plantlets. **(A)** Occurrence of fungi in roots of grapevine cv. Syrah grafted onto rootstocks of cv. IAC766. **(B)** Visual aspect of the plantlet crown, defined as the area between the root and the trunk. **(C)** Crown of a diseased plantlet. A total of 25 healthy and 25 diseased plantlets of grafted cv. Syrah on cv. IAC766 were used in the isolations. Identifications were performed through morphological analysis using a microscope and the number of colonies was determined on PDA plates.

A total of 156 strains were analysed from healthy plantlets, from which *Fusarium* represented 19%, whereas 236 strains were analysed from diseased plantlets, with *Fusarium* representing 39% of the total.

3.3. *Fusarium* Strains from Diseased Plantlets Were Highly Diverse

The genus *Fusarium* was the most prevalent in the roots of both healthy and diseased plantlets and therefore was the focus of our diversity studies. A total of 86 strains of *Fusarium* from diseased plantlets were randomly selected for a diversity study with BOX-PCR fingerprinting. These strains were grouped into 45 genetic groups on the basis of 53 polymorphic bands. From these genetic groups, 10 were composed of more than one strain, whereas 35 were formed of one unique strain (Figure 4).

In order to identify the strains of *Fusarium* from diseased plantlets, at least one strain from each BOX group was selected for the sequencing of a fragment of the *tef1* gene. A total of 78 strains were used in these identifications, 49 from diseased plantlets and 30 strains isolated from healthy plantlets. The strains from diseased plantlets included 45 from each BOX group and 5 other strains from BOX groups containing more than 1 strain (Figure 4). Sequence and phylogenetic analyses allowed the identification of 14 *Fusarium* species among the 49 strains from the roots of diseased plantlets (Figure 5). These 14 *Fusarium* species belong in four species complexes as follows: 23 strains in the *Fusarium oxysporum* species complex (FOSC), 21 strains in the *F. fujikuroi* species complex (FFSC), 3 strains in the *F. tricinctum* species complex (FTSC) and 2 strains in the *F. solani* species complex (FSSC) (Table 2, Figure 5). These species were identified by comparing their *tef1* sequences with the type strains deposited in public databases (Table S1) and most of them were 98.47–100% identical. However, some strains, such as LMF0441, LMF0442 and LMF0427 that were closest to *F. chongqingense*, and LMF0417, LMF0420, LMF0414, LMF0421, LMF041, LMF046 and LMF0432 that were close to *F. foetens*, showed identities of 97.48–98.2% when compared

to type strains deposited in databases. Although we have named them based on their closest related sequences they may represent novel *Fusarium* species.

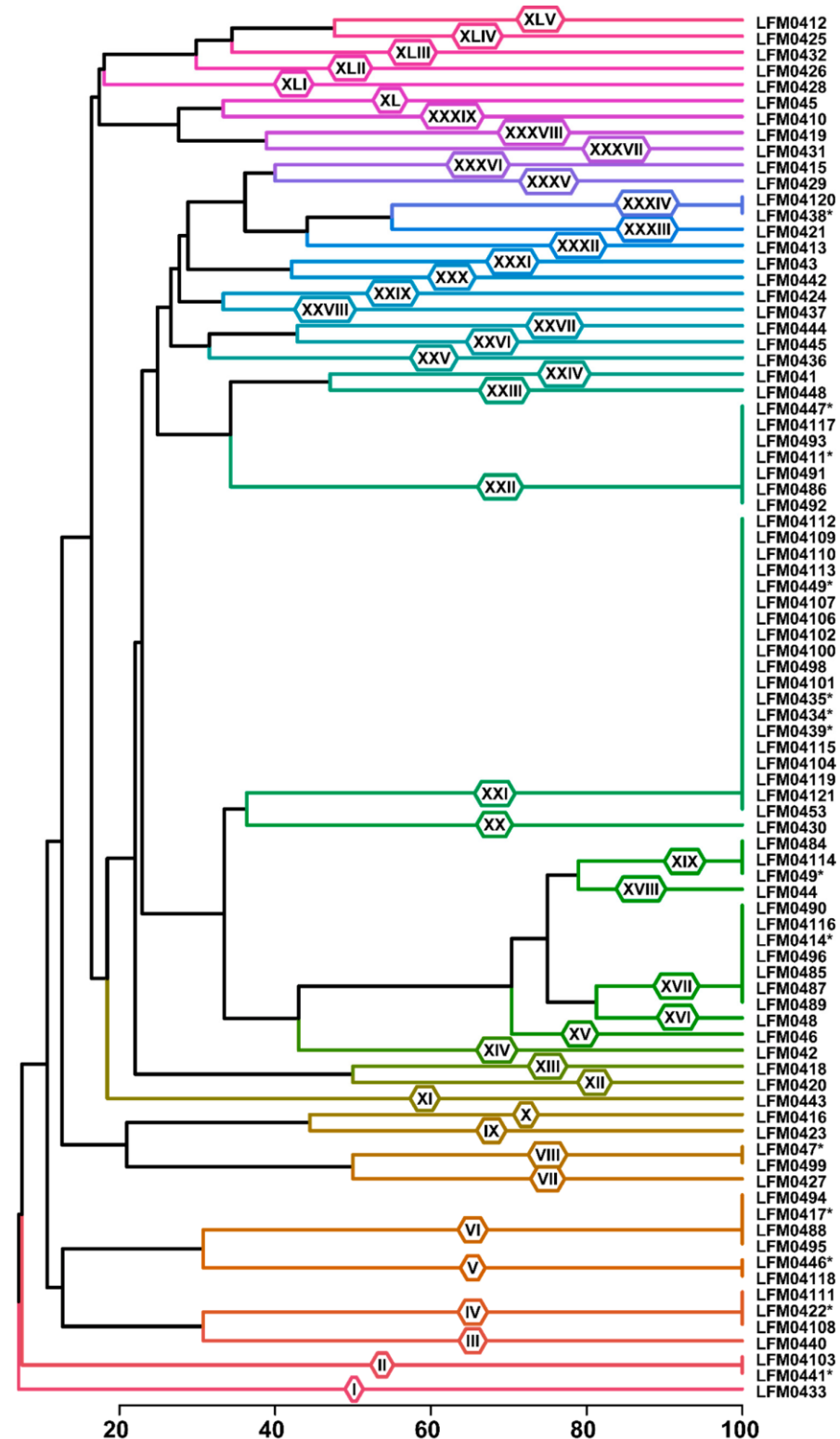


Figure 4. BOX-PCR dendrogram of *Fusarium* strains generated with Jaccard's distance in the un-weighted pair group method with arithmetic averages (UPGMA). The roman numerals on the branches represent the BOX-PCR groups. The scale represents the similarity in percentage and the BOX groups were defined on the basis of 100% similarity. Strains marked with asterisks were used in the pathogenicity tests in groups composed of more than one strain.

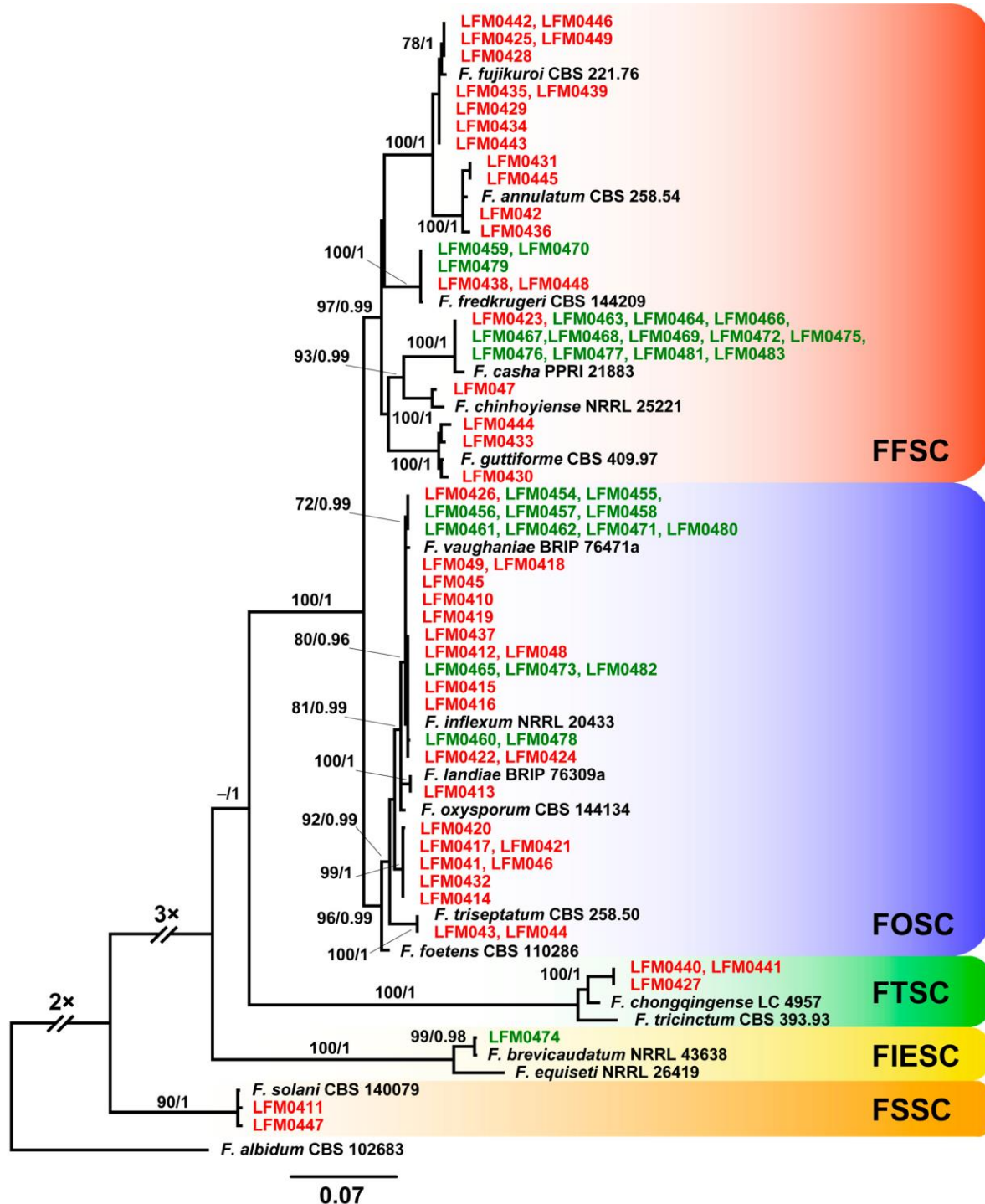


Figure 5. Phylogenetic tree of the *Fusarium* species inferred with the maximum likelihood (ML) and Bayesian inference (BI) methods with *tef1* sequences and the GTR + G model for both methods. The bootstrap analysis was performed with 1000 resamplings. The numbers on the nodes represent the bootstraps in ML analysis followed by the posterior probabilities (PP) in the Bayesian inference. Values lower than 70% in ML and lower than 0.95 PP in BI were not taken into consideration for branch support. Although the *tef1* gene of 79 strains was sequenced and all strains are shown in the phylogenetic tree, only one representative of identical sequences was deposited in the databases. Therefore, the evolutionary analysis involved a total of 65 distinct sequences with 673 bps of aligned nucleotides. Only sequences from type material available in the databases were used for comparison purposes and are listed in Table S1. Strains from healthy plantlets are indicated in green and the ones from diseased plantlets are in red. *Fusarium albidum* (syn. *Luteonectria albida*) was used as an outgroup and the species complexes are indicated with different colours. The scale indicates the number of substitutions per site.

Table 2. Identification and pathogenicity of *Fusarium* strains isolated from diseased roots of grapevine plantlets. The strains were selected based on their BOX groups, identification was performed by sequencing a fragment of the *tef1* gene, and pathogenicity was performed by the trunk inoculation method. Virulence was determined by quantifying the number of diseased inoculated plantlets according to a grading scale shown in Table 1. A total of 15 plantlets per treatment were evaluated 30 days after the inoculations. Identical accession numbers indicate that the strains had the same sequence, and in these cases only one representative sequence was deposited.

Strains	BOX-PCR Group	Closest Species (Type Material)	<i>tef1</i> - α (bp)	Accession Number	Identity (%)	Species Complex	Pathogenicity /Virulence ¹
LFM0433	I	<i>F. guttiforme</i>	650	PV081890	99.23	FFSC	+++
LFM0441	II	<i>F. chongqingense</i>	645	PV081913	97.51	FTSC	+
LFM0440	III	<i>F. chongqingense</i>	645	PV081913	97.51	FTSC	+
LFM0422	IV	<i>F. inflexum</i>	651	PV081876	99.68	FOSC	++
LFM0446	V	<i>F. fujikuroi</i>	648	PV081893	99.53	FFSC	++
LFM0417	VI	<i>F. foetens</i>	658	PV081883	97.88	FOSC	+++
LFM0427	VII	<i>F. chongqingense</i>	639	PV081914	97.48	FTSC	+
LFM047	VIII	<i>F. chinohyense</i>	656	PV081900	98.92	FFSC	-
LFM0423	IX	<i>F. casha</i>	630	PV081901	99.21	FFSC	++
LFM0416	X	<i>F. inflexum</i>	669	PV081877	100	FOSC	+++
LFM0443	XI	<i>F. fujikuroi</i>	639	PV081894	99.37	FFSC	+++
LFM0420	XII	<i>F. foetens</i>	668	PV081884	97.91	FOSC	++
LFM0418	XIII	<i>F. vaughaniae</i>	661	PV081870	99.54	FOSC	++
LFM042	XIV	<i>F. annulatum</i>	654	PV081904	99.54	FFSC	+
LFM046	XV	<i>F. foetens</i>	667	PV081885	98.20	FOSC	+++
LFM048	XVI	<i>F. inflexum</i>	671	PV081878	99.69	FOSC	+
LFM0414	XVII	<i>F. foetens</i>	673	PV081886	97.93	FOSC	+++
LFM044	XVIII	<i>F. triseptatum</i>	655	PV081888	99.51	FOSC	+++
LFM049	XIX	<i>F. vaughaniae</i>	661	PV081870	99.54	FOSC	++
LFM0430	XX	<i>F. guttiforme</i>	651	PV081891	99.23	FFSC	++
LFM0439	XXI	<i>F. fujikuroi</i>	656	PV081895	99.39	FFSC	++
LFM0434	XXI	<i>F. fujikuroi</i>	656	PV081896	99.39	FFSC	+++
LFM0435	XXI	<i>F. fujikuroi</i>	656	PV081895	99.39	FFSC	+++
LFM0449	XXI	<i>F. fujikuroi</i>	693	PV081897	99.54	FFSC	+++
LFM0411	XXII	<i>F. solani</i>	688	PV081912	99.41	FSSC	++
LFM0447	XXII	<i>F. solani</i>	706	PV081911	99.86	FSSC	++
LFM0448	XXIII	<i>F. fredkrugeri</i>	706	PV081908	98.67	FFSC	+++
LFM041	XXIV	<i>F. foetens</i>	667	PV081885	98.20	FOSC	++
LFM0436	XXV	<i>F. annulatum</i>	654	PV081905	98.93	FFSC	++
LFM0445	XXVI	<i>F. annulatum</i>	658	PV081906	99.09	FFSC	+
LFM0444	XXVII	<i>F. guttiforme</i>	653	PV081892	98.47	FFSC	+++
LFM0437	XXVIII	<i>F. inflexum</i>	651	PV081879	99.68	FOSC	++
LFM0424	XXIX	<i>F. inflexum</i>	651	PV081876	99.68	FOSC	++
LFM0442	XXX	<i>F. fujikuroi</i>	648	PV081893	99.53	FFSC	+++
LFM043	XXXI	<i>F. triseptatum</i>	655	PV081888	99.51	FOSC	++
LFM0413	XXXII	<i>F. landiae</i>	664	PV081889	100	FOSC	++
LFM0421	XXXIII	<i>F. foetens</i>	658	PV081883	97.88	FOSC	++
LFM0438	XXXIV	<i>F. fredkrugeri</i>	706	PV081908	98.67	FFSC	+
LFM0429	XXXV	<i>F. fujikuroi</i>	644	PV081898	99.38	FFSC	++
LFM0415	XXXVI	<i>F. inflexum</i>	653	PV081880	100	FOSC	+
LFM0431	XXXVII	<i>F. annulatum</i>	645	PV081907	99.07	FFSC	+
LFM0419	XXXVIII	<i>F. vaughaniae</i>	661	PV081871	99.69	FOSC	++
LFM0410	XXXIX	<i>F. vaughaniae</i>	639	PV081872	99.69	FOSC	+
LFM045	XL	<i>F. vaughaniae</i>	650	PV081873	99.69	FOSC	+++
LFM0428	XLI	<i>F. fujikuroi</i>	639	PV081899	99.53	FFSC	+
LFM0426	XLII	<i>F. vaughaniae</i>	660	PV081869	99.54	FOSC	+++
LFM0432	XLIII	<i>F. foetens</i>	655	PV081887	97.87	FOSC	+
LFM0425	XLIV	<i>F. fujikuroi</i>	693	PV081897	99.54	FFSC	+
LFM0412	XLV	<i>F. inflexum</i>	671	PV081878	99.69	FOSC	-

¹ Defined according to Table 1. FOSC = *Fusarium oxysporum* species complex, FTSC = *Fusarium tricinctum* species complex, and FSSC = *Fusarium solani* species complex.

The 30 strains isolated from healthy grapevine plantlets were not analysed by BOX- PCR and all of them were identified by sequencing a fragment of the *tef1* gene. These 30 strains were identified as five species in three species complexes, 15 strains in the FFSC, 14 strains in the FOSC and 1 strain in the FIESC (Figure 5).

The species *F. casha* and *F. fredkrugeri* in the FFSC and *F. vaughaniae* and *F. inflexum* in the FOSC occurred in both healthy and diseased plantlets, whereas *F. brevicaudatum* was only obtained from healthy plantlets (Figure 5). Some strains identified in the same species were classified in more than one BOX group, but there were no cases where strains in the same BOX group were identified in different species (Figures 4 and 5).

3.4. *Fusarium* from Diseased Plantlets Are Pathogenic to Grapevine

In this study, three methods were used to evaluate the pathogenicity and virulence of the *Fusarium* strains to grapevines. The trunk inoculation method was adopted for at least one strain per BOX group, whereas the inoculation at the base of rootless plantlets and on rooted plantlets were used for a limited number of strains (see below). From a total of 49 strains obtained from diseased plantlets and classified into 13 species in 4 species complexes, 12 species were able to cause trunk rot in grapevine plantlets (Table 2). These 12 pathogenic species are distributed in 43 BOX groups, from a total of 45 BOX groups obtained in the study (Table 2). There were no relationships between virulence and BOX group or phylogenetic identity of the strains (Table 2). Non-inoculated plantlets showed no trunk rot symptoms.

To reproduce the symptoms of root and trunk rot, the trunk, and rooted and unrooted plantlets were inoculated with randomly selected strains (Figure 6). The inoculation of rooted plantlets followed by storage at 4 °C for four months did not result in any disease symptoms. On the other hand, the inoculation of the trunk resulted in trunk rot by all strains at various degrees (Figure 6A). These nine strains were also re-isolated from the diseased plantlets and morphologically compared to the inoculated strains, completing Koch's postulates. The inoculation of the base of unrooted plantlets followed by 1 year of growth in the field and 3 months of storage at 4 °C resulted in root and trunk rot by the five most virulent strains (Figure 6B) as determined in the trunk inoculation experiment (Figure 6A). Rooting in the field nursery was not influenced by the inoculation of the different *Fusarium* strains. The disease was not observed after the removal of inoculated plantlets from the soil where they were cultivated for one year for rooting; therefore, cold storage was essential for disease development.

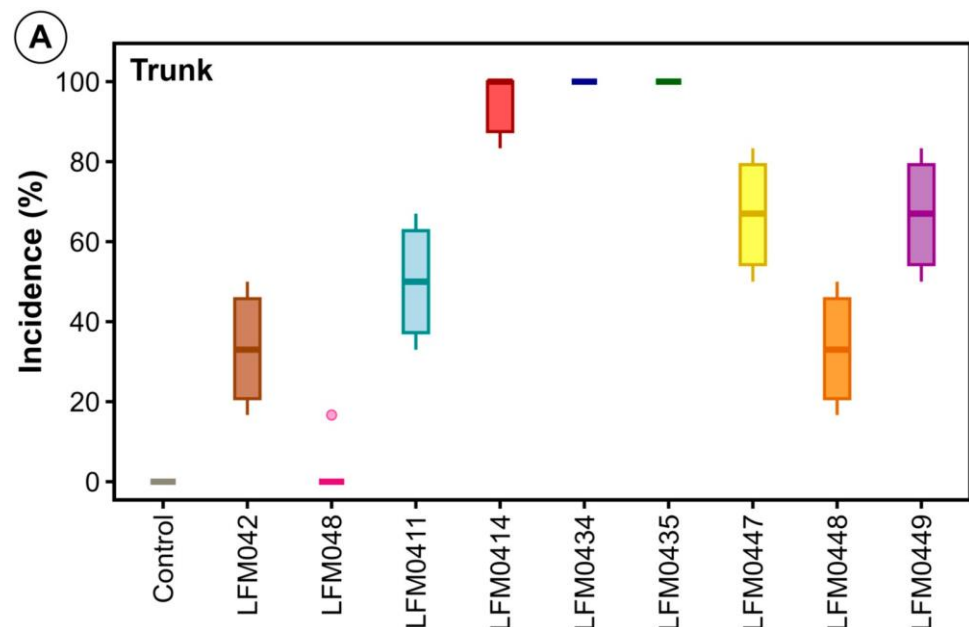


Figure 6. Cont.

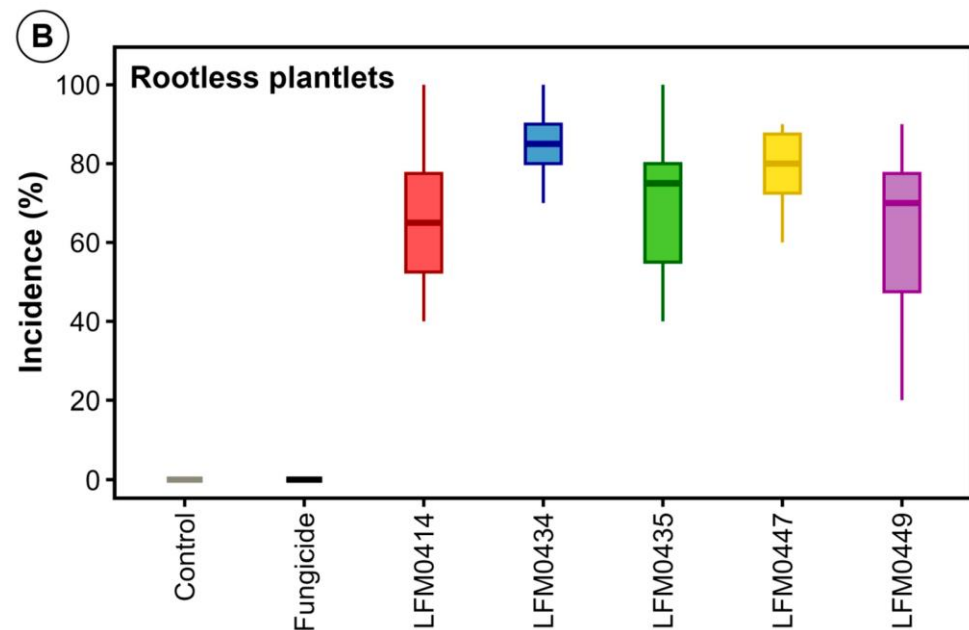


Figure 6. Trunk rot and root rot after cold storage in cv. Syrah grafted onto IAC766 rootstock. (A) Incidence of trunk rot. Plantlets were inoculated with a mycelial disc on the trunk wounded with a sterile cork borer and symptoms were evaluated 30 days later. (B) Incidence of root rot under cold storage. Rootless plantlets were inoculated with spore suspensions, grown in the field nursery for 12 months, removed from the soil, cleaned, and stored in a cold chamber for 3 months before evaluating the symptoms of root rot. The control treatments were non-inoculated plantlets and a fungicide treatment. Strain LFM0414 = *F. foetens*, LFM0434, LFM0435 and LFM0449 = *F. fujikuroi* and LFM0447 = *F. solani*. Data are averages of 100 plantlets per treatment.

There was a correspondence between the incidence of vascular trunk rot (Figure 6A) and root rot after cold storage (Figure 6B). The fungicide treatment of the unrooted plantlets was effective in inhibiting the pathogen and the non-inoculated negative control did not show disease symptoms (Figure 6).

3.5. Comparative Analysis of Healthy and Diseased Tissues

Further studies with scanning electron microscopy (SEM) and X-ray spectroscopy were used to understand the relationship between plant and pathogen. The SEM images showed that healthy plantlets had normal xylem vessels in roots (Figure 7A,B), whereas diseased plantlets had xylem vessels clogged with tyloses and gum (Figure 7C,D). *Fusarium* hyphae was observed in diseased tissues (Figure 7E,F), but not in healthy vascular tissues (Figure S1).

Energy-dispersive X-ray spectroscopy (EDS) analyses showed differences in the mineral composition of healthy and diseased plantlet roots. Healthy root tissues had lower levels of calcium (Ca), aluminium (Al), and magnesium (Mg), and had higher levels of potassium (K) when compared to diseased root tissues (Figure 8A,B). In healthy roots, Ca was more evenly distributed and part of it was concentrated in the epidermis (Figure 8C), whereas in diseased plantlets it was in the xylem vessels (Figure 8D). Silicon was not detected in healthy tissues but was uniformly distributed in diseased tissues (Figure 8E,F).

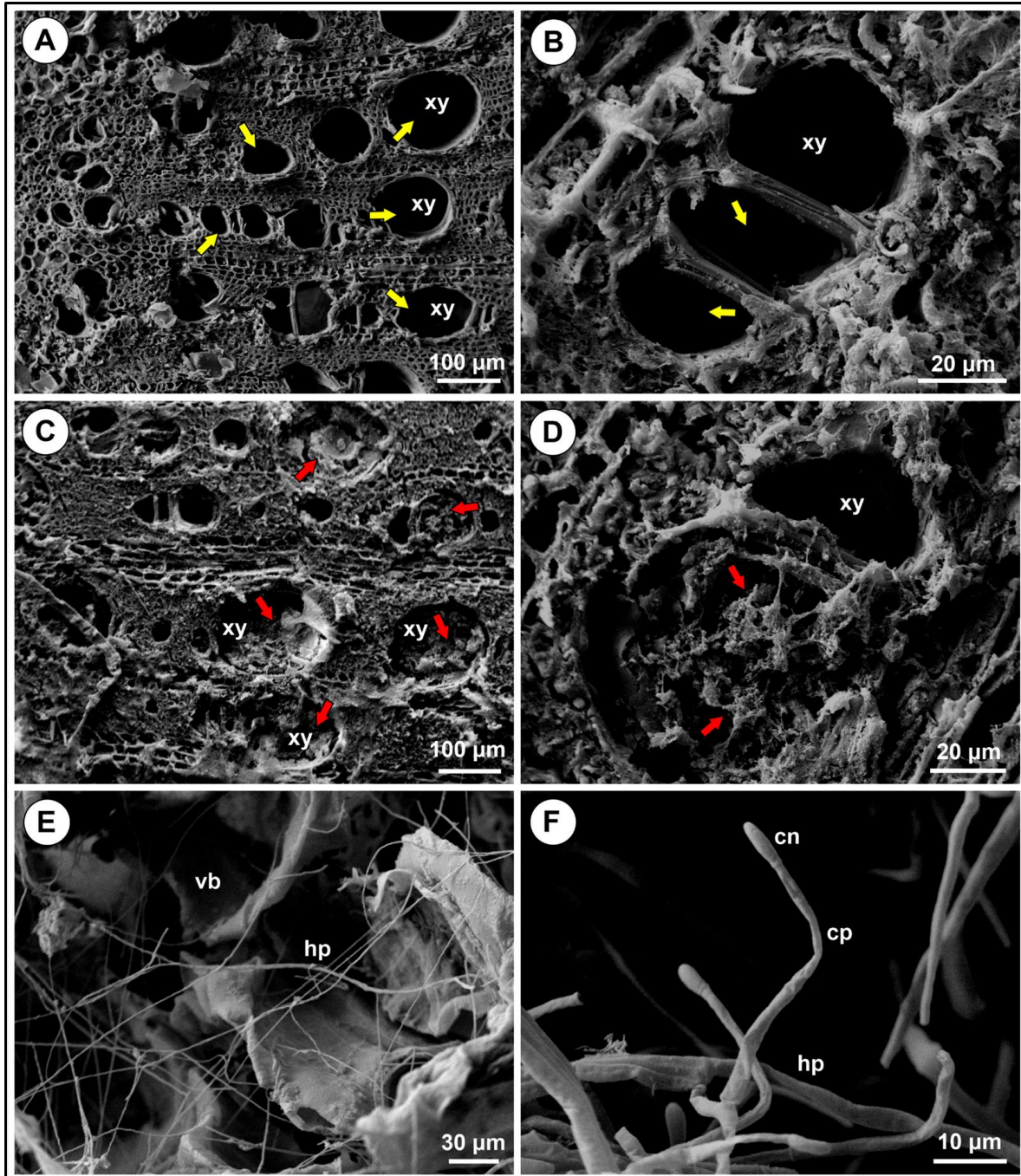


Figure 7. Scanning electron microscopy (SEM) images of xylem vessels of cv. Syrah grafted onto IAC766 rootstock. (A,B) Root xylem vessels of healthy plantlets showing the lumen of the vessels. (C,D) Root xylem of diseased plantlets. (E,F) Xylem vessels of diseased plantlets colonised by *Fusarium*. Abbreviations are xy = xylem, hp = hyphae, vb = vascular bundle, cn = conidium, and cp = conidiophore. Yellow arrows indicate the xylem in healthy tissues and red arrows show clogged xylem vessels in diseased plantlets. Scale bars are shown at the lower right corner of the figures.

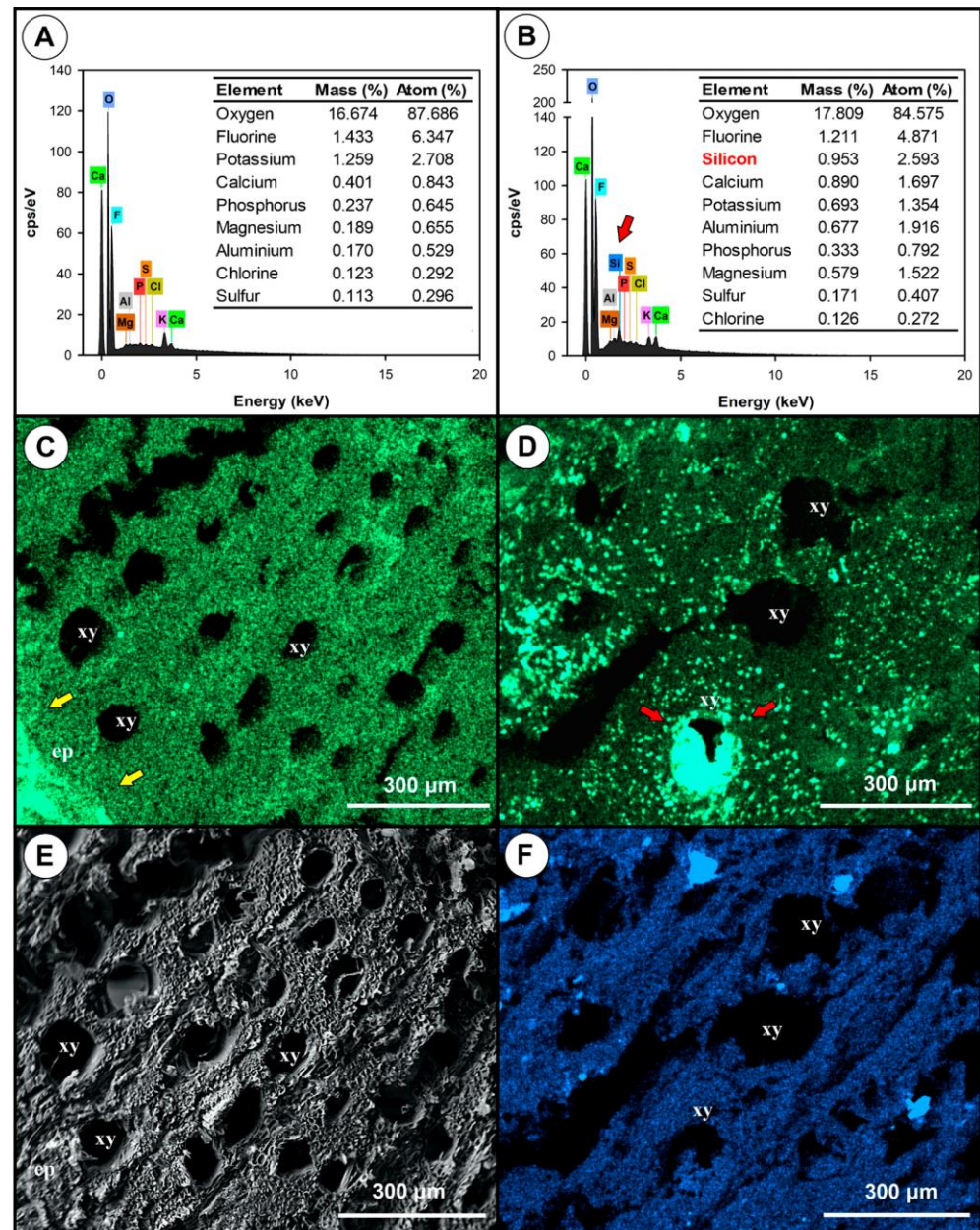


Figure 8. Energy-dispersive X-ray spectroscopy (EDS) of healthy and diseased root tissues of grapevine cv. Syrah grafted onto IAC766 rootstock. (A,B) EDS quantification of minerals in healthy and diseased tissues. (C,D) Distribution of Ca in healthy (C) and diseased (D) tissues; yellow arrows show concentration in the epidermis of healthy tissue and red arrows show concentration in the xylem. (E,F) Presence and distribution of Si in healthy and diseased tissue; lack of Si in healthy tissue (E) and Si evenly distributed in diseased tissue is indicated by the blue colour (F). Abbreviations are xy—xylem and ep—epidermis; scale bars are shown at the lower right corner of the microscopy figures.

3.6. *Fusarium* Colonises Roots of Different Species, but Does Not Show Symptoms

Fusarium strains obtained from diseased plantlets and previously shown to cause root and/or trunk rot (Table 2, Figure 6) were inoculated in grapevine roots and in tomato and common bean seedlings by root immersion to verify if they could colonise these plants and cause symptoms (Table 3). The results showed that all strains were re-isolated from the surface-sterilised roots of all plant species without causing any symptoms (Table 4). The strains were from six BOX groups, six species, and three species complexes, indicating that

this is a common behaviour in a diverse range of *Fusarium* species. The percentages of re-isolations were high for all strains in all plant species and the non-inoculated plantlets showed no contamination (Table 3).

Table 3. Colonisation and pathogenicity of *Fusarium* strains obtained from diseased grapevine plantlets. These strains were inoculated on surface-sterilised roots of grapevine plantlets and seedlings of common bean and tomato, and after a period of 7 days of incubation these roots were surface-sterilised again and plated on culture media to determine the percentage of re-isolation. Disease symptoms were analysed visually on the roots. Controls were non-inoculated plantlet roots. The absence of symptoms is indicated by the sign “–”.

Strains ¹	BOX-PCR Group	Closest Species (Type Material)	Grapevine		Tomato		Common Bean	
			Re-Isolation (%)	Symptoms	Re-Isolation (%)	Symptoms	Re-Isolation (%)	Symptoms
LFM042	XIV	<i>F. annulatum</i>	100.0	–	94.0	–	88.0	–
LFM048	XVI	<i>F. inflexum</i>	91.6	–	92.0	–	89.0	–
LFM0411	XXII	<i>F. solani</i>	75.0	–	95.0	–	97.0	–
LFM0414	XVII	<i>F. foetens</i>	91.6	–	93.0	–	94.0	–
LFM0434	XXI	<i>F. fujikuroi</i>	100.0	–	96.0	–	93.0	–
LFM0435	XXI	<i>F. fujikuroi</i>	100.0	–	100.0	–	91.0	–
LFM0447	XXII	<i>F. solani</i>	75.0	–	92.0	–	92.0	–
LFM0448	XXIII	<i>F. fredkrugeri</i>	91.6	–	96.0	–	87.0	–
LFM0449	XXI	<i>F. fujikuroi</i>	91.6	–	90.0	–	85.0	–
Control	–	–	0.0	–	0.0	–	0.0	–

Table 4. *Fusarium* strains isolated from different parts of healthy plantlets. The *tef1* gene was sequenced for all strains, and identical sequences as indicated by the same length in base pairs (bps) and by the same sequence identity (%) were deposited only once, and, therefore, received only one accession number. Pathogenicity and virulence were determined by the trunk inoculation method with 15 plantlets per treatment and evaluations performed 30 days after the inoculations by using the descriptive scale shown in Table 1.

Strains	Isolation Source	Species (Type Material)	<i>tef1</i> - α (bp)	Accession Number	Identity (%)	Species Complex	Pathogenicity /Virulence ¹
LFM0464	Trunk	<i>F. casha</i>	657	PV081902	99.39	FFSC	++
LFM0466	Trunk	<i>F. casha</i>	657		99.39	FFSC	+
LFM0468	Root	<i>F. casha</i>	657		99.39	FFSC	++
LFM0469	Trunk	<i>F. casha</i>	657		99.39	FFSC	+
LFM0472	Root	<i>F. casha</i>	657		99.39	FFSC	+++
LFM0475	Trunk	<i>F. casha</i>	657		99.39	FFSC	–
LFM0476	Root	<i>F. casha</i>	657		99.39	FFSC	++
LFM0477	Root	<i>F. casha</i>	657		99.39	FFSC	++
LFM0481	Trunk	<i>F. casha</i>	657		99.39	FFSC	+
LFM0483	Trunk	<i>F. casha</i>	657		99.39	FFSC	+
LFM0463	Root	<i>F. casha</i>	654	PV081903	99.38	FFSC	+++
LFM0467	Trunk	<i>F. casha</i>	654		99.38	FFSC	–
LFM0459	Trunk	<i>F. fredkrugeri</i>	658	PV081909	98.62	FFSC	+++
LFM0470	Trunk	<i>F. fredkrugeri</i>	658		98.62	FFSC	++
LFM0479	Trunk	<i>F. fredkrugeri</i>	655	PV081910	98.61	FFSC	++
LFM0460	Root	<i>F. inflexum</i>	581	PV081881	99.66	FOSC	++
LFM0478	Root	<i>F. inflexum</i>	581		99.66	FOSC	++
LFM0462	Root	<i>F. vaughaniae</i>	660	PV081874	99.54	FOSC	–
LFM0454	Root	<i>F. vaughaniae</i>	657		99.54	FOSC	–
LFM0455	Root	<i>F. vaughaniae</i>	657	PV081875	99.54	FOSC	++
LFM0456	Root	<i>F. vaughaniae</i>	657		99.54	FOSC	–
LFM0457	Root	<i>F. vaughaniae</i>	657		99.54	FOSC	+
LFM0458	Root	<i>F. vaughaniae</i>	657		99.54	FOSC	–
LFM0461	Root	<i>F. vaughaniae</i>	657		99.54	FOSC	++
LFM0471	Root	<i>F. vaughaniae</i>	657		99.54	FOSC	++
LFM0480	Root	<i>F. vaughaniae</i>	657		99.54	FOSC	++
LFM0465	Root	<i>F. inflexum</i>	613	PV081882	100	FOSC	+
LFM0473	Root	<i>F. inflexum</i>	613		100	FOSC	++
LFM0482	Root	<i>F. inflexum</i>	613		100	FOSC	+++
LFM0474	Trunk	<i>F. brevicaudatum</i>	651	PV081915	99.68	FIESC	–

¹ Defined according to Table 1. FFSC = *Fusarium fujikuroi* species complex, FOSC = *Fusarium oxysporum* species complex, and FIESC = *Fusarium incarnatum-equiseti* species complex.

3.7. *Fusarium* Strains from Healthy Plantlets Are Also Pathogenic to Grapevine

Strains of *Fusarium* obtained from the roots and trunks of healthy plantlets were inoculated in the trunk of grapevines to study their capacity to cause disease. None of

the strains were isolated from the leaves and petioles of healthy plantlets. Interestingly, all strains in the FOSC were from the roots, while strains in the FFSC were from the roots and from the trunks (Table 4). The strains from healthy plantlets were identified as five species in three species complexes (Table 4). The identity of the *tef1* fragment obtained for the strains from healthy plantlets varied from 98.6% to 100% when compared to the sequences of type strains deposited in public databases. The results showed that most strains were able to cause trunk rot in grapevines. *Fusarium* strains from the FFSC and FOSC were able to cause disease, whereas *F. brevicaudatum* in the FIESC was not pathogenic (Table 4). Strains from the same species varied in their pathogenicity and also in the amount of disease symptoms in grapevines (Table 4).

4. Discussion

In this study, we report a novel root and trunk rot disease of grapevine plantlets under cold storage caused by *Fusarium* species. This disease only shows symptoms when the plantlets are exposed to the cold after being cultivated with the pathogen for a whole year in the commercial process of producing grafted grapevine plantlets. This disease invariably leads to plant death within a period of 90 days after field transplantation. There were 14 different *Fusarium* species in four species complexes associated with healthy and diseased plantlets. Of these 14 species, 12 were able to cause disease and 9 are being reported for the first time as pathogenic to grapevines. *Fusarium* species obtained from both diseased and healthy plantlets were pathogenic to grapevines, indicating that healthy infected plantlets can carry the pathogens that may later be responsible for plant decline in the field.

The *Fusarium* genus is commonly associated with grapevine trunk disease (GTD) in several countries as it is part of the fungal complex that causes it, but this genus is not considered to be the main causative agent of GTD [21–23,42–46]. In our study, *Fusarium* was the only fungal genus considered in the pathogenicity assays due to the high populations found in healthy and diseased grapevine plantlets before transplantation to the field. Symptoms of root and trunk rot include a general loss of vigour and the vascular rot of the root and trunk of diseased plantlets. Despite the damage to the root system, sprouting occurred due to the accumulation of starch and sugar reserves in the plantlets [47,48]. Marked differences between these diseases are the rapid plant death (90 days) in root and trunk rot and the main role of *Fusarium*, whereas GTD has the involvement of a high number of pathogens, including *Fusarium* as a secondary pathogen, and it occurs in young orchards (<5 years) and develops slowly, allowing plants to grow and be productive for years before death [11,12,18,19,21,49]. Root and trunk rot under cold storage seems to be an overload of *Fusarium* on diseased plantlets that rapidly leads to plant death. Other pathogens than *Fusarium* were not detected in our isolations and further studies with deep sequencing (NGS) need to be performed to verify their association in plants with root and trunk rot symptoms. Moreover, it will be interesting to study whether plantlets infected with *Fusarium* without root and trunk rot symptoms may develop GTD after transplantation to the field.

Our pathogenicity tests were performed with three different methods, and only in the inoculation of unrooted plantlets followed by cultivation in the field nursery for one year and storage for 3–4 months at 4 °C the symptoms of root rot were observed. However, due to the length and cost of this process, we only tested five strains in three species classified in three species complexes. The inoculation of rootless plantlets method correlated well with the trunk inoculation method, which was used to test the pathogenicity of all *Fusarium* strains in this study. The trunk inoculation method was fast, reproducible, and easy to perform and became our standard pathogenicity test.

Nine *Fusarium* species, from a total of fourteen found in this study, were identified for the first time as pathogenic to grapevines, including *F. chongqingense* in the FTSC, *F. foetens*, *F. inflexum*, *F. landiae*, *F. vaughaniae*, *F. triseptatum*, and *F. inflexum* in the FOSC, and *F. fujikuroi*, *F. guttiforme*, and *F. casha* in the FFSC. The species *F. brevicaudatum* and *F. chinhyoiense* in the FIESC and FFSC, although not pathogenic in our assays, were also found for the first time in association with grapevines. These results demonstrated that the species associated with root rot and trunk disease in Minas Gerais state are diverse and distinct from the ones reported by other authors elsewhere [21–23,42–46]. Among the species reported in our study, the ones close to *F. foetens* and to *F. chongqingense* (Figure 5) may be novel species due to the relatively low levels of identity of their *tef1* gene as compared to type strains deposited in public databases. However, sequencing of other markers such as *rpb2* and calmodulin is required to corroborate this postulation.

The pathogenicity and virulence of the strains tested in our study varied considerably, even in strains from the same species. Some of these differences were detected in our BOX-PCR analysis (Figure 4), a fingerprinting technique that locates variations in the whole genome [28]. BOX-PCR is sensitive enough to detect differences among strains that are not detected when a single fragment of DNA, such as *tef1*, is analysed. Therefore, there was no relationship between our phylogenetic placement (Figure 5) and BOX-PCR groupings (Figure 4). For example, strains LFM0410, LMF0412, and LMF0426 of *F. vaughaniae*, classified in three different BOX groups, markedly differed in pathogenicity and virulence to grapevines (Table 2). These differences are probably due to virulence factors that may be located in accessory chromosomes [50–59]. Alternatively, the loss and gain of pathogenicity and virulence in *Fusarium* may be due to horizontal transfer of genetic material through conidial anastomosis tubes [60,61] and subculture in laboratory artificial culture media [62]. The occurrence of *Fusarium* in healthy grapevine plantlets was reported by other authors [22,23,63,64], but their pathogenicity has not been confirmed. In our study, four *Fusarium* species, *F. casha*, *F. fredkrugeri*, *F. inflexum*, and *F. vaughaniae*, were isolated from healthy and diseased plantlets and their pathogenicity to grapevines has been confirmed with the trunk inoculation method (Tables 2 and 4). These results show that *Fusarium*, like many other pathogens with a necrotrophic phase, may remain inside healthy tissues for long periods of time without showing any visible symptoms [21,65]. This endophytic colonisation occurs because the pathogen is unable to overcome the resistance of the plant and may remain incognito until the level of local resistance lowers [66]. Stresses such as the storage of plantlets with exposed roots to cold temperatures for 3–4 months are thought to be responsible for lowering the levels of resistance and the consequent manifestation of grapevine root and trunk rot symptoms. Experiments to confirm this hypothesis would involve the quantification of the resistance and colonisation levels, either by qPCR or enzymatic determinations [67].

Root and trunk rot of grapevine plantlets has similarities with the canker disease of cold-stored fruit and tree nuts [68], where plantlets were exposed to 5 to 10 °C for approximately 2 months. However, the *Fusarium* species inciting the disease was among the differences observed in our study. Despite the fact that old nomenclature still adopts *formae speciales* for some *Fusarium* species [69], there is increasing evidence that most species in this genus present very little host specificity and high levels of opportunism [70]. An example from our own results is *F. guttiforme*, which was found to be pathogenic to grapevine, but is only known for its association with pineapple fruit rot [71]. The high amount of *Fusarium* species associated with grapevine diseases was reported by other authors [21–23,42–46], and because of the broad host range of *Fusarium* species in general it appears that at least in some instances the local diversity of species influences the aetiology of any given disease incited by this genus. For example, grapevine trunk disease was

reported to be caused by different *Fusarium* species according to the geographical region it is reported [42,44,46]. Our attempts to inoculate grapevines and other plant species with *Fusarium* spp. from diseased plantlets did not lead to root rot symptoms, instead, they resulted in extensive endophytic colonisation (Table 3). Further studies will be pursued by our research group to verify if different stresses would induce root rot symptoms in these plant species.

The rootstock IAC766 used in our pathogenicity tests is reported to possess a certain level of resistance against *Fusarium* spp. [72]. In fact, we observed the production of tyloses and gum in the xylem of diseased plantlets, translocation of Ca, and accumulation of Si (Figures 7 and 8). These are typical manifestations of resistance [73–81] that were overcome by *Fusarium* under cold storage. An important requirement for disease development is the level of colonisation of the internal tissues by *Fusarium*, as suggested by [64,82]. In our study, *Fusarium* probably reached high levels of colonisation only when the inoculations were performed on the base of rootless plantlets. This occurred due to the approximately one year of plant growth in the field with the pathogen. By contrast, in inoculations performed on rooted plantlets, the pathogen had not enough time to grow together with the roots and reach high populations. This would explain why the disease only developed in inoculations performed in unrooted plantlets. Other factors that probably influence disease development are the composition and diversity of the internal and external microbiomes of the plantlets, their nutritional status and consequently their general resistance level, and the time in cold storage. Diverse and resilient microbiomes in well-nourished plantlets will restrict colonisation and favour resistance reactions against pathogens in general [83,84]. Further studies are needed to understand the influence of *Fusarium* colonisation levels, plant resistance, and the associated microbiomes in disease development. The presence of beneficial fungi, such as *Trichoderma*, *Epicoccum*, and *Chaetomium*, in addition to pathogens in our isolations (Figure 3) indicates that changing the population balance toward increased numbers of beneficial microorganisms is a promising strategy to manage this disease. Observations performed by one of us (de Souza, C.R., personal observations) indicated that shorter periods under cold storage led to a decreased incidence of root and trunk disease in susceptible material, which is currently around 5 to 10% in the last 5 years. The treatment with the fungicide, although effective in disease control, comes with all the disadvantages of these molecules, including environmental, human, and animal health hazards, and the risk of selecting resistant pathogen populations [85].

In conclusion, *Fusarium* species are present in both healthy and diseased plantlets, but the symptoms of the root and trunk rot of grapevines only appear under certain conditions, including cold storage and possibly a minimum threshold level of root colonisation. This study adds information on the grapevine root rot and trunk disease, including symptoms, diversity of the pathogen, identity and mode of action of the causative agents, disease development, and plant resistance reactions. This information will be important to develop control strategies against this disease.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/jof11030230/s1>, Table S1: Accession numbers of *tef1* from type strains of *Fusarium* species deposited in public databases and used in the phylogenetic analysis; Figure S1: Scanning Electron Microscopy (SEM) image of healthy root vascular tissues of cv. Syrah grafted onto IAC766 rootstock. Xylem vessels without obstruction are shown.

Author Contributions: Conceptualization, J.M.F.d.L.C. and J.T.d.S.; methodology, J.M.F.d.L.C., C.R.d.S. and J.T.d.S.; software, J.M.F.d.L.C.; validation, J.M.F.d.L.C. and B.C.L.A.; formal analysis, J.M.F.d.L.C., O.R.d.F., B.C.L.A., A.V.R. and J.T.d.S.; investigation, J.M.F.d.L.C., O.R.d.F., B.C.L.A., A.V.R. and J.T.d.S.; resources, C.R.d.S. and J.T.d.S.; data curation, J.M.F.d.L.C.; writing—original draft preparation, J.M.F.d.L.C. and J.T.d.S.; writing—review and editing, J.M.F.d.L.C., O.R.d.F., B.C.L.A.,

A.V.R. and J.T.d.S.; visualisation, J.M.F.d.L.C., O.R.d.F., B.C.L.A., A.V.R. and J.T.d.S.; supervision, J.T.d.S. and C.R.d.S.; project administration, J.T.d.S. and C.R.d.S.; funding acquisition, J.T.d.S. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Minas Gerais State Research Support Foundation (FAPEMIG) through project APQ-00949-21.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The original contributions presented in the study are included in the article, further inquiries can be directed to the corresponding author.

Acknowledgments: We thank the technicians of the Agricultural Research Company of Minas Gerais (EPAMIG) for propagating the plant material, providing space, and sharing their knowledge throughout these four years of research, and to Eduardo Alves for his assistance with the electron microscopy analyses. J.T.d.S. thanks CNPq for providing a productivity scholarship and J.M.F.d.L.C. acknowledges the PhD scholarship received from FAPEMIG.

Conflicts of Interest: The authors declare no conflicts of interest.

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Table S1. Accession numbers of *tef1* from type strains of *Fusarium* species deposited in public databases and used in the phylogenetic analysis.

Species	Type strain	Accession number (<i>tef1</i>)
<i>F. fujikuroi</i>	CBS 221.76	MN534010.1
<i>F. annulatum</i>	CBS 258.54	MT010994.1
<i>F. fredkrugeri</i>	CBS 144209	LT996097.1
<i>F. casha</i>	PPRI 21883	MF787261.1
<i>F. chinhoiense</i>	NRRL 25221	MN534050.1
<i>F. guttiforme</i>	CBS 409.97	MT010999.1
<i>F. vaughaniae</i>	BRIP 76471a	PQ393368.1
<i>F. inflexum</i>	NRRL 20433	AF008479.1
<i>F. landiae</i>	BRIP 76309a	PQ393366.1
<i>F. oxysporum</i>	CBS 144134	MH485044.1
<i>F. triseptatum</i>	CBS 258.50	MH484964.1
<i>F. foetens</i>	CBS 110286	MT011001.1
<i>F. chongqingense</i>	LC 4957	MW620138.1
<i>F. tricinctum</i>	CBS 393.93	AB674263.1
<i>F. brevicaudatum</i>	NRRL 43638	GQ505665.1
<i>F. equiseti</i>	NRRL 26419	GQ505599.1
<i>F. solani</i>	CBS 140079	KT313611.1
<i>F. albidum</i>	CBS 102683	MW834283.1

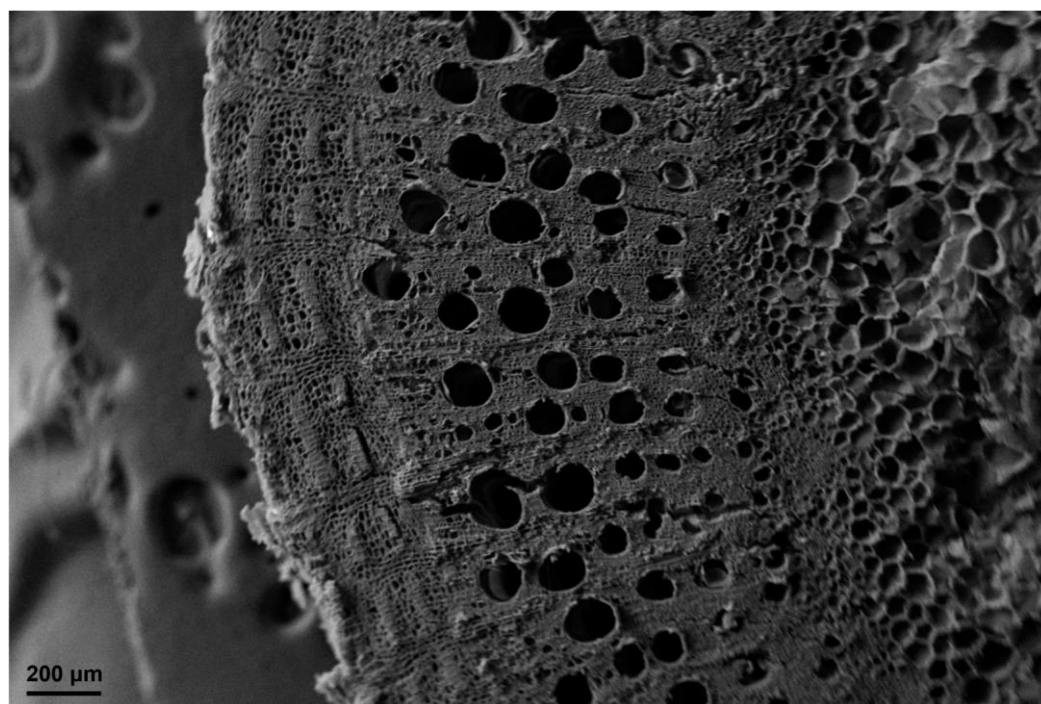


Figure S1. Scanning Electron Microscopy (SEM) image of healthy root vascular tissues of cv. Syrah grafted on IAC766 rootstock. Xylem vessels without obstruction are shown.

Article 2 – Manuscript prepared for submission to Fungal Biology (Not Published)	
Article title:	<i>Trichoderma harenae</i> sp. nov. with biotechnological potential in grapevine
Authors:	José Manoel Ferreira de Lima Cruz Luciana Cordeiro do Nascimento Otilia Ricardo de Farias Patrick Materatski Carla Marisa Reis Varanda Cláudia Rita de Souza Jorge Teodoro de Souza
Journal:	Fungal Biology
ISSN	1878-6162

***Trichoderma harenae* sp. nov. with biotechnological potential in grapevine**

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Abstract

The low rooting rate of grafted grapevine cuttings represents a significant challenge in viticulture, driving the search for *Trichoderma* strains with biostimulant activity as a promising alternative to enhance root development and seedling quality. In this study, we conducted a survey of *Trichoderma* isolates from forest soils within the Atlantic Forest biome, located in a protected conservation area. Phylogenetic analyzes based on the *tefl* and *rpb2* genes enabled the identification of 33 isolates belonging to six distinct species (*T. breve*, *T. spirale*, *T. koningiopsis*, *T. camerunense*, *T. zelobreve*, and *T. afarasin*), distributed across three major clades (*Viride*, *Spirale*, and *Harzianum*). A novel species was formally described based on additional molecular evidence and comparative morphological features with its sister species, *T. koningiopsis*. Among the isolates evaluated, LFM004 exhibited notable biostimulant activity, with high calcium phosphate solubilization capacity (20.32 µg/mL) and indole-3-acetic acid production (6.54 µg/mL). When applied to grapevine seedlings, this strain promoted rooting, increased shoot emergence, and enhanced root biomass accumulation. The average improvements in these traits were approximately 2-fold and 1-fold higher compared to water and commercial hormone treatments, respectively. These findings highlight the biotechnological potential of the newly described species, for which we proposed the name *T. harenae* sp. nov.

1. Introduction

Grapevine (*Vitis vinifera* L.) is one of the oldest and most widely cultivated fruit crops in the world, grown for both table grapes and wine production, and stands out for its economic, social, and cultural importance (Terral et al., 2010; McGovern et al., 2017; Margaryan et al., 2021). The propagation through cuttings and grafting is widely used, especially in commercial plantlet production (Regina, 2002; Mudge et al., 2009). This method of multiplication allows for the preservation of the mother plant's genetic traits, ensuring uniformity and early production. Moreover, asexual propagation enables the combination of scions and rootstocks with desirable agronomic traits, such as resistance to pests, diseases, and abiotic stresses, vigor, and adaptability to

different soil types, thereby optimizing vineyard management and yield (Habibi et al., 2022; Tedesco et al., 2022; Yang et al., 2022). However, viticultural success is often hindered by challenges in propagation (Carbone et al., 2022; Cruz et al., 2025). One of the main obstacles is the low rooting rate of grafted cuttings (Goldschmidt, 2014; Assunção et al., 2019; Wurz et al., 2022; Boeno and Zuffellato-Ribas, 2023), which leads to significant economic losses. These are typically associated with factors such as cutting vigor, substrate quality, hormonal balance, pest and disease incidence, and the limited availability of essential nutrients during the early stages of plant development (Pearse, 1943; Gramaje and Armengol, 2011; Vršič et al., 2015; Waite et al., 2015; Boeno and Zuffellato-Ribas, 2023; Chen et al., 2024). Considering these physiological and sanitary challenges associated with grapevine propagation, the use of beneficial microorganisms, such as species of the genus *Trichoderma*, has emerged as a promising and sustainable alternative (Grohs et al., 2017; Csótó et al., 2022; Zañfano et al., 2024).

Trichoderma is a filamentous fungus widely distributed in terrestrial ecosystems, particularly in the rhizosphere, and is recognized for its dual role as a biocontrol agent against phytopathogens and as a plant growth promoter (Woo et al., 2023). Numerous studies have highlighted the ability of *Trichoderma* strains to produce phytohormones such as indole-3-acetic acid (IAA), solubilize inorganic phosphorus, mobilize essential nutrients, and stimulate root morphogenesis through the activation of endogenous hormonal pathways (Kapri and Tewari, 2010; Pelagio-Flores et al., 2017; Bader et al., 2020; Chen et al., 2024). These traits are particularly advantageous during the rooting stage of grafted cuttings—a critical phase in propagation—where robust root development is essential for successful plantlets establishment. Furthermore, *Trichoderma* is known for its rapid colonization of the root system, offering plant protection against soil-borne pathogens through mechanisms such as competition for nutrients and space (Oszust et al., 2020), antibiosis (Li et al., 2019; Jiménez-Bremont et al., 2024), mycoparasitism (Mukherjee et al., 2022), and systemic resistance induction (Nawrocka and Małolepsza, 2013; Sawant et al., 2020). The implementation of soil management strategies that promote the conservation and restoration of soil health in vineyards has been a consistent focus of recent research (Jindo et al., 2022; Visconti, López, and Olego, 2024; Liu et al., 2024). In addition, the identification and development of new *Trichoderma* strains with a broad spectrum of activity—particularly those with proven plant biostimulant capabilities—represent a promising strategy to overcome the challenges associated with root development in commercial nurseries. In this context, selecting strains in underexplored natural environments, such as conservation areas and diverse ecosystems, becomes essential for expanding the biotechnological potential of the genus, contributing not only to the reduction of synthetic inputs but also to the advancement of more sustainable viticultural practices (Fiorentino et al., 2018; Rush et al., 2021; Bononi et al., 2020).

During a bioprospecting survey of *Trichoderma* isolates from forest soils in northeastern Brazil, we reported the occurrence of six species distributed across different clades and described a novel species associated with Atlantic Forest biome. We also demonstrated the biotechnological potential of this new species in promoting grapevine rooting, paving the way toward a more sustainable viticulture.

2. Materials and Methods

2.1. Sample collection and isolation

Isolates of *Trichoderma* were obtained from five composite soil samples collected in the Mata do Pau-Ferro State Park, an ecological reserve and conservation unit of the Atlantic Forest biome, located in the city of Areia, Paraíba, Brazil (6° 59' 28" S, 35° 45' 4" W). Each composite sample consisted of ten subsamples of 50 g of leaf litter-covered soil, collected at a depth of 0–20 cm, placed in sterilized polyethylene bags, and stored at $\pm 4^{\circ}\text{C}$ for seven days. The geographic coordinates of the sampling sites were georeferenced using a Garmin GPSmap 60CSx device and QGIS Geographic Information System v. 3.16.0 (Fig. 1).

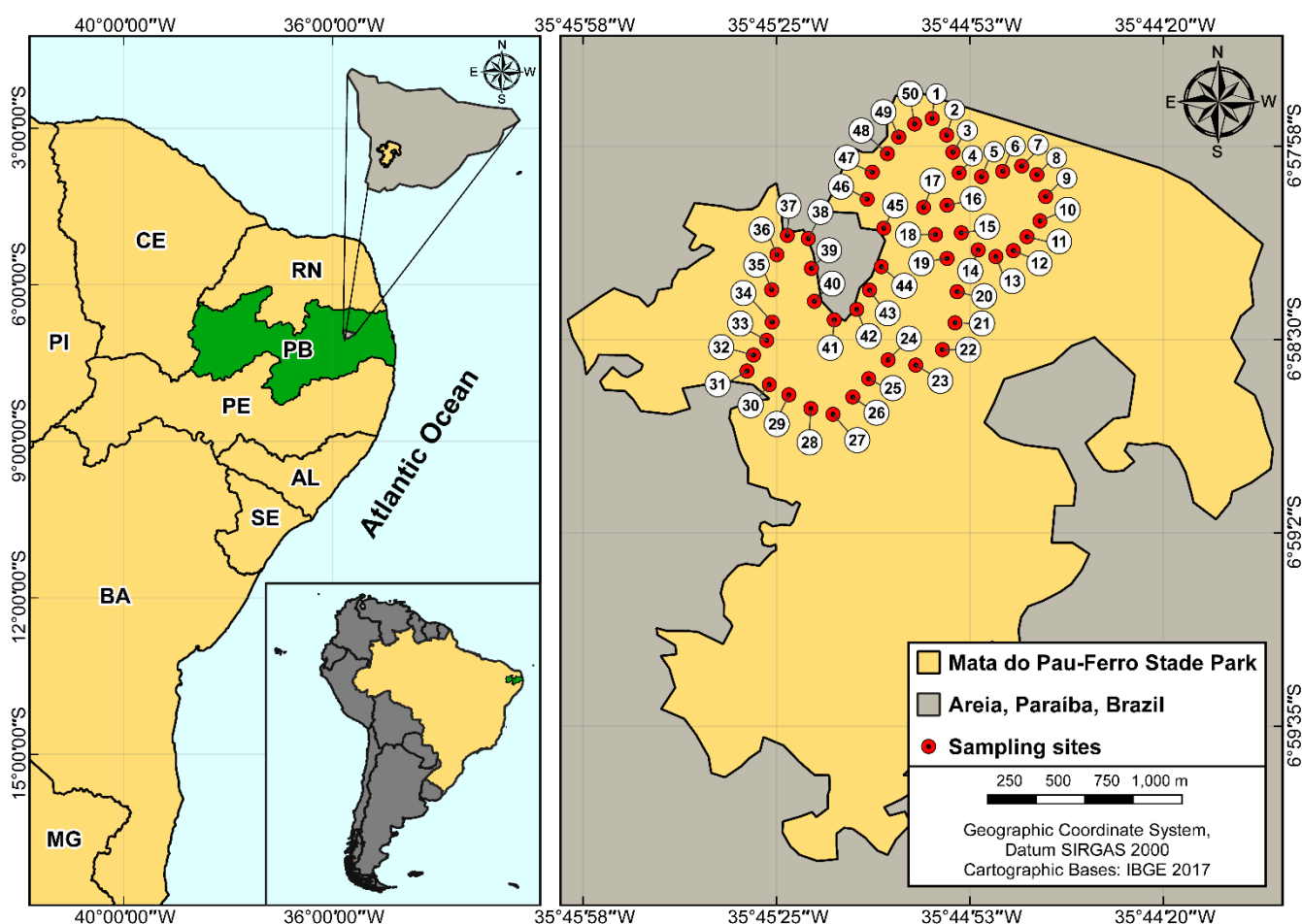


Fig. 1. Sampling location in Mata do Pau-Ferro State Park, a conservation unit of the Atlantic Forest biome located in the municipality of Areia, Paraíba, Brazil. On the left, Paraíba (in green) is highlighted within the Northeast region of Brazil and the South American continent. On the right, red dots represent the 50 georeferenced soil sampling sites within the park area used for the isolation of *Trichoderma*. The geographic coordinates of the sampling sites were recorded using a Garmin GPSmap 60CSx device and processed using QGIS software v. 3.16.0.

Samples of 10g of soil suspended in 90 mL of sterilized distilled water were shaken on an orbital shaker at 180g for 30 min, and subjected to serial dilutions ranging from 10^{-2} to 10^{-4} . An aliquot of 1000 μL was spread using a Drigalski spatula onto Petri dishes ($\varnothing = 9\text{ cm}$) containing a selective medium for soil fungi isolation

(Martin, 1950), adjusted to pH 5.5, and incubated at $25 \pm 2^\circ\text{C}$ for seven days under a 12-hour photoperiod. Colonies with reproductive structures typical of *Trichoderma* were purified and preserved in sterile distilled water (Castellani, 1939) at $\pm 5^\circ\text{C}$ and in 40% glycerol at -80°C in the ‘Phytopathogenic Fungi Collection’ of the Plant Pathology Laboratory at the Federal University of Paraiba. The type strains of the newly described species were deposited in the collection of the Molecular Plant Pathology Laboratory at the Federal University of Lavras.

2.2. DNA extraction, PCR amplification and sequencing

Total genomic DNA from *Trichoderma* strains was extracted using a pre-wash step with Sorbitol, followed by extraction with high-salt CTAB buffer (Inglis et al., 2018). The purity of the extracted DNA was estimated, and its concentration was quantified using a spectrophotometer (Nanodrop Lite UV-Vis; Thermo Fisher Scientific, San Francisco, CA, USA). The DNA was then stored at -20°C for further analyses. *Trichoderma* strains were subjected to amplification and sequencing a fragment of the *tefl* (translation elongation factor 1- α), *rpb2* (RNA polymerase II second largest protein subunit), and *cal* (calmodulin) genes. Polymerase chain reaction (PCR) amplification of the gene fragments was carried out at the University of Évora using the primers *tef71F*, *tef1080R*, *RPB2_210up*, *RPB2_1450low*, *CAL-228F*, and *CAL-737R*, following the recommendations described in Table 1S. The amplified fragments were purified and sequenced by Macrogen Inc. (Seoul, South Korea).

2.3. Phylogenetic analyses and species delimitation

Sequence quality was assessed, and consensus sequences were generated through assembly and editing using Sequencher v.5.4.6 (Gene Codes Corporation, Ann Arbor, MI, USA). These sequences were then compared with the closest related type material sequences (Table 1) available in public databases (Benson et al., 2013) using the BLASTn algorithm (Altschul et al., 1990). Alignments were conducted using MAFFT (multiple alignment using fast Fourier transform) v. 7 (Katoh and Standley, 2013; Katoh et al., 2019) and refined using GBlocks v. 0.91b (Castresana, 2000), eliminating low-quality regions and preserving only the conserved blocks. The best evolutionary models were selected with the program jModelTest2 v. 2.1.6 (Darriba et al., 2012), using the Akaike Information Criterion (AIC). The phylogenetic analyses were performed in the CIPRES Science Gateway (Miller et al., 2010), and evolutionary relationships were inferred through Maximum Likelihood (ML) and Bayesian Inference (BI). ML analysis was performed with 1000 bootstrap replications implemented in RAxML-HPC v. 8.2.12 (Stamatakis, 2014). BI analysis was conducted with four simultaneous chains in two independent runs for 50000000 generations, sampling every 1000 generations. Phylogenetic trees were summarized, and posterior probabilities (PP) were estimated using Markov Chain Monte Carlo (MCMC) methods after discarding the first 25% of generations as “burn-in”, using MrBayes v.3.2.7a (Ronquist et al., 2012). Final phylogenetic trees were visualized using FigTree v. 1.4.4 (Rambaut, 2018) and edited in Adobe Illustrator 2025 v. 29.3.

Species delimitation was carried out using three complementary approaches: ASAP (Assemble Species by Automatic Partitioning), ABGD (Automatic Barcode Gap Discovery), and BPP (Bayesian Phylogenetics and Phylogeography). ASAP Puillandre (et al., 2021) and ABGD (Puillandre et al., 2012) analyses were conducted using the online platform (<https://spartexplorer.mnhn.fr/delimitation>), based on genetic distances calculated under the Kimura 2-parameter model (K80), with input file containing aligned single-gene sequences. The ASAP method was run using default parameters, whereas in ABGD, the model criteria were defined as follows: default prior intraspecific divergence values ($P_{min} = 0.001$, $P_{max} = 0.1$), a relative gap width (X) of 0.5, and 50 screening steps. For BPP analyses, multilocus sequence data (*tef1* and *rpb2*) were used. The analysis was run under the A11 model (A11: species delimitation = 1, and species tree = 1) in BPP v.4.8.4 (Yang, 2015).

Table 1

Name, strain number, origin, and accession numbers of *tef1*, *rpb2*, and *cal* from type strains of *Trichoderma* species deposited in public databases and used in the phylogenetic analysis. Species

Species	Strain	Origin	Accession number		
			<i>tef1</i>	<i>rpb2</i>	<i>cal</i>
<i>Trichoderma ochroleucum</i>	CBS 119502	United Kingdom	FJ860659.1	FJ860556.1	–
<i>T. albofulvopsis</i>	HMAS 273760	China	KU529127.1	KU529138.1	–
<i>T. intricatum</i>	GJS 97.88	Thailand	AY376060.1	–	DQ367680.1
<i>T. caribbaeum</i>	GJS 97.3	France	DQ284977.1	KJ665246.1	DQ367688.1
<i>T. istrianum</i>	S 310	Croatia	KJ665523.1	KJ665281.1	–
<i>T. dorotheae</i>	GJS 99.202	New Zealand	DQ307536.1	EU248602.1	DQ367706.1
<i>T. tibetica</i>	YMF 1.05583	China	MK779179.1	MK779178.1	–
<i>T. taiwanense</i>	GJS 95.93	Taiwan	DQ284973.1	–	–
<i>T. koningii</i>	CBS 457.96	Netherlands	AF456909.1	–	DQ367686.1
<i>T. texanum</i>	LESF 551	USA	KT278988.1	KT278920.1	–
<i>T. ovalisporum</i>	Dis 70a	Ecuador	KJ871200.1	FJ442742.1	DQ379005.1
<i>T. harenae</i> sp. nov.	LFM001	Brazil	not submitted	not submitted	not submitted
<i>T. harenae</i> sp. nov.	LFM003	Brazil	not submitted	not submitted	not submitted
<i>T. harenae</i> sp. nov.	LFM004	Brazil	not submitted	not submitted	not submitted
<i>T. koningiopsis</i>	GJS 93.20	Cuba	DQ284966.1	EU241506.1	–
<i>T. merleae</i>	MST-FP 3586	Australia	PQ572712.1	PQ572711.1	–
<i>T. canadense</i>	SuRDC 1422	Canada	MZ189377.1	–	–
<i>T. arenarium</i>	TUCIM 10301	China	MT242303.1	MT242310.1	–
<i>T. amoenum</i>	YMF 1.06209	China	MT070146.1	MT052192.1	–
<i>T. dorotheopsis</i>	HZA5	China	MK850827.1	MH647795.1	–
<i>T. albofulvum</i>	GJS 01.265	Thailand	–	KR094870.1	–
<i>T. vinosum</i>	GJS 99.158	New Zealand	AY376047.1	KC285779.1	DQ122158.1

<i>T. viridarium</i>	S 136	Italy	KC285658.1	KC285760.1	DQ122157.1
<i>T. atroviride</i>	CBS 142.95	Slovenia	AF456891.1	EU341801.1	DQ122166.1
<i>T. martiale</i>	GJS 04.40	Brazil	EU248618.1	EU248597.1	FJ442425.1
<i>T. austrokonigii</i>	GJS 99.146	Australia	DQ307561.1	KJ842161.1	DQ379001.1
<i>T. strigosum</i>	DAOM 166121	USA	EU280019.1	AF545556.1	JN133544.1
<i>T. erinaceum</i>	DAOM 230019	Thailand	AY750880.1	EU248603.1	DQ122163.1
<i>T. spirale</i>	DAOM 183974	Thailand	EU280049.1	AF545553.1	FJ442395.1

Note: All *Trichoderma* strains are type materials, and the strains of the species described during this study are indicated in bold.

2.4. Phenotypic characterization

Phenotypic analysis of *Trichoderma* strains was carried out following the methodologies detailed by Sales et al. (2020), with all assays performed in triplicate for each strain and repeated five times. The strains were cultivated on PDA (potato dextrose agar), CMD (cornmeal dextrose agar), and SNA (synthetic nutrient agar) media at five different temperatures (15, 20, 25, 30, and 35°C) for 7–14 days, under alternating cycles of 12 h of cool white fluorescent light and 12 h of darkness (Samuels et al., 2002; Chaverri et al., 2003). Growth rate was assessed after 72 h of incubation. Macroscopic characteristics, including the onset of conidiation, pustule formation, colony coloration (Kornerup and Wanscher, 1978), aerial mycelium development, growth pattern, exudate production, and soluble pigment release, were examined using a Nikon SNZ1500 stereomicroscope. For micromorphological analysis, the length and width of phialides, conidia, conidiophores, chlamydospores, and pustules were measured. Samples were stained with 3% potassium hydroxide (KOH) and examined under a Carl Zeiss Axio Observer Z1 epifluorescence microscope, equipped with a Carl Zeiss AxioCam 105 color camera, as well as by scanning electron microscopy (SEM), following the protocol described by Alves et al. (2013).

2.5. Phosphate solubilization and indole-3-acetic acid production assays

To assess the biotechnological potential of isolate LFM004, *in vitro* assays were conducted to evaluate their ability to solubilize inorganic phosphate and produce indole-3-acetic acid (IAA). For both assays, fungal isolates were initially cultured on PDA plates at $25 \pm 2^\circ\text{C}$ under a 12-hour photoperiod for eight days. For IAA production, mycelial plugs ($\varnothing = 5$ mm) were transferred from the actively growing colonies to 250 mL Erlenmeyer flasks containing 50 mL of BD broth supplemented with 100 mg L^{-1} of L-tryptophan. Cultures were incubated on an orbital shaker at 150g and $25 \pm 2^\circ\text{C}$ for eight days. After incubation, mycelial biomass was separated by centrifugation at 12000g for 15 minutes. IAA presence was detected qualitatively by the appearance of a pink color following reaction with Salkowski's reagent (0.5 M FeCl_3 in $35\% \text{ HClO}_4$) at a 1:2 reagent-to-supernatant ratio (Gordon and Weber, 1951). The reaction was carried out in the dark at $28 \pm 2^\circ\text{C}$ for 20 minutes. Quantification was performed using a spectrophotometer at 530 nm, and concentrations were determined from a standard curve constructed with synthetic IAA ($0\text{--}100 \text{ } \mu\text{g mL}^{-1}$). For phosphate solubilization, mycelial plugs were inoculated into 250 mL Erlenmeyer flasks containing 50 mL of modified

NBRIP broth (Nautiyal, 1999), prepared with 10% (w/v) K_2HPO_4 and 10% (w/v) CaCl_2 to induce the formation of insoluble CaHPO_4 , with the medium pH adjusted to 6.5. Cultures were incubated at $25 \pm 2^\circ\text{C}$ on an orbital shaker at 150g for eight days. Following incubation, the cultures were centrifuged at 12000g for 15 minutes, and the supernatant was used for soluble phosphorus quantification. The colorimetric assay followed the method of Murphy and Riley (1962), in which 0.5 mL of the reagent was mixed with 0.5 mL of the supernatant and 5 mL of deionized distilled water. After a 20-minute reaction, absorbance was measured at 725 nm, and phosphate concentrations were estimated using a KH_2PO_4 standard curve. Statistical analyses were performed using R software v.4.3.3 (R Core Teams, 2025). Data normality was assessed using the Shapiro–Wilk test, and homogeneity of variances was tested using Bartlett’s test. Differences between treatments were evaluated by Tukey's test at $p \leq 0.05$.

2.6. Grapevine plantlets assay

The experiment was conducted at the experimental field of the Agricultural Research Company of Minas Gerais (EPAMIG), located in Caldas, Minas Gerais, during the 2023–2024 plantlet production cycle. Cuttings of the 'Syrah' cultivar grafted onto the rootstock 'Pausen 1103' (*Vitis berlandieri* Planch. $\times$ *Vitis rupestris* Scheele.) were used, following the standardized methodology described by Regina (2002). *Trichoderma* strains were cultured and multiplied in transparent polypropylene bags (20×30 cm) containing sterilized parboiled rice grains (200 g of rice and 100 mL of distilled water), incubated at 25°C for 10 days (Steffen and Maldaner, 2019). After incubation, the colonized rice was washed with sterile distilled water to release the conidia, filtered through a double layer of sterile gauze, and the conidial concentration was adjusted to 10^9 conidia/mL using a Neubauer counting chamber. For the inoculation process, rootless grapevine plantlets were immersed in the conidial suspension for 24 h, as previously described, and then transplanted into a nursery setting where they remained for 12 months. The experiment was arranged in a randomized block design with four replicates of 25 plantlets each, totaling 100 plants per treatment. For comparison purposes, a negative control (immersion in sterile distilled water) and a positive control (immersion in naphthaleneacetic acid at 0.0042%) were included under the same experimental conditions. Budburst percentage, rooting percentage, and root dry mass were measured at the end of the experiment. Statistical analyses were performed using R software v.4.3.3 (R Core Teams, 2025). Data normality was assessed using the Shapiro–Wilk test, and homogeneity of variances was tested using Bartlett’s test. Differences between treatments were evaluated using the paired Student’s *t*-test, with significance levels set at $p \leq 0.05$ and $p \leq 0.01$.

3. Results

3.1. Isolates obtained

From soil samples collected in the Mata do Pau-Ferro State Park, 250 fungal strains belonging to the genus *Trichoderma* were isolated. Of these, 33 strains were selected based on distinct macro- and micromorphological characteristics and subjected to molecular identification through sequencing of the *tef1*,

214 *rpb2*, and *cal* genes (Table 2). Molecular analyses revealed six distinct species: *T. breve* (1 isolate), *T. spirale*
 215 (3 isolates), *T. koningiopsis* (4 isolates), *T. camerunense* (6 isolates), *T. zelobreve* (7 isolates), and *T. afarasin*
 216 (12 isolates), distributed among three major clades: *Spirale*, *Viride*, and *Harzianum*. The *Harzianum* clade was
 217 the most prevalent, encompassing 78% of the identified isolates, which suggests a predominance of species
 218 from this lineage in the Atlantic Forest soils of the Mata do Pau-Ferro State Park.

219

220 Table 2

221 Identification of *Trichoderma* Strains isolated from soils of the Atlantic Forest biome in the Mata do Pau-Ferro
 222 State Park.

Strain	<i>tefl</i>			<i>rpb2</i>			<i>cal</i>		
	Length (bp)	Identity (%)	Closest Species (Type Material)	Length (bp)	Identity (%)	Closest Species (Type Material)	Length (bp)	Identity (%)	Closest Species (Type Material)
LFM001	853	96.23%	<i>Trichoderma koningiopsis</i>	1147	98.85%	<i>Trichoderma koningiopsis</i>	491	97.35	<i>Trichoderma ovalisporum</i>
LFM002	853	96.23%	<i>T. koningiopsis</i>	—	—	—	491	97.15	<i>T. ovalisporum</i>
LFM003	853	96.23%	<i>T. koningiopsis</i>	1148	98.97%	<i>T. koningiopsis</i>	491	97.56	<i>T. ovalisporum</i>
LFM004	853	96.23%	<i>T. koningiopsis</i>	1148	98.97%	<i>T. koningiopsis</i>	491	97.56	<i>T. ovalisporum</i>
LFM005	859	98.09%	<i>T. spirale</i>	1116	99.53%	<i>T. spirale</i>	—	—	—
LFM006	874	99.30%	<i>T. afarasin</i>	1108	99.00%	<i>T. azevedoi</i>	—	—	—
LFM007	873	99.65%	<i>T. afarasin</i>	—	—	—	—	—	—
LFM008	850	98.09%	<i>T. spirale</i>	—	—	—	—	—	—
LFM009	853	99.31%	<i>T. zelobreve</i>	1114	99.24%	<i>T. zelobreve</i>	—	—	—
LFM0010	874	99.30%	<i>T. afarasin</i>	1108	99.00%	<i>T. azevedoi</i>	—	—	—
LFM0011	903	99.39%	<i>T. zelobreve</i>	—	—	—	—	—	—
LFM0012	903	99.39%	<i>T. zelobreve</i>	—	—	—	—	—	—
LFM0013	903	99.39%	<i>T. zelobreve</i>	1114	99.24%	<i>T. zelobreve</i>	—	—	—
LFM0014	874	99.30%	<i>T. afarasin</i>	1110	99.49%	<i>T. inhamatum</i>	—	—	—
LFM0015	879	99.48%	<i>T. camerunense</i>	—	—	—	—	—	—
LFM0016	879	99.48%	<i>T. camerunense</i>	—	—	—	—	—	—
LFM0017	874	99.47%	<i>T. afarasin</i>	1108	99.00%	<i>T. azevedoi</i>	—	—	—
LFM0018	874	99.30%	<i>T. afarasin</i>	—	—	—	—	—	—
LFM0019	874	99.30%	<i>T. afarasin</i>	1114	99.62%	<i>T. inhamatum</i>	—	—	—
LFM0020	874	99.47%	<i>T. afarasin</i>	—	—	—	—	—	—
LFM0021	903	99.39%	<i>T. zelobreve</i>	—	—	—	—	—	—
LFM0022	879	99.48%	<i>T. camerunense</i>	—	—	—	—	—	—
LFM0023	873	99.47%	<i>T. afarasin</i>	—	—	—	—	—	—
LFM0024	879	99.48%	<i>T. camerunense</i>	—	—	—	—	—	—
LFM0025	879	99.48%	<i>T. camerunense</i>	—	—	—	—	—	—

LFM0026	903	99.39%	<i>T. zelobreve</i>	—	—	—	—	—	—
LFM0027	873	99.65%	<i>T. afarasin</i>	—	—	—	—	—	—
LFM0028	853	99.67%	<i>T. breve</i>	—	—	—	—	—	—
LFM0029	903	99.39%	<i>T. zelobreve</i>	—	—	—	—	—	—
LFM0030	850	97.85%	<i>T. spirale</i>	1117	99.53%	<i>T. spirale</i>	—	—	—
LFM0031	879	99.48%	<i>T. camerunense</i>	—	—	—	—	—	—
LFM0032	873	98.95%	<i>T. afarasin</i>	1118	98.87%	<i>T. azevedoi</i>	—	—	—
LFM0033	873	99.65%	<i>T. afarasin</i>	—	—	—	—	—	—

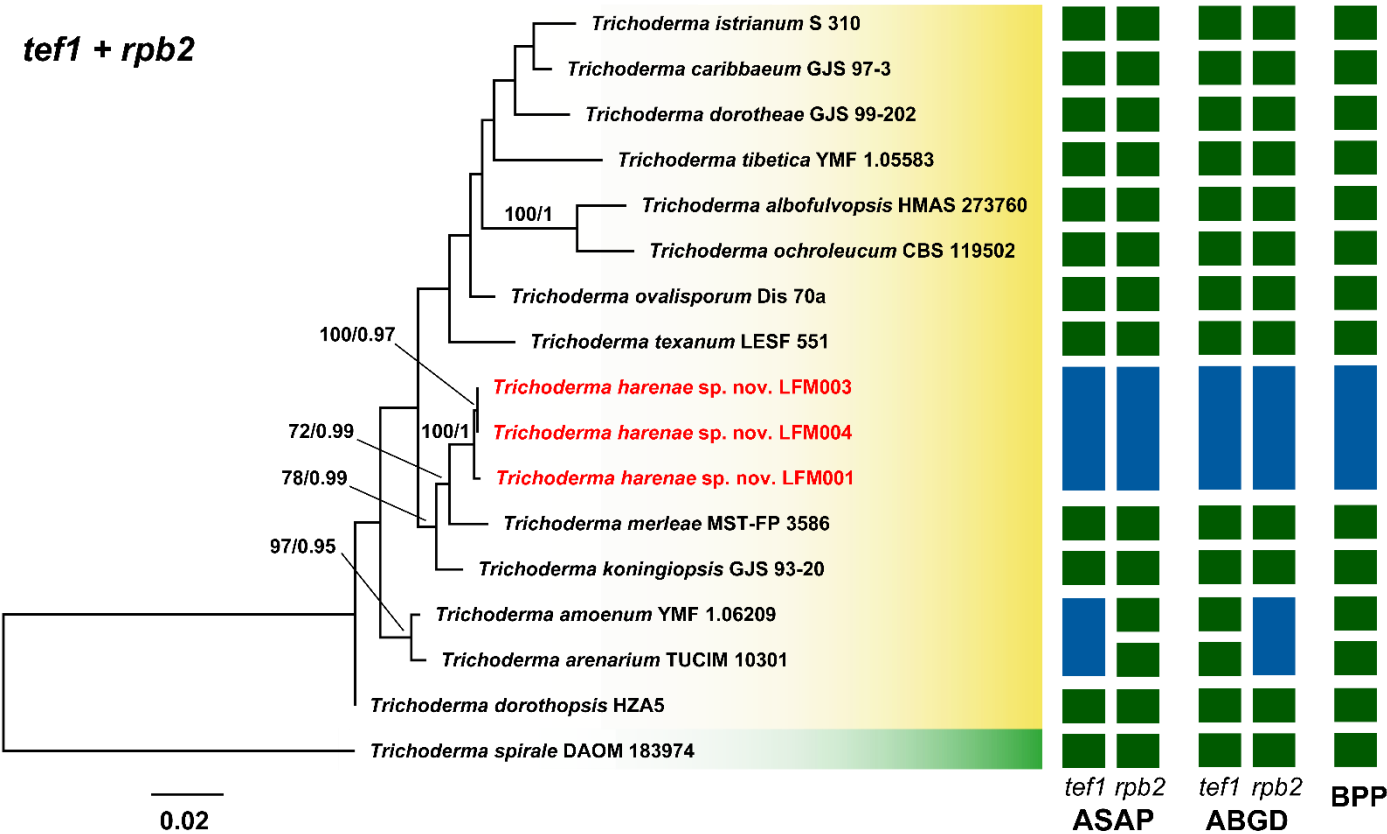
Note: The strains of the species described during this study are indicated in bold.

The isolates LFM001 to LFM004, belonging to the *Viride* clade and representing 12% of the total strains isolated, showed low sequence identity to type species for two molecular markers commonly used in *Trichoderma* species identification (*tefl* and *rpb2*). Additionally, the *cal* gene — another marker known for its high phylogenetic resolution — was also sequenced. However, identity values remained below the species-level threshold (<99%), consistent with the results from the other genes, suggesting that these isolates are distinct from any currently described species. In contrast, isolates LFM005, LFM008, and LFM030, which belong to the *Spirale* clade and represent 9% of all isolates, exhibited relatively low identity for the *tefl* gene (>98.5%), but high identity for the *rpb2* gene (>99.5%), both matching *T. spirale*, thus supporting a reliable identification.

3.2. Phylogenetic analyses and species delimitation

Phylogenetic analyses based on individual gene sequences (*tefl*, *rpb2*, and *cal*), as well as a combined *tefl+rpb2* matrix, were conducted to determine the taxonomic placement of isolates LFM001, LFM003, and LFM004. Strain LFM002 was not included due to the absence of *rpb2* gene sequencing. Both individual and concatenated alignments resulted in similar topologies in Maximum Likelihood (ML) and Bayesian Inference (BI) analyses, with high statistical support for most clades. The concatenated tree supports the hypothesis that isolates LFM001 to LFM004, with sequences closest to *T. koningiopsis* (GJS 93-20) and *T. merleae* (MST-FP 3586) (Table 2), represent a new species, *Trichoderma harenae* sp. nov. (Fig. 2). These two species form distinct clades, positioned as sister groups to *Trichoderma harenae* sp. nov., with strong statistical support (ML = 100%; BI = 1.0), corroborating the hypothesis that this is a novel and well-delimited lineage. Based on the evolutionary analysis of individual genes, in the phylogenetic tree of the *tefl* gene (Fig. S1), isolates LFM001, LFM003, and LFM004 formed a monophyletic clade with high support values (ML = 99%; BI = 0.99). A topology similar to that of the concatenated gene tree was observed in the tree for *rpb2* (Fig. S2), where *T. harenae* sp. nov. again clustered as a distinct lineage (ML = 97%; BI = 0.98), forming an exclusive clade closely related to *T. merleae* (MST-FP 3586) and *T. koningiopsis* (GJS 93-20). In the analysis of the *cal* gene (Fig. S3), topological variation was observed due to the limited availability of sequences from closely related species, particularly the absence of *T. merleae* (MST-FP 3586) and *T. koningiopsis* (GJS 93-20).

253 Nevertheless, isolates LFM001, LFM003, and LFM004 formed a well-supported, independent clade (ML =
 254 98%; BI = 0.96), positioned as a sister group to *T. koningii* (CBS 457.96).
 255



256 **Fig. 2.** Phylogenetic tree of *Trichoderma* species inferred using Maximum Likelihood (ML) and Bayesian
 257 Inference (BI) methods, with the TIM1ef+G substitution model applied both methods, based on the *tef1+rpb2*
 258 sequences matrix. Bars represent species delimitation results from ASAP-, ABGD- and BPP-based algorithmic
 259 (green: delimited as the different species; blue: delimited as the same species). The bootstrap analysis was
 260 performed with 1000 resamplings. The numbers on the nodes represent the bootstraps values from the ML
 261 analysis, followed by the posterior probabilities (PP) from the BI. Values lower than 70% in ML and lower
 262 than 0.95 PP in BI were not considered for branch support. The evolutionary analysis involved a total of 17
 263 distinct sequences, with 1196 bp of aligned nucleotides (*tef1* = 424 bp and *rpb2* = 772 bp). Only sequences
 264 from type material available in the databases (in black bold) were used for comparison purposes and are listed
 265 in Table S1. *Trichoderma spirale* (DAOM 183974) was used as an outgroup (green background). The strains
 266 reported in this study are highlighted in red bold. The scale indicates the number of substitutions per site.

268
 269 The species delimitation methods based on genetic distance (ASAP and ABGD) and coalescence (BPP)
 270 were performed to assess congruence with the multilocus phylogenetic tree. The BPP analysis, conducted
 271 using individual *tef1* and *rpb2* datasets, supported the recognition of 15 distinct species, corroborating the
 272 species boundaries from phylogenetic inference. In contrast, the distance-based methods ASAP and ABGD
 273 showed incongruent results regarding the delimitation of *T. amoenum* (YMF 1.06209) and *T. arenarium*
 274 (TUCIM 10301). In the *tef1* gene analysis, the ASAP method failed to distinguish between the two species,

whereas the ABGD method successfully recovered them as distinct molecular units. Conversely, in the *rpb2* gene analysis, the ASAP method identified them as separate species, while the ABGD method failed to detect any differentiation. Despite these discrepancies, all three methods (ASAP, ABGD, and BPP) consistently supported the recognition of *T. harenae* sp. nov. as a distinct lineage. The congruent results across both distance-based and coalescent-based approaches reinforce the validity of this taxon as a novel and well-delimited species within the genus *Trichoderma*.

3.3. Phosphate solubilization and indole-3-acetic acid production

All isolates of *T. harenae* sp. nov. were able to synthesize indole-3-acetic acid (IAA) in medium supplemented with L-tryptophan and to solubilize dicalcium phosphate (CaHPO_4) in NBRIP culture medium (Fig. 3). The ability to solubilize phosphate and produce IAA indicated significant intraspecific physiological variation within *T. harenae* sp. nov., with phosphate solubilization ranging from 9.55 mg L^{-1} to 20.32 mg L^{-1} and IAA production from $1.25 \text{ } \mu\text{g mL}^{-1}$ to $6.54 \text{ } \mu\text{g mL}^{-1}$ among the isolates.

Regarding phosphate solubilization, isolates LFM003 and LFM004 exhibited increases of 87% and 113%, respectively, compared to LFM002. For IAA production, isolate LFM004 demonstrated a high synthesis capacity, showing increases of 63%, 90%, and 422% in comparison to isolates LFM001, LFM002, and LFM003, respectively. While most isolates exhibited a proportional trend between phosphate solubilization and IAA production, isolate LFM003 displayed contrasting physiological performance across these parameters, with high solubilization capacity and low IAA synthesis.

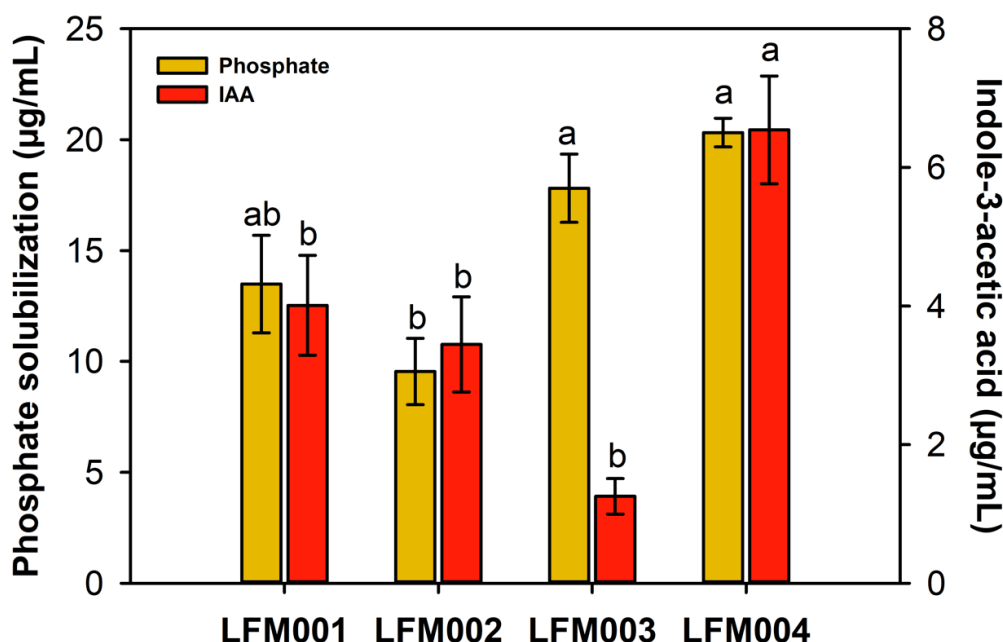


Fig. 3. Indole-3-acetic acid (IAA) production and phosphate solubilization by four *Trichoderma harenae* isolates (LFM001–LFM004) under in vitro conditions. The isolates were cultured in potato dextrose broth supplemented with L-tryptophan (100 mg L^{-1}) for IAA production and in modified NBRIP broth to induce solubilization of dibasic calcium phosphate (CaHPO_4). Metabolite concentrations were quantified after 8 days

by spectrophotometry (530 nm for IAA; 725 nm for phosphate). Means followed by the same letter do not differ significantly according to Tukey's test at the 5% significance level.

3.4. Biotechnological potential in grapevine

The isolate LFM004 was selected based on its physiological traits, including high phosphate solubilization and IAA synthesis, for field trials to evaluate the potential of *T.harenae* sp. nov. as a biofertilizer and biostimulant in grapevine plantlets. The application of *T. harenae* sp. nov. significantly improved grapevine plantlets development compared to control the control (water, CTL) and phytohormone (naphthaleneacetic acid, NAA) treatments. (Fig. 4). Rooting rates increased by 98% and 55% when compared to treatments with water and a commercial phytohormone, respectively. Additionally, *T. harenae* sp. nov. promoted a 92% increase in the number of shoots relative to the water treatment and a 56% increase compared to the phytohormone treatment. Root dry biomass accumulation was enhanced by *T. harenae* sp. nov., resulting in increases of 124% and 49% compared to the water and commercial phytohormone treatments, respectively.

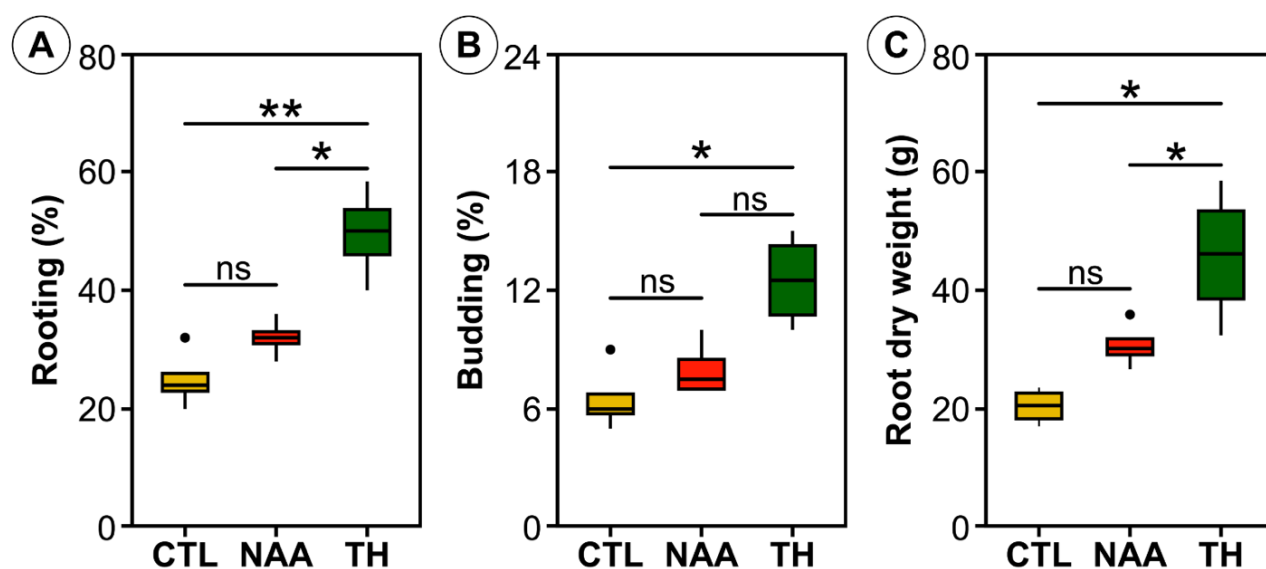


Fig. 4. Effects of *Trichoderma harenae* sp. nov. strain LFM004 (TH) treatment compared to the negative control (CTL) and the positive phytohormone control (NAA) on (A) rooting rate (%), (B) budding (%), and (C) root dry weight (g), evaluated after 12 months in the field using 100 Syrah cultivar cuttings grafted onto 'Pausen 1103' rootstock. All treatments involved immersing the cuttings for 24 h: in water for the negative control (CTL); in a 0.0042% naphthaleneacetic acid solution for the positive control (NAA); and in a conidial suspension of *T. harenae* at 10^9 conidia/mL (TH). Statistical analysis was performed using paired *t*-tests. Significant differences between treatments are indicated by $p < 0.05$ (*), $p < 0.01$ (**), ns = not significant.

3.5. Taxonomy

Trichoderma harenae J.M.F.L. Cruz, and J.T. De Souza, **sp. nov.** Fig.5.

MycoBank: not submitted.

Etymology: Named after the city of Areia, Paraíba, Brazil, the locality where the species was found.

Classification: *Hypocreaceae*, *Hypocreales*, *Hypocreomycetidae*, *Sordariomycetes*.

Diagnosis: Phylogenetic analyses based on genealogical concordance (Taylor et al., 2000), supported by the programs ASAP, ABGD, and BPP, demonstrate that *T. harenae* sp. nov. is distinct from *T. koningiopsis*, *T. dorotheopsis*, *T. ovalisporum*, *T. dorotheae*, *T. merleae*, and *T. amoenum*. The microscopic features of *T. harenae* sp. nov. differ from those of these species, including the size and shape of conidia, conidiophores, and phialides, as well as growth rates on different media. *T. harenae* sp. nov. has so far been found only in the Atlantic Forest biome of Brazil, with strong evidence suggesting that it is predominantly distributed in tropical latitudes (Samuels et al., 2006).

Typification: Brazil, Paraíba, Areia (6° 58' 36.34"S, 35° 45' 1.41"W), isolated from 'Mata do Pau-Ferro' (State Park) on leaf litter covered soil, 14 May 2020, Cruz J.M.F.L., Holotype: LFM004, preserved as a metabolically inactive culture at -80 C at the Laboratory of Molecular Phytopathology, Federal University of Lavras. GenBank: *tefl*: **not submitted**, *rpb2*: **not submitted**, *cal*: **not submitted**.

Sexual morph: Unknown.

Asexual morph: Mycelium on synthetic low-nutrient agar (SNA) initially smooth, hyaline and adhered to the medium, later developing aerial hyphae. Chlamydospores infrequently formed, intercalary, solitary or in pairs, thick-walled, globose to subglobose, and hyaline; measuring $(6.0-6.6 (-7.2) \times (5.4-6.0 (-6.2)) \mu\text{m}$ (L×W), with a L/W ratio of $(0.83-1.08 (-1.42))$ (n = 40). Conidiation starting after 72 h and occurring predominantly in small, dark green, aerial pustules, solitary or rarely aggregated, densely formed with a cushion-like appearance, mostly along the colony margin; measuring $(0.8-1.0 (-1.2) \times (2.7-3.0 (-3.5)) \text{ mm}$ at 15 days (n = 30). Conidiophores trichoderma-like, sinuous when large and straight when small, with fertile branches arising from the main axis, paired or rarely solitary. Phialides symmetrically arranged, ampulliform, curved toward the apex (forming an angle of approximately 30 to 60°), produced in pairs and typically forming terminal clusters of 3–4 phialides; measuring $(6.7-7.7 (-8.4) \times (2.2-2.9 (-3.7)) \mu\text{m}$ (L×W), with an apical width of 0.6–1.1 μm and a basal width of 1–2 μm , typically showing a distinct basal ring visible under SEM; arising from a cell measuring $(4.7-5.7 (-7.4) \times (2.1-2.3 (-2.5)) \mu\text{m}$ (n = 60). Conidia asymmetrically ellipsoidal, smooth, often with a slightly tapered base, occasionally subglobose or oblong, bluish-green to green in color; measuring $(2.3-3.4 (-4.7) \times (1.7-2.4 (-3.5)) \mu\text{m}$ (L×W), with a L/W ratio of $(0.66-1.42 (-2.76))$ (n = 200).

Colony characters: Optimum temperature for growth 25°C, with the mycelium covering the entire plate within 5 days. No growth at 35°C in potato dextrose agar (PDA), cornmeal dextrose agar (CMD) and synthetic low-nutrient agar (SNA). On PDA, after 72 h, colonies have a radius of 39.5–45.6 mm at 30°C, 40.3–58.4 mm at 25°C, 30.9–36.1 mm at 20°C, and 5.35–11.1 at 15°C; Colony initially dense and white, exhibiting irregular circular growth, and gradually turning deep green. The mycelium is diffuse, forming alternating lighter and darker concentric zones (2-3). Conidiation starting after 48 h on abundant aerial hyphae arising from the center of the plate, which display a crinkled to wavy appearance and a velvety texture. No pustules were observed, likely due to the profuse mycelial growth covering the colony surface. No autolytic exudates or diffused

pigments were observed. Odor indistinct. Colony margin is regular and well-defined, with a thin, lighter-colored border (whitish to light green), suggesting actively growing, young aerial mycelium. On CMD, after 72 h, colonies have a radius of 45.22–48.2 mm at 30°C, 55.3–59.8 mm at 25°C, 22.2–30.5 mm at 20°C, and 7.4–9.4 mm at 15°C; Colony initially smooth, hyaline, and adherent to the medium, with partially sterile hyphae forming a halo of irregular, circular growth. The thin mycelium expands diffusely, subsequently developing aerial hyphae and forming alternating concentric zones (2-3) that gradually become dark green, later transitioning to olive-yellow. Conidiation starting after 96 h, with cushion-like pustules dispersed throughout the medium and abundant aerial hyphae emerging beyond the initial halo of sterile hyphae, exhibiting a wavy appearance and floccose texture. No autolytic exudates or diffused pigments were observed. Odor indistinct. Colony margin is regular, with a clearly visible color transition. On SNA, after 72 h, colonies have a radius of 39.8–45.9 mm at 30°C, 37.8–49.4 mm at 25°C, 21.6–27.2 mm at 20 °C, 5.93–10.2 mm at 15°C; Colony initially smooth, hyaline, and adherent to the medium, with partially sterile hyphae exhibiting irregular circular growth. The thin mycelium expands diffusely, developing aerial hyphae. Conidiation begins after 72 h, with cushion-like pustules dispersed along the colony margin and the development of abundant aerial hyphae in the periphery. No autolytic exudates or diffused pigments were observed. Odor indistinct. The colony margin is regular, with a visible color transition, where a concentric zone is formed due to high conidial production.

Distribution: Brazil, Paraíba, Areia.

Additional strains examined: Brazil, Paraíba, Areia (6° 58' 36.34"S, 35° 45' 1.41"W), isolated from 'Mata do Pau-Ferro' (State Park) on leaf litter covered soil, 14 May 2020, Cruz J.M.F.L. Strains LFM001, LFM002, and LFM003 preserved at the Laboratory of Molecular Phytopathology, Federal University of Lavras. GenBank identification LFM001 = *tefl*: **not submitted**, *rpb2*: **not submitted**, *cal*: **not submitted**. GenBank identification LFM002 = *tefl*: **not submitted**, *cal*: **not submitted**. GenBank identification LFM003 = *tefl*: **not submitted**, *rpb2*: **not submitted**, *cal*: **not submitted**.

Notes: Phylogenetic analysis of *Trichoderma* species based on the *tefl*, *rpb2* and *cal* genes provides highest resolution for identification of species of the genus. Based on a megablast search of NCBI's GenBank nucleotide database, the closest hits using the *tefl* sequence are *Trichoderma koningiopsis* (GenBank DQ284966.1; Identities = 511/531 (96%), 3 gaps (0 %)), *Trichoderma amoenum* (GenBank MT070146.1; Identities = 455/473(96%), 5 gaps (1 %)) and *Trichoderma merleae* (GenBank PQ572712.1; Identities = 578/601(96%), 5 gaps (0 %)). Closest hits using the *rpb2* sequence had highest similarity to *Trichoderma koningiopsis* (GenBank EU241506.1; Identities = 765/772(99%), no gaps), *Trichoderma merleae* (GenBank PQ572711.1; Identities = 878/887(99%), no gaps) and *Trichoderma dorotheopsis* (GenBank MH647795.1; Identities = 848/867(98%), no gaps). Closest hits using the *cal* sequence had highest similarity to *Trichoderma ovalisporum* (GenBank Q379005.1; Identities = 479/491(98%), 1 gap (0 %)), *Trichoderma dorotheae* (GenBank DQ367706.1; Identities = 461/473(97%), no gaps) and *Trichoderma koningii* (GenBank DQ367671.1; Identities = 466/482(97%), 4 gaps (0 %)).

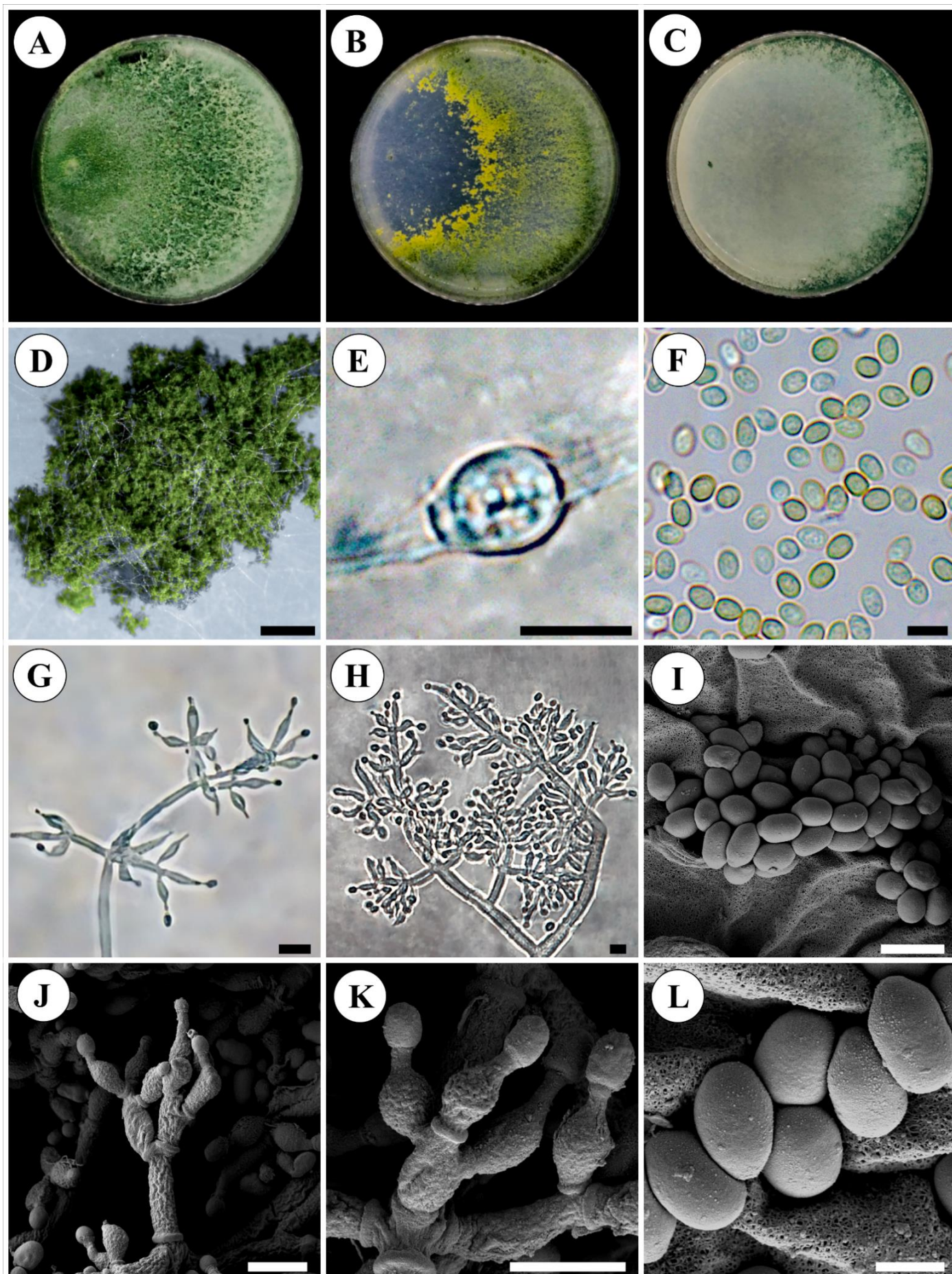


Fig. 5. *Trichoderma harenae* (LFM004). A. Colony on PDA (1wk). B. Colony on CMD (1wk). C. Colony on SNA (1wk). D. Pustule (CMD). E. Chlamydospore (CMD). F, I, L. Conidia (SNA). G, H. Conidiophores

(SNA). J, K. Phialides and cell that phialides appear (SNA). I– L = Scanning electron micrograph (SEM).
Scale bars: D = 500 µm, E – K = 5 µm. L = 2 µm.

4. Discussion

In this study, we conducted a survey for novel *Trichoderma* strains in the Atlantic Forest biome, specifically in a protected area designated as an ecological reserve and conservation unit. *Trichoderma* isolates obtained from forest soils and identified based on partial sequences of the *tefl* gene revealed the predominance and broad distribution of species belonging to the *Harzianum* clade in this ecosystem. Our sampling uncovered six species inhabiting this environment, one of which is described here as a new species. The biological richness and microbial diversity of these ecosystems offer unique opportunities for the discovery of microorganisms with ecological functions adapted to complex and competitive environmental conditions (Pacchioni et al., 2014; Bail et al., 2022). The *Harzianum* clade is one of the most widely distributed and ecologically versatile groups within the *Trichoderma* genus, comprising species that inhabit a wide range of environments, particularly soil ecosystems (Druzhinina et al., 2010; Ismaiel et al., 2024). This ecological plasticity reflects the ability of these species to adapt to diverse physicochemical soil conditions, interact with a broad spectrum of hosts, and effectively compete with other microorganisms. Factors such as rapid substrate colonization, production of hydrolytic enzymes and antimicrobial compounds, and the capacity to modulate plant development through secondary metabolites contribute to the ecological success and biotechnological relevance of this group (Druzhinina et al., 2011; Chaverri and Samuels, 2013; Kubicek et al., 2019).

Several species identified in this study belong to groups recognized for their broad geographic distribution and significant biotechnological potential. The most frequently detected species, *T. afarasin* Chaverri & Rocha, has been reported as both an endophyte and soil inhabitant in tropical regions, with records from Brazil (Peixoto et al., 2025), Argentina (Barrera et al., 2021), and Cameroon (Chaverri et al., 2015). Recent studies have also demonstrated its potential as a biocontrol agent against phytopathogens (Contreras-Soto et al., 2025). *T. camerunense* Chaverri & Samuels, originally described from natural soils in Cameroon (Chaverri et al., 2015), has also been reported in South Africa (Du Plessis et al., 2018) and Brazilian coastal ecosystems such as mangroves, where it exhibits marked cellulolytic and xylanolytic activities, as well as biosurfactant production (Martinho et al., 2019). Furthermore, this species has shown biotechnological potential for the control of *Agroathelia rolfii* (Sacc.) Redhead & Mullineux in agricultural soils in India (Jambhulkar et al., 2024) and nematicidal activity against *Meloidogyne incognita* Kofoed & White in Nepal (Jaiswal et al., 2025). *T. breve* Chen & Zhuang, originally isolated from soil in China (Chen and Zhuang, 2017), has gained attention for its hyperparasitic capabilities (Liu et al., 2023) and plant growth promotion via the production of indole-3-acetic acid (IAA) and siderophores (Intana et al., 2024). It also demonstrates efficacy in controlling phytopathogenic fungi and nematodes (Nyang'au et al., 2023; Liu et al., 2023; Intana et al., 2024). Its sister species, *T. zelobreve* Sun & Liu, recently reported in Chinese soils (Gu et al., 2020), has also shown strong biocontrol potential (Nourian et al., 2024), suggesting it is an emerging lineage with promising applications in

sustainable agriculture. *T. spirale* Bissett, a member of the homonymous clade, is typically associated with tropical forest soils (Kubicek et al., 2003). Studies suggest that its biocontrol activity is mediated through the production of both volatile and non-volatile antifungal metabolites (Li et al., 2012; Baiyee et al., 2019; Li et al., 2019; Chóez-Guaranda et al., 2023). Among the species with the highest biotechnological relevance is *T. koningiopsis* Samuels, Suarez & Evans, a cosmopolitan member of the *Viride* clade extensively studied for its diverse applications. This species has been associated with enhanced soil fertility, modulation of rhizosphere microbiota, and suppression of plant diseases through the production of hydrolytic enzymes and antifungal volatile compounds (Yu and Luo, 2020; Kong et al., 2022; Luo et al., 2023; Osorio-Guerro et al., 2024). Additionally, *T. koningiopsis* has been explored in industrial contexts, including as a bioherbicide (Bordin et al., 2018; Ulrich et al., 2023; Camargo et al., 2024), in biofuel production (Mendoza-López et al., 2017), and for the bioremediation of industrial effluents (Kubeneck et al., 2025), highlighting its versatility across various technological sectors.

We described *T. harenae* sp. nov., a new species found in this biome, based on morphological characteristics and molecular evidence derived from sequence fragments of the *tefl*, *rpb2*, and *cal* genes. Additionally, strains isolated from the *Spirale* clade (LFM005, LFM008, and LFM030) exhibited a high mutation rate in the *tefl* gene region, suggesting the possible presence of an undescribed species in our samples. These findings underscore the importance of further studies, as recently conducted by Sales et al. (2025), who redefined the *Spirale* clade as monophyletic, thereby significantly improving the understanding of its diversity by employing phylogenetically informative regions within the *tefl* gene. A multigene approach using *tefl* and *rpb2*, combined with phenotypic characteristics, remains essential and widely applied in the identification of novel *Trichoderma* species (Souza et al., 2023; Zhao et al., 2023; Ye et al., 2024; Sales et al., 2025; Zhao et al., 2025; Peixoto et al., 2025). Recently established and validated protocols provide a standardized framework for species delimitation within this genus (Cai and Druzhinina, 2021). Furthermore, the inclusion of the *cal* gene fragment is recommended to increase the robustness and reliability of phylogenetic analyses due to its high interspecific variability within *Trichoderma* (Chaverri et al., 2015; Sales et al., 2025; Peixoto et al., 2025). However, its broader application is currently limited by the low representation of this marker in public databases (Sales et al., 2025). In addition to the observed molecular variation, clear morphological and physiological differences were found between *T. harenae* sp. nov. (LFM004) and *T. koningiopsis* (GJS 93-20), a phylogenetically related sister species supported by robust statistical values (ML = 78%; BI = 0.99). *T. koningiopsis* (GJS 93-20) is characterized by predominantly pachybasium-like conidiophores and phialides that are narrowly lageniform or conspicuously inflated in the middle region (Samuels et al., 2006), whereas *T. harenae* sp. nov. displays trichoderma-like conidiophores and clearly ampulliform phialides. Regarding mycelial growth, *T. koningiopsis* (GJS 93-20) exhibits optimal radial growth at 30 °C on PDA between (20–) 52–72 mm and on SNA between (15–) 32–54 (–64) mm. It can also grow at 35 °C, with diameters between (0–) 2–10 (–12) mm on PDA and (0–) 1.5–6.2 (–14) mm on SNA (Samuels et al., 2006). In contrast, *T. harenae* sp. nov. (LFM004) shows optimal growth at 25 °C and no growth at 35 °C, indicating higher thermal sensitivity

and ecological adaptation to humid environments with moderate temperatures. We also note that *T. merleae* (MST-FP 3586), another sister group of *T. harenae* sp. nov. (ML = 72%; BI = 0.99), although present in public genetic databases and listed as a type species, has not yet been formally described in accordance with the criteria established by the International Code of Nomenclature for Algae, Fungi, and Plants – ICN (Turland et al., 2018).

The delimitation of this new taxon, *T. harenae* sp. nov., was strongly supported by complementary species delimitation methods based on genetic distance (ASAP and ABGD) and coalescent theory (BPP). Analyses employing these approaches have become increasingly common as supporting tools in the delimitation and formal description of new *Trichoderma* species (Bustamante et al., 2021; Sales et al., 2025). During our analyses, *T. amoenum* Yu & Lv and *T. arenarium* Cai, Ding & Druzhinina—two formally described and distinct species—exhibited taxonomic incongruence in species boundary recognition. We observed that both species were described concurrently, which precluded cross-validated polyphasic characterization of these lineages. This limitation is further compounded by the fact that *T. arenarium* is represented by a short fragment of the *tefl* gene and a shallow morphological and physiological description based on limited character sampling, making accurate comparisons with closely related species such as *T. amoenum* difficult (Ding et al., 2021; Zheng et al., 2021). In this study, we chose to treat *T. amoenum* and *T. arenarium* as distinct species, based on the topology of phylogenetic trees and the available information. Nevertheless, we emphasize the need for future taxonomic revisions incorporating more detailed morphological analyses, more complete multilocus sequencing, and representative sampling of *T. arenarium*, in order to clarify its true phylogenetic position within the genus *Trichoderma*.

Our results highlight the remarkable ability of *T. harenae* sp. nov. in phosphate solubilization and indole-3-acetic acid (IAA) synthesis, underscoring the potential of the Atlantic Forest biome as an important source for the discovery and selection of novel native microbial strains with agricultural applications. Additionally, we demonstrated the promising performance of the LFM004 isolate as a bioinput, acting as a biostimulant in the rooting and budding of grapevine plantlets, further supporting its potential application in sustainable viticulture. The prospecting of *Trichoderma* strains across different biomes is essential for the development of new biofertilizers with agricultural potential (e.g., Bononi et al., 2020). Phosphorus is a macronutrient vital for root development, energy metabolism, signaling, and the structural components of plant cells (Shen et al., 2011; Lambers, 2022). However, the natural deficiency of phosphorus in soils represents one of the major global challenges for agriculture (Du et al., 2020). Even when soluble phosphate fertilizers are applied, much of the nutrient can be rapidly immobilized in the soil, becoming unavailable to plants due to adsorption onto clay particles or precipitation with cations such as Ca^{2+} , Fe^{2+} , and Al^{3+} (Zhu et al., 2018). Microorganisms capable of solubilizing inorganic phosphate, such as *T. harenae* sp. nov., increase nutrient availability, promoting enhanced root growth and overall plant vigor (Bedine et al., 2022; Galeano et al., 2025). Simultaneously, IAA is a well-known plant hormone associated with the stimulation of lateral root formation, cell elongation, and differentiation (Zhao, 2010; Mano and Nemoto, 2012; Luo et al., 2018). The production

of IAA analogs by rhizospheric or endophytic fungi is a key mechanism for modulating root architecture and increasing plant biomass (Jaroszuk-Ścisiel et al., 2018; Bader et al., 2020; Intana et al., 2024), particularly in nurseries, where uniform rooting is desirable. These aspects are particularly relevant to viticulture, as low rooting success in nurseries remains one of the main obstacles in large-scale plantlets production, resulting in significant economic losses (Waite et al., 2015; Wurz et al., 2022; Boeno and Zuffellato-Ribas, 2023). The high rooting of grapevine plantlets promoted by *T. harenae* sp. nov. may be closely linked to the synergistic effect of IAA analog production and phosphate solubilization, two traits widely recognized for their roles in promoting plant growth (Guzmán-Guzmán et al., 2019). Increased phosphorus availability in the soil, coupled with the hormonal stimulation of rhizogenesis, creates a favorable environment for root system development, which is essential for the initial vigor of young plants. Furthermore, the biocontrol activity of *Trichoderma* contributes to protecting seedlings from root pathogens, a factor often associated with poor rootstock quality (El-Mohamedy et al., 2010; Silva-Valderrama, 2020; Martínez-Diz et al., 2021; Cruz et al., 2025).

In this context, considering the high economic relevance of grapevine cultivation and the growing demand for sustainable viticultural practices, the use of *Trichoderma* represents a promising sustainable strategy for seedling propagation and more ecological soil management. This not only highlights the functional versatility of *T. harenae* sp. nov. but also underscores the untapped biotechnological potential of fungal biodiversity within the Atlantic Forest biome.

CRedit authorship contribution statement

José Manoel F. de L. Cruz: Writing – original draft, Visualization, Validation, Software, Resources, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Luciana C. do Nascimento:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Funding acquisition, Conceptualization. **Otilia R. de Farias:** Writing – review & editing, Validation, Investigation. **Patrick Materatski:** Writing – review & editing, Validation, Investigation. **Carla Marisa R. Varanda:** Writing – review & editing, Validation. **Claudia Rita de Souza:** Writing – review & editing, Validation, Supervision, Funding acquisition. **Jorge T. De Souza:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

Submission declaration

All authors have edited the manuscript and approved to publish the research findings. This research has not been published, and is not under consideration for publication elsewhere.

Funding

This research was funded by the Minas Gerais State Research Support Foundation (FAPEMIG) through project APQ-00949-21.

546 Data availability

547 The authors affirm that the data supporting the conclusions of this study are included within the article.

549 Declaration of competing interest

550 The submission of this manuscript has been approved by all co-authors and by the institutions where the
551 research was conducted.

552 I affirm that all authors have no competing interests and confirm that this work is original. It has not been
553 published elsewhere and is not currently under consideration for publication elsewhere

555 Acknowledgments

556 We thank the technicians of the Agricultural Research Company of Minas Gerais (EPAMIG) for
557 propagating the plant material, providing space, and sharing their knowledge throughout these four years of
558 research, and to Professor Eduardo Alves for his assistance with the electron microscopy analyses. J.T.S.
559 thanks CNPq for providing a productivity scholarship and J.M.F.L.C. acknowledges the PhD scholarship
560 received from FAPEMIG.

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Supplementary material

Table S1

Primers and PCR conditions for the amplification of *tef1*, *rpb2*, and *cal* genes in *Trichoderma* strains.

Primers			
Region	Primer	Sequence 5' - 3'	Reference
<i>tef1</i>	tef71F	CAA AAT GGG TAA GGA GGA SAA GAC	Hoyos-Carvajal et al. (2009)
	tef1080R	GAT ACC AGC CTC GAA CTC ACC	Inglis et al. (2020)
<i>rpb2</i>	RPB2_210up	TGG GGW GAY CAR AAR AAG G	Inglis et al. (2020)
	RPB2_1450low	CAT RAT GAC SGA ATC TTC CTG GT	Inglis et al. (2020)
<i>cal</i>	CAL-228F	GAG TTC AAG GAG GCC TTC TCC C	Carbone and Kohn (1999)
	CAL-737R	CAT CTT TCT GGC CAT CAT GG	Carbone and Kohn (1999)

PCR conditions						
Region	Initial denaturation	Denaturation	Annealing	Extension	Final extension	N°. cycles
<i>tef1</i>	3min at 95°C	30s at 95°C	30s at 50°C	90s at 72°C	5min at 72°C	35
<i>rpb2</i>	3min at 94°C	30s at 94°C	30s at 48°C	90s at 72°C	7min at 72°C	35
<i>cal</i>	8min at 95°C	15s at 95°C	20s at 55°C	60s at 72°C	5min at 72°C	35

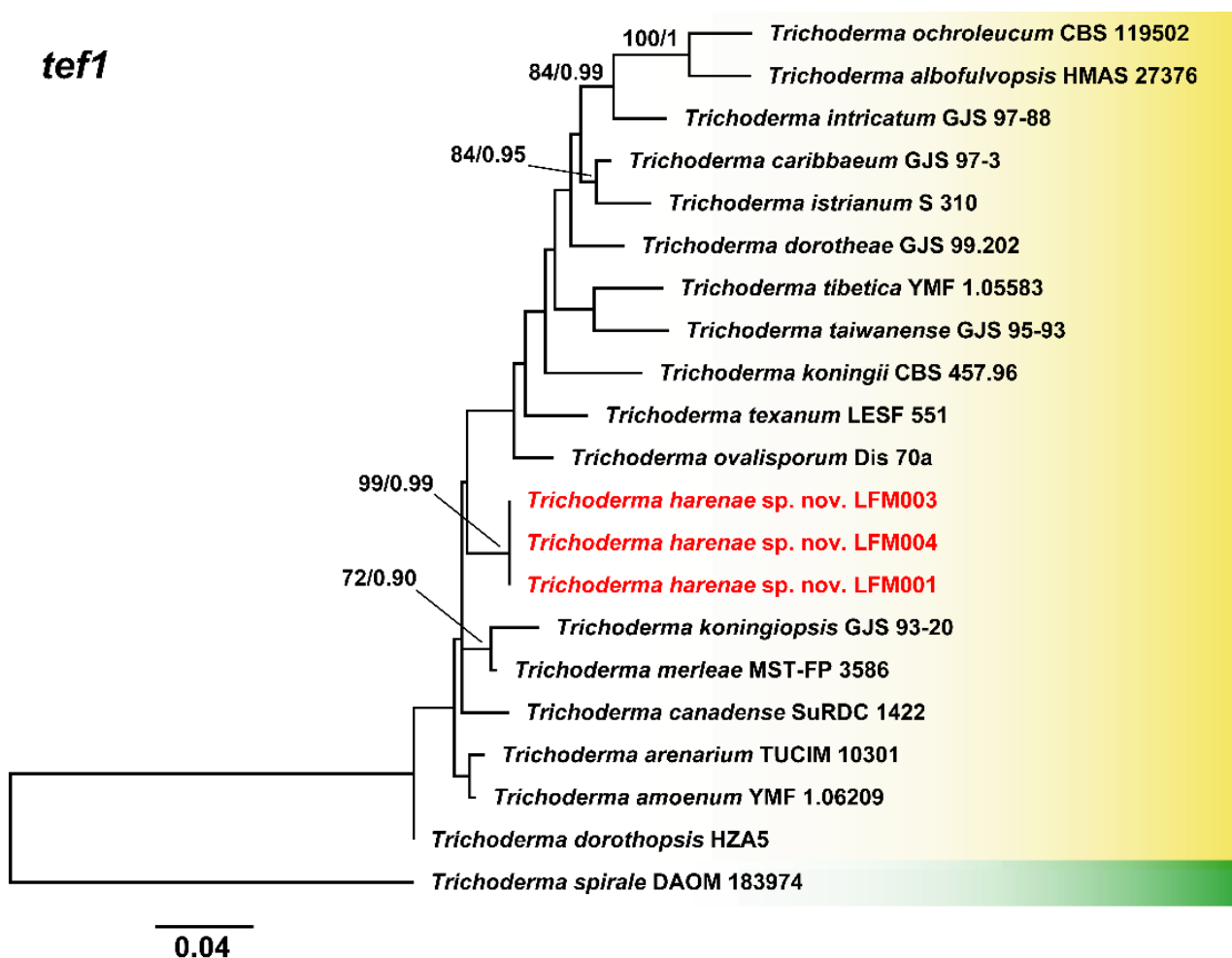


Fig. S1. Phylogenetic tree of *Trichoderma* species inferred using Maximum Likelihood (ML) and Bayesian Inference (BI) methods, with the HKY+G substitution model applied both methods, based on the *tef1*

sequences. The bootstrap analysis was performed with 1000 resamplings. The numbers on the nodes represent the bootstraps values from the ML analysis, followed by the posterior probabilities (PP) from the BI. Values lower than 70% in ML and lower than 0.95 PP in BI were not considered for branch support. The evolutionary analysis involved a total of 21 distinct sequences, with 424 bp of aligned nucleotides. Only sequences from type material available in the databases (in black bold) were used for comparison purposes and are listed in Table 1. *Trichoderma spirale* (DAOM 183974) was used as an outgroup (green background). The strains reported in this study are highlighted in red bold. The scale indicates the number of substitutions per site

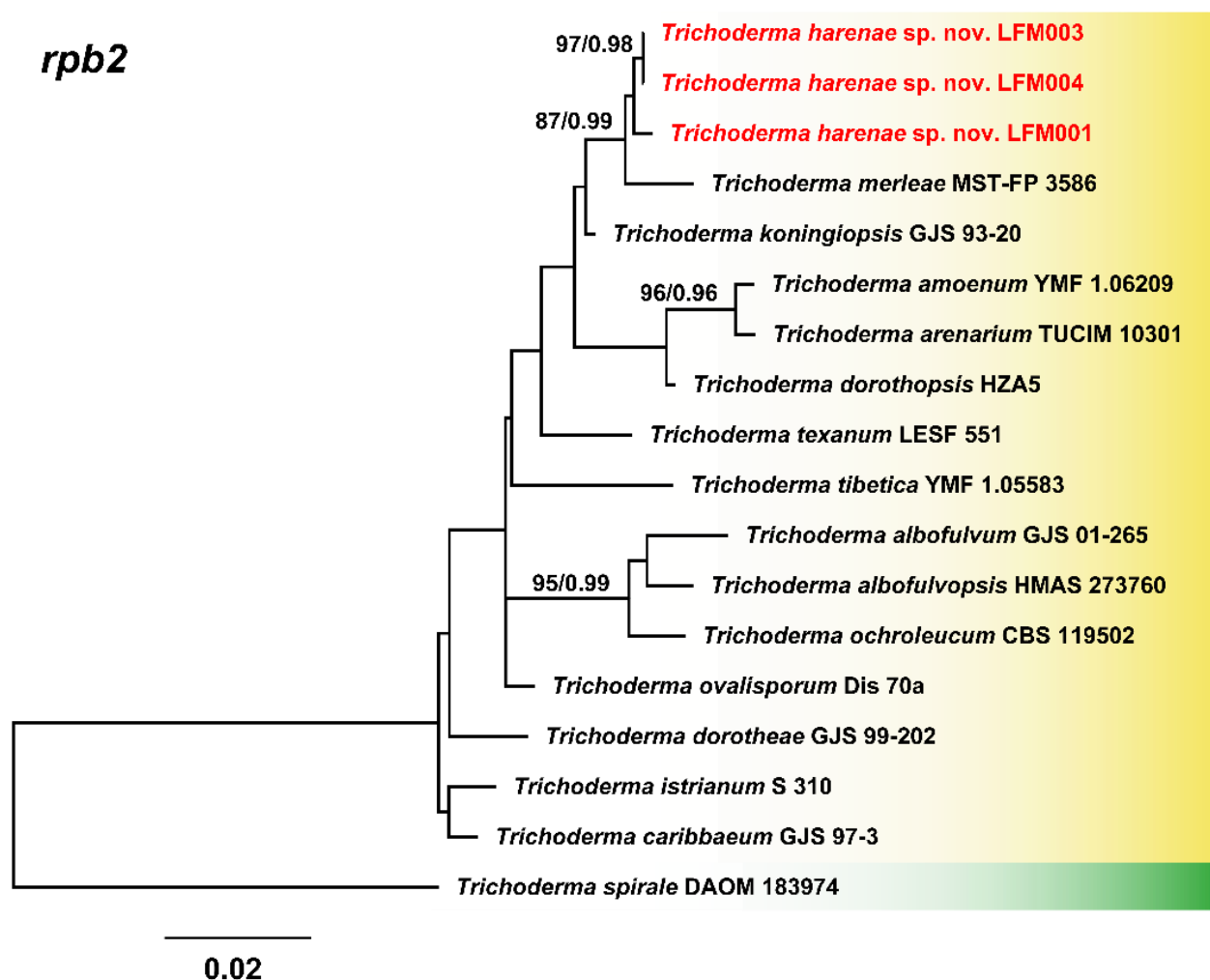


Fig. S2. Phylogenetic tree of *Trichoderma* species inferred using Maximum Likelihood (ML) and Bayesian Inference (BI) methods, with the TIM1ef+I+G substitution model applied both methods, based on the *rpb2* sequences. The bootstrap analysis was performed with 1000 resamplings. The numbers on the nodes represent the bootstraps values from the ML analysis, followed by the posterior probabilities (PP) from the BI. Values lower than 70% in ML and lower than 0.95 PP in BI were not considered for branch support. The evolutionary analysis involved a total of 18 distinct sequences, with 772 bp of aligned nucleotides. Only sequences from type material available in the databases (in black bold) were used for comparison purposes and are listed in Table 1. *Trichoderma spirale* (DAOM 183974) was used as an outgroup (green background). The strains reported in this study are highlighted in red bold. The scale indicates the number of substitutions per site

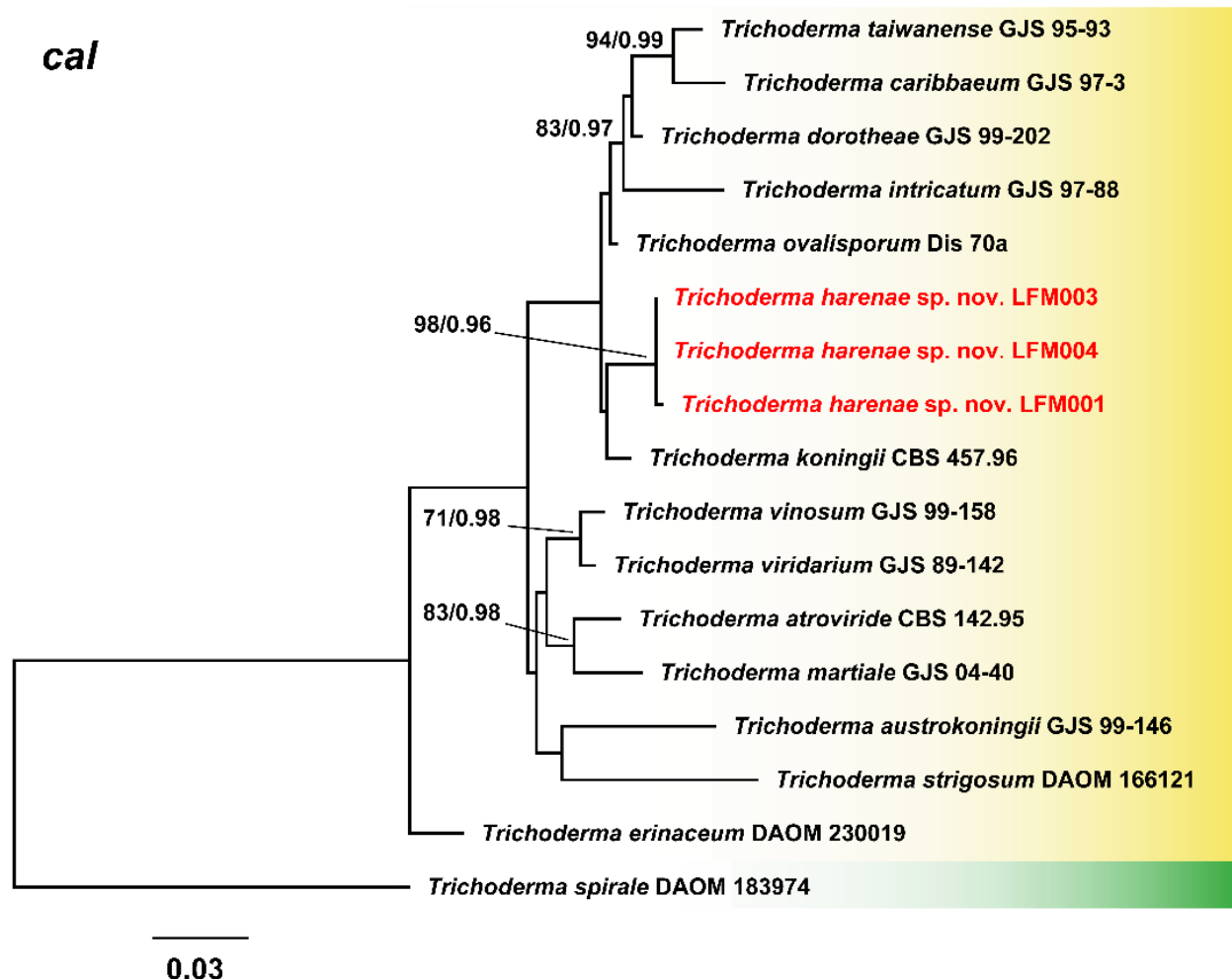


Fig. S3. Phylogenetic tree of *Trichoderma* species inferred using Maximum Likelihood (ML) and Bayesian Inference (BI) methods, with the TrNef+G substitution model applied both methods, based on the *cal* sequences. The bootstrap analysis was performed with 1000 resamplings. The numbers on the nodes represent the bootstraps values from the ML analysis, followed by the posterior probabilities (PP) from the BI. Values lower than 70% in ML and lower than 0.95 PP in BI were not considered for branch support. The evolutionary analysis involved a total of 17 distinct sequences, with 421 bp of aligned nucleotides. Only sequences from type material available in the databases (in black bold) were used for comparison purposes and are listed in Table 1. *Trichoderma spirale* (DAOM 183974) was used as an outgroup (green background). The strains reported in this study are highlighted in red bold. The scale indicates the number of substitutions per site.