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Exploring common but untapped fungal strains from olive trees to be used as biocontrol agents or plant-growth promoting

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List of abbreviation

ACT – Actin

ACCD – 1-aminocyclopropane-1-caroxylate deaminase

ANOVA – Analysis of Variance

BCA – Biological control agents

β-tubulin – Beta tubulin

BLAST – Basic Local Alignment Search Tool

CAS – Chrome Azurol S

CHS-1 – Chitin synthase 1

CIMO – Mountain Research Center

CIMOCC - Microorganism culture collection of the Mountain Research Center

DNA – Deoxyribonucleic acid

EU – Europa Union

HCN – Hidrocyanic acid

HIS – Histone H3

IAA – Indoleacetic acid

IPB – Instituto Politécnico de Bragança

IPM – Integrated pest management

ITS – Internal transcribed spacer

MEGA X – Molecular Evolutionary Genetics Analysis

ML – Maximum Likelihood

PAST – Paleontological Statistic

PCR – Polimerase chain reaction

PDA – Potato Dextrose Agar

PDB – Potato Dextrose Broth

PGPF – Plant growth-promoting fungi

PGPR – Plant growth-promoting rhizobacteria

Pi – Inorganic phosphorus

PVK – Pikovskaya Agar

RPB2 – RNA polymerase II

TEF-1α – Translation elongation factor 1-alpha

Abstract

The use of plant growth promoting microorganisms, which act directly promoting growth or indirectly as agents of biocontrol of diseases, has been gaining importance as a sustainable tool for increasing agricultural production. In this regard, microorganisms that live in association with plants, either on the surface (epiphytes) or inside (endophytes) their tissues, are seen as the most promising. These beneficial properties have been seen mainly at the level of fungi of the order Phaeomoniellales. Therefore, in this work a collection of fourteen isolates of this order previously obtained from olive leaves was characterized. In particular, we proceeded to their molecular identification using a multi-locus approach and to the evaluation of their biotechnological potential to act as biocontrol agent and plant growth promoting fungi in olive tree. The phylogenetic analysis of six loci, namely internal transcribed spacer region (ITS), actin (*ACT*), chitin synthase 1 (*CHS-1*), beta tubulin (*β-tubulin*), translation elongation factor 1-alpha (*TEF-1α*) and histone H3 (*HIS*), allowed to assigned isolates into Phaeomoniellales (eight isolates) and Eurotiales (five isolates) order. Curiously, three of the identified species (*Celerioriella umnquma*, *Pseudophaeomoniella globosa* and *Pseudophaeomoniella oleae*) were reported for the first time in Portugal. The same analysis indicated that five isolates, assigned to *Penicillium*, were previously misidentified. *Penicillium* species separation was better when *ACT*, *β-tubulin* and/or ITS are simultaneously used; while ITS, *TEF-1α* and *β-tubulin* was the best set of loci for resolving *Pseudophaeomoniella* species. The five *Penicillium* strains were screened for their ability to produce compounds frequently associated to biocontrol and plant growth promotion traits. Although none of the isolates were able to reduce significantly the *in vitro* growth of the causal agent of olive anthracnose, *Colletotrichum fioriniae*, it was found that some isolates increased significantly the production of cellulase, amylase and siderophores, when challenged by the pathogen. All five *Penicillium* isolates were able to solubilize phosphate and to produce indole acetic acid (IAA). Overall, N172, N162 (*Penicillium* sp. 2) and N299 (*Penicillium* sp. 3) isolates were the most promising as biocontrol agents and plant growth promotors. Their effect in *in planta* assays must be conducted in the future.

Keywords: Fungal diversity; Phaeomoniellales; taxonomy; phylogeny; multi-locus.

Resumo

A utilização de microrganismos promotores do crescimento das plantas, que atuam diretamente promovendo o crescimento ou indiretamente como agentes de biocontrolo, têm vindo a ganhar importância como instrumento sustentável para aumentar a produção agrícola. Neste âmbito, os microrganismos que colonizam as plantas, quer na sua superfície (epífitos) quer no interior dos seus tecidos (endófitos), são vistos como os mais promissores. Estas propriedades benéficas foram observadas principalmente ao nível dos fungos da ordem Phaeomoniellales. Assim, neste trabalho caracterizou-se uma coleção de catorze isolados desta ordem previamente obtidos a partir de folhas de oliveira. Em particular, procedeu-se à sua identificação molecular utilizando uma abordagem multi-locus e à avaliação do seu potencial biotecnológico como agente de biocontrolo e promotor de crescimento na oliveira. A análise filogenética de seis loci, nomeadamente região espaçadora transcrita interna (ITS), actina (*ACT*), quitina sintase 1 (*CHS-1*), beta tubulina (*β-tubulina*), fator de alongamento de tradução 1-alfa (*TEF-1α*) e histona H3 (*HIS*), permitiu classificar os isolados na ordem Phaeomoniellales (oito isolados) e Eurotiales (cinco isolados). Curiosamente, três das espécies identificadas (*Celerioriella umnquma*, *Pseudophaeomoniella globosa* e *Pseudophaeomoniella oleae*) foram reportadas, pela primeira vez, em Portugal. A mesma análise indicou que cinco isolados, classificados como sendo *Penicillium*, foram previamente mal identificados. A separação das espécies de *Penicillium* foi melhor quando os loci *ACT*, *β-tubulina* e/ou ITS foram simultaneamente utilizados; enquanto que a combinação ITS, *TEF-1α* e *β-tubulina* foi a melhor para separar as espécies de *Pseudophaeomoniella*. Os cinco isolados de *Penicillium* foram estudados quanto à sua capacidade de produzir compostos frequentemente associados ao biocontrolo e à promoção de crescimento das plantas. Apesar de nenhum dos isolados ter mostrado capacidade de reduzir significativamente o crescimento do agente causal da gafa, *Colletotrichum fioriniae*, em condições *in vitro*, verificou-se que alguns isolados aumentaram significativamente a produção de celulase, amilase e sideróforos, quando estimulados pelo agente patogénico. Todos os cinco isolados de *Penicillium* foram capazes de solubilizar fosfato e de produzir ácido indolacético (IAA). No geral, N172, N162 (*Penicillium* sp. 2) e N299 (*Penicillium* sp. 3) parecem ser os isolados mais promissores como agentes de biocontrolo e promotores de crescimento das plantas. O seu efeito em ensaios com plantas deve ser testado no futuro.

Palavras-chave: Diversidade fúngica; Phaeomoniellales; taxonomia; filogenia; multi-locus.

Framework and objectives

Plants are colonized by a great diversity of fungi that live within (termed as endophytes) or on the surface (termed as epiphytes) of their tissues (Trivedi et al., 2020). Some of these fungi play an important role on host plant fitness, by protecting them from both biotic and abiotic stresses and by improving plant growth (Trivedi et al., 2020). Because of their recognized importance, numerous studies have been performed in the last decade aiming to identify and explore these fungi in agriculture (Bacon & White, 2016). Some of such possible applications are as biological control agents, by protecting the plant against pests and pathogens (Gao et al., 2010), and as plant growth promotors by improving nutrient supplies to crop plants (Hossain & Sultana, 2020).

In the recent years, we have been isolating fungal endophytes and epiphytes from the olive tree aiming to explore these fungi as bioinoculants in olive groves (Costa et al., 2020). The olive tree (*Olea europaea* L.) is one of the most economically important crops for the Mediterranean countries, including Portugal (FAO, 2019). However, this crop is subject to attack by a number of pests and diseases, causing important yield losses. Among diseases, the anthracnose, is considered one of the most damaging (Talhinhas et al., 2018). Therefore, the isolated endophytes and epiphytes can be very useful for the development of alternatives to synthetic pesticides to control anthracnose. In particular, a set of fungal endophytes and epiphytes, belonging to the order Phaeomoniellales, were found in two olive tree cultivars (cv. Madural and cv. Cobrançosa) at high frequency and abundance (Costa et al., 2020). Hence, we hypothesize that they must play a relevant ecological relationship with the host, and thus might be interesting to be studied as potential microbial inoculants in olive trees. Nevertheless, these fungal strains from the order Phaeomoniellales, which have been previously isolated and are kept at the Mountain Research Center (CIMO) Culture Collection (CIMOCC) belong to yet uncharacterized and unexploited taxonomic groups.

This work aims to thoroughly characterize those Phaeomoniellales strains at phylogenetic level and evaluated some features related to biocontrol and promoting of plant growth. Specific objectives are:

(i) to evaluate their phylogenetic diversity and if they represent novel taxa within the group, by using a multi-locus sequence approach;

(ii) to assess their ability to antagonize the phytopathogen *Colletotrichum* spp., the causal agent of anthracnose, by using the dual-culture method;

(iii) to investigate if they produce compounds frequently associated to biocontrol traits, namely lytic enzymes, antimicrobial compounds (hydrogen cyanide) and iron-chelating agents (siderophores);

(iv) to investigate if they produce compounds frequently associated to plant growth promoting traits, namely indolacetic acid and phosphate solubilization.

Overall, this study will allow to determine the appropriateness of the fungal strains to be used as biocontrol agents and/or plant-growth promoters in olive trees.

1. Introduction

1.1. Olive trees: distribution and economic importance

Olive tree (*Olea europaea* L.) is considered one of the most important crops worldwide, because of their high economic value and health benefits for the importance of olive oil and table olives (Costa et al., 2021).This crop tree belongs to the order Lamiales and the family *Oleaceae*, which contains about 24 genera and 600 different species (Besnard et al., 2009; Ray et al., 2015).

Approximately 98% of olive orchards are localized in the Mediterranean region, but there are now many olive cultivars growing in more than 47 countries, some in other regions of the world (Costa et al., 2019; Rallo et al., 2018). Indeed, nowadays the olive tree crop has also been introduced in other countries such as South Africa, the Australian coast, New Zealand and China, becoming one of the most widely cultivated crops in the world (Sebastiani & Busconi, 2017).

The products derived from the olive tree, namely olive oil and table olives, are of extreme economic importance. Both products are important to the European Union (EU) economy, with this region contributing for 64% of world production, being Spain the largest producer with 39.1% (IOC, 2020). It is followed by Italy with 10.8%, Greece with 9.5%, and finally Portugal with 4% of world production (IOC, 2020). In Portugal, olive growing represents about 95.5 million euros, with the Alentejo region being the largest growing region with 50% of national production, followed by Trás-os-Montes with 22%, the Center with 18%, Ribatejo with 8% and the Algarve with 2% (Arias-Calderón et al., 2017; GPP, 2018). Moreover, the olive oil sector represents an important source of employment in these rural, depressed regions, playing a crucial role on the reduction of rural depopulation (Besnard et al., 2018). The emerging olive-oil tourism, has also been a vital instrument for rural development (Pulido-Fernández et al., 2019). These aspects are very important, in particular for some regions of the Mediterranean basin that are expected to undergo important land abandonment processes (Perpiña Castillo et al., 2020). In addition to the economic benefits, these products are beneficial to health and their consumption is recognized for aiding in wound healing, for their cardiovascular benefits and for their anti-cancer properties (Guasch-Ferré et al., 2014; Ray et al., 2015). In fact, they are characterized as very nutritious products because of their richness in

antioxidants, monounsaturated fatty acids and phenolic compounds (Lorenzo et al., 2019).

1.2. Production limitation due to diseases and pests

Pests and diseases are among the biotic factors that cause the most damage on olive crop, both in the Mediterranean region and in Portugal, and can lead to 30% in production loss (Haniotakis, 2005). Estimates point to a worsening of this scenario, because of climate change impact on the development of pests and diseases (Ponti et al., 2014). Among the pests, the olive moth and the olive fly are the most important in the olive grove, while at the level of diseases, the most important are anthracnose, peacock spot and olive knot (Barranco & Rallo, 2017).

The olive fly, *Bactrocera oleae* (Rossi, 1790) (Diptera: *Tephritidae*), is considered a key pest of olive worldwide, having as its only host plants of the genus *Olea* (Mansour et al., 2015; Tzanakakis, 2006). With a biological cycle perfectly synchronized with the phenology of the olive tree, this pest causes damage by the consumption of olive pulp by the larvae (Fig. 1) (Daane & Johnson, 2010). Thus, the losses resulting from the attack of this pest can be quantitative, resulting mainly from the premature fall and destruction of the attacked fruits, and qualitative by affecting the quality of the oil (Abdelfattah et al., 2018; Talhinhas et al., 2018). This pest can cause losses of 80% in olive oil production and in some cases total loss of production when the final product is table olives (Daane & Johnson, 2010).

Figure 1. Olive fly, *Bactrocera oleae* (Rossi, 1790). (a) Adult; (b) Larval damage on olive pulp; (c) Olive with exit hole of the adult. Source José Pereira, IPB-ESA.

Among the diseases that affect the olive tree, anthracnose is considered one of the most damaging in the countries where this crop is grown, including Portugal (Martins et al., 2019; Talhinhas et al., 2018). Olive anthracnose was first described in Portugal by Veríssimo de Almeida in 1899 (Cacciola et al., 2012). This disease, caused by various fungal species of the genus *Colletotrichum*, affects mainly the fruit, but also other organs of the olive tree, including flowers, leaves and branches (Fig. 2) (Martins et al., 2019). Thus, the characteristic symptoms of the disease are blackish spots, generally in depression, with abundant production of orange mucilaginous masses of conidia on the epicarp of the fruit,which appear mainly when they are almost ripe (Cacciola et al., 2012). The appearance of these spots causes mummification, rotting and premature fall of the fruit, leading to significant crop losses. Infection can occur in any part of the fruit, but is more frequent in the apex, because it remains wetter for longer periods. In the vegetative organs (leaves and branches), the symptoms include leaf chlorosis, dehydration, followed by defoliation and apical death of the branches, contributing to weakening of the tree, and consequently affecting the production of the following year. Infected flowers dry up and tend to fall off (Cacciola et al., 2012; Moral et al., 2008). Besides being responsible for considerable losses in olive production, this disease also contributes to a marked reduction in olive oil quality (Moral et al., 2014; Silva, 2016; Talhinhas et al., 2018). Usually, olive oil derived from infected olives is of poor quality due to increased acidity and peroxidase, causing a decrease in oxidative stability and phenolic compounds (Carvalho et al., 2008). At national level, this disease is very common and often reaches epidemic levels, sometimes causing total loss of income (Talhinhas et al., 2011). In Trás-os-Montes (Northeast of Portugal), there has been an increase in the incidence and severity of anthracnose in recent years (Silva, 2016). This fact may be related to the change in environmental conditions that favor the development of the disease, requiring a relative humidity above 90% and temperature between 17-20ºC (Cacciola et al., 2012). In addition to environmental conditions, there is also a variable susceptibility to the disease at the level of the different cultivars. As far as Portuguese cultivars are concerned, the cv. Cobrançosa variety has a low incidence and severity of the disease, while the cv. Galega Vulgar variety is very susceptible, generally showing higher levels of disease severity (Moral et al., 2017).

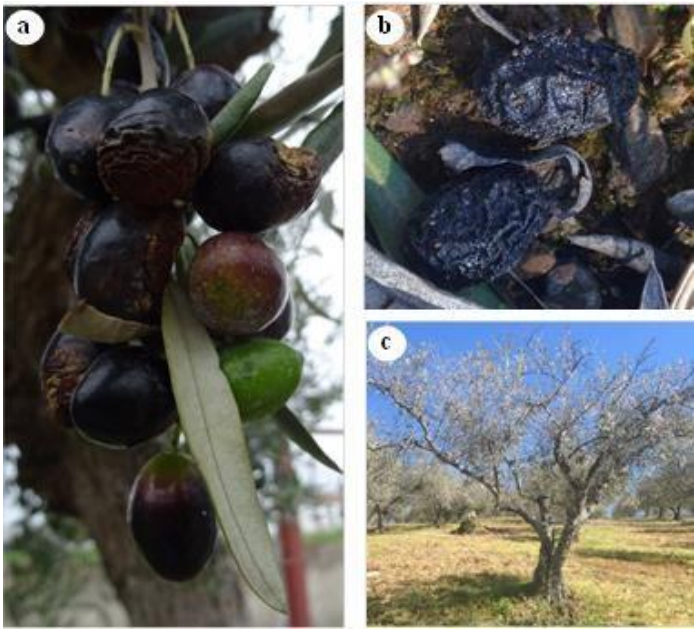

Figure 2. Typical symptoms of olive anthracnose. (a) Appearance on the surface of the olive of an orange mass of conidia in depressed necrotic patches; (b) under dry conditions, infected fruit dehydrate and mummify; (c) infected leaves may fall off causing defoliation of the tree. Source Paula Baptista, ESA-IPB.

In Trás-os-Montes region, *C. godetiae* is the most frequent causal agent of anthracnose, although *C. nymphaeae* and *C. rhombiforme* have also been isolated (Talhinhas et al., 2011). There are some reports that associate the severity of anthracnose with the intensity of olive fly attack (Cacciola et al., 2012). In fact, it is believed that this insect favors the spread of *Colletotrichum* spp. and aids in the infection process by creating holes in the fruit, which may act as an entry point for the pathogen (Cacciola et al., 2012).

1.3. Methods for the control of pests and diseases of the olive tree, and the case of anthracnose

The management of olive pests and diseases has led to the widespread use of pesticides (Daane & Johnson, 2010; Loureiro et al., 2018). The control of the olive fly is primarily done with chemical insecticides, but their use raises relevant problems such as the contamination of the environment, development of resistance to insecticides, and negative impacts on the health of farmers and consumers (Daane & Johnson, 2010).

The control of olive anthracnose is very difficult because its spreading and development relies greatly on the climatic conditions. Hence, no effective control measures have been proposed so far to manage this disease. At present, management of anthracnose is based on the use of a combination of several approaches, including

cultural, chemical and biological control. In disease prevention, the aim is to reduce the inoculum levels of the causal agents and their spread (Martins et al., 2019). This can be achieved using various agricultural practices, including removing diseased branches and increasing canopy aeration through pruning, better management of fertilizer application and soil drainage through irrigation, use of anthracnose tolerant/resistant varieties, and control of insects, such as the olive fruit fly, that could potentially spread the pathogen, among others (Loureiro et al., 2019; Moral et al., 2014). In direct methods, copper-based fungicides, such as copper sulphate, copper hydroxide and copper oxychloride are used (Cacciola et al., 2012). This strategy is ineffective since under favorable environmental conditions the disease develops, because *Colletotrichum* species are resistant to copper (Loureiro et al., 2019). Moreover, since the widespread use of these fungicides poses potential environmental and human health risks (Komárek et al., 2010), the European authorities is about to ban copper-based fungicides in conventional and organic farming across the EU (La Torre et al., 2018). Thus, the use of alternative methods of control in crop protection is favored, giving priority to non-chemical methods and practices, within integrated pest management (IPM) programs. IPM is an effective and environmentally approach that relies on a combination of indirect (e.g. preventive) and direct (e.g. control) means for disease and pest control (Boller et al., 1998). In the first case, it aims to manage the crop by using natural resources and farming practices with no negative impact on ecosystems, in order to protect and enhance natural antagonists. This first line of pest control aims to prevent pests from becoming a threat. When preventive methods are no longer effective, then proper control methods must be employed (direct control). Effective and less risky pest control that act exclusively on the target organisms, by applying more selective means, are the most frequently chosen methods (Moral et al., 2018). In the case of olive groves in organic or integrated production modes, a set of techniques are used that are first preventive, then direct control with low risks and, as a last solution, chemical control (DGAV, 2017).

One of alternative for the control of pests and diseases of olive crop may be the use and exploitation of biocontrol agents (Moral et al., 2014, 2015), including microorganisms. These microorganisms could play an important role in the control of pests as entomopathogens (e.g. parasites and insects), and diseases as antagonists (generally called biological controlagents - BCA). There are some successful examples of the application of these microorganisms in the control of olive tree pests and diseases.

For example, some entomopathogenic fungi of the order Hypocreales have been shown under laboratory conditions to be effective against olive fruit fly (Yousef et al., 2013). Currently there are a scarce number of studies that have evaluated the efficacy of antagonists in the control of olive anthracnose, particularly under field conditions. Only recently, it was reported that the application in olive groves of the endophyte *Aureobasidium pullulans*, a ubiquitous yeast widely used as BCA in several plants (Freimoser et al., 2019), significantly reduced the severity of anthracnose (Nigro et al., 2018).

1.4. Microorganisms associated to the phyllosphere and their potential as biocontrol and plant-growth promoting agents

Phyllosphere refers to the aerial or above ground parts of plants colonized by microbial communities that live associated to those plants, including on the leaves (Legein et al., 2020). Some of these microorganisms, termed as pathogens, have a deleterious effect on host plant causing diseases. Pathogens usually first have an epiphytic phase before passing into plant tissues, where they will occupy the cells and/or the intercellular space (Legein et al., 2020). In contrast, other microorganisms inhabiting plant tissues possess biocontrol properties which help sustaining plant health, for example by inhibiting the growth of microbial pathogens. The ability to protect host plant from biotic stresses are more commonly described to microorganisms that inhabit the internal tissues of the plant (termed as endophytes) than those that colonized the surface of host plants (termed as epiphytes). The protection conferred specifically by fungal endophytes against pests and pathogens is largely attributed to endophytic production of secondary metabolites in colonized plants. These compounds may suppress pathogen infections either directly, by antibiosis, mycoparasitism and competition, or indirectly by induction of plant defense system (Latz et al., 2018). In the case of direct effect, fungal endophytes directly suppress pathogens by producing antibiotics (terpenoids, alkaloids, aromatic compounds and polypeptides) and secreting lytic enzymes, such as chitinases, cellulases and β-1,3-glucanases, to degrade the cell wall of the pathogen during the process of the mycoparasitism (reviewed by Gao et al., 2010). In what concerns volatile compounds, the hydrocyanic acid (HCN), has been reported as highly effective in suppressing the growth of phytopathogens (Prasad et al., 2017). This compound can degrade the cell wall of the fungal pathogen and render its pathogenicity (Hussain et al., 2016). In the competition, the endophyte competes with the pathogen by acquiring the essential nutrients and space in the plant,

or inclusively infection sites, and thus preventing the colonization of the host plant by pathogens (Latz et al., 2018). The production of siderophores is one of the major mechanisms of fungal endophytes to inhibit the growth of pathogens via competition of nutrients. Siderophores are small, high-affinity iron-chelating molecules, that are produced by the endophyte in iron-limited conditions to sequester the less-available iron from the environment and thereby deprive the pathogen of iron (Kannan & Bastas, 2015). Based on their functional groups, siderophores are categorized into three families, namely, catecholate, hydroxamates, and carboxylates (Kannan & Bastas, 2015). Endophytes can also reduce the infection of plant tissues by pathogens indirectly by triggering a defense response in the host, in which occurs the differential expression of genes, protein synthesis and specific metabolic changes (Latz et al., 2018). This induced resistance can be local or systemic (Latz et al., 2018).

Protection of plants against pest is mainly due to the production of compounds by endophytes that are toxic to insects and/or may repel the insect (Latz et al., 2018). Ergot alkaloids and neurotoxins are considered the main compounds produced against pests (Latz et al., 2018). These compounds produced by endophytes are able to reduce insect weight, growth and survival, and even make the plant less attractive and unpalatable to insect pests (Azevedo et al., 2000). Endophytes can also alter the composition of the host organic volatile compounds making its olfactory properties more attractive to natural enemies of the insect pest (Fuchs & Krauss, 2019). However, up to date, it remains unclear whether the endophyte produces volatiles or induces a change in plant derived volatiles (Fuchs & Krauss, 2019).

Currently, numerous biocontrol researches are being conducted studies in many commercial plants, aiming to evaluate the use of endophytic and/or epiphytic microbial strains as BCAs (defined similarly as probiotics) and elucidate their mechanism of action when interacting with pathogens and/or the host plant (Köhl et al., 2019; Massart et al., 2015). Some relevant examples include the fungal species from the genera *Aureobasidium*, *Beauveria* and *Trichoderma*, which are ubiquitous, broad host-range BCAs showing dissimilar effectiveness in different plants and/or diseases (Köhl et al., 2019; Legein et al., 2020). Another possibility to bioprospecting for microbial BCAs aimed to be used for a particular plant-pathogen system, is to seek for more host-specialized microorganisms, thus preventing their propagation into other plant species (Brodeur, 2012).

Beside these fungi with antagonistic properties, plant growth-promoting fungi (PGPF) are also biotechnologically relevant since they enhance plant growth and indirectly protect plants from pests and abiotic stresses (Compant et al., 2005; Hossain

et al., 2017). PGPF mechanisms to promote plant growth are not fully understood. It is widely accepted that PGPF can promote plant growth directly due to the production of (i) the enzyme 1-aminocyclopropane-1-caroxylate deaminase (ACCD) that depress the ethylene level in the plant, (ii) of phytohormones (auxins, cytokinins, gibberellins, ethylene, and abscisic acid), (iii) of volatile compounds that affect the plant signaling pathways, or (iv) by facilitating the uptake of mineral nutrients, such as nitrogen, phosphorus, potassium, zinc, and iron (Argumedo-Delira et al., 2022; Maraghy et al., 2021). The solubilizing and mineralizing of nutrients by PGPF involve the production of several metabolites, including organic acids, enzymes, such as phosphatases, and iron-chelating siderophores (Maraghy et al., 2021). For example, the organic acids produced by PGPF increase the release of inorganic phosphorus (Pi) into soil solutions and thereby improve phosphorus utilization in soils. Tri- and di-carboxylic acids, such as citric and oxalic, are stronger P-solubilizers than monocarboxylic acid (gluconic acid) (Mendes et al., 2021). Additionally, PGPF can benefit plant growth indirectly, by protecting the plant against pathogens via antibiosis, competition for space and nutrients and induction of systemic resistance (Hossain et al., 2017). This process relies on the production of metabolites by the PGPF, including antibiotics, siderophores, HCN, and cell wall-degrading enzymes (Naziya et al., 2019). Several fungi belonging to the genera *Trichoderma*, *Penicillium*, *Aspergillus*, *Fusarium*, *Mortierella*, *Phoma*, and *Piriformospora* have been reported to be efficient plant growth promoters (reviewed by Argumedo-Delira et al., 2022).

Hence, the application of PGPR-based formulations may be a better environmental friendly approach to increase crop production and health when compared to synthetic fertilizers and agrochemicals. PGPR-based formulations can increase soil fertility, promote plant growth, and suppress phytopathogens, and thus maintaining agricultural productivity.

1.5. Microorganisms from and applied to olive trees, and the case of Phaemoniellales

Bizos et al. (2020) revise the use of microbial inoculants for olive tree plant protection and growth, including PGPF and endophytes, and recognize that *Trichoderma* spp. is the most effective BCA against olive tree diseases, including anthracnose. Other fungal endophytes that shown some promising results to control anthracnose in olive trees include *Alternaria* spp., several *Aspergillus* spp., *Chaetomium*

globosum, *Chondrostereum purpureum*, *Diaporthe* spp., *Epicoccum nigrum*, *Quambalaria cyanescens*, and *Nigrospora oryzae* (Poveda & Baptista, 2021), some of them (e.g. *C. purpureum,Diaporthe*, *Q. cyanescens*) regarded as plant pathogens in other species.

In addition to these broad-spectrum species, unique, olive-tree native microorganisms may be interesting as a source of strains to be applied as BCAs and/or PGPF. Following a high throughput ITS metabarcoding approach, Costa et al. (2021) studied the fungal endophytic community of the phyllosphere of five Portuguese cultivars, exposing the microbial diversity associated to the olive tree. One of the fungal groups that has received enhanced interest in recent years is the Phaeomoniellales. This order was recently established by Chen et al. (2015) and is placed within the *Eurotiomycetes* (Pezizomycotina, Ascomycota), encompassing mainly endophytes but also plant pathogens. For example, *Phaeomoniella chlamydospora*, which can cause Petri disease by blocking plant host vessels (e.g. in grapevine crops; Kraus et al., 2020), has also been isolated from healthy and dead plant tissues and so it is suggested to be an endophyte or saprotroph (Chen et al., 2015). Based on a seven-locus phylogeny, Chen et al. (2015) named five genera in the Phaeomoniellales – *Dolabra*, *Moristroma*, *Celothelium*, *Xenocylindrosporium* and *Phaeomoniella*. Some more genera were recently erected such as *Aequabiliella*, *Minutella*, *Nothophaeomoniella*, *Pseudophaeomoniella* and *Neophaeomoniella* (Crous et al., 2021; Kraus et al., 2020). There are two *Pseudophaeomoniella* species, *P. oleae* and *P. oleicola*, that so far were uniquely described for olive trees, indicating possible host specialization of some of Phaeomoniellales taxa, something already suggested by Chen et al. (2015). Besides these two *Pseudophaeomoniella* spp., very little is known about Phaeomoniellales in olive trees. In the study of Costa et al. (2021), the authors found that Phaeomoniellales represents less than 1.5% of the total fungal community. However, in a distinct, culture-dependent study from Costa et al. (2020) it was observed that strains from Phaemoniellales, either endophytes or epiphytes, appeared in high abundance in the studied olive trees. Interestingly, most of the isolates could not be taxonomically assigned to any of the already described genera from this fungal group (Costa et al., 2020).

2. Material and methods

2.1. Fungal isolates and inocula production

In total, fourteen (14) fungal strains (10 epiphytic and 4 endophytic) were characterized in this study (Table 1). These fungi were previously isolated from symptomless leaves of two different olive tree cultivars, cv. Madural and cv. Cobrançosa, growing in an organic olive grove in *Vale de Telhas* (41º36'28.6 "N7º13'26.7 "W), Mirandela (Northeast of Portugal) as referred in Bahri et al. (2020) and Costa et al. (2020). The ability of these fungal strains to suppress phytopathogens growth was tested using the causal agent of olive anthracnose, *Colletotrichum fioriniae* (CIMO 15FM001). This phytopathogen was previously isolated from the inner tissues of naturally infected olives and identified by sequencing the internal transcribed spacer region (ITS) of ribosomal nuclear DNA, in particular the region ITS1–5.8S–ITS2, as well as the protein-coding genes translation elongation factor 1-alpha (*TEF-1α*), RNA polymerase II (*RPB2*), beta tubulin (*β-tubulin*) and histone H3 (*HIS*). All these fungal strains are deposited at CIMOCC, the microorganism culture collection of the Mountain Research Center (CIMO) of the Instituto Politécnico de Bragança (IPB) and are preserved in 30% glycerol at -86 ºC.

The fungal inoculum used in the present study for molecular characterization and for evaluation of biocontrol/plant growth promotion traits were prepared from frozen stocks by transferring fungal spores/mycelium to Potato Dextrose Agar (PDA) culture medium. The fungi were grown at room temperature for 10-15 days, in the dark, and the spores produced were then scraped from the agar plates with a sterile rod, and suspended on 30 mL of sterile 0.025% (v/v) Tween 80. The concentration of fungal spores was adjusted to $1x10^6$ spore/mL with sterile 0.025% (v/v) Tween 80, in a Neubauer haemocytometer, under light microscope (Leica DM500), and further used as inoculum.

Table 1. Fungal strains characterized in the present study.

Strains (CIMO code) / (Abbreviation)	**Host cultivar**	**Leaf habitat**
Unassigned *Phaeomoniellaceae* (CIMO 19DM028) / (N028)	Cobrançosa	Epiphytic
Unassigned *Pseudophaeomoniellaceae* sp.5 (CIMO 19DM032) / (N032)	Madural	Epiphytic
Pseudophaeomoniella sp.1 (CIMO 19DM058) / (N058)	Madural	Endophytic
Pseudophaeomoniella sp.2 (CIMO 19DM060) / (N060)	Madural	Endophytic
aff. Celerioriella sp. (CIMO 19DM162b) / (N162)	Cobrançosa	Epiphytic
Unassigned *Phaeomoniellaceae* sp.16 (CIMO 19DM163) / (N163)	Madural	Epiphytic
Unassigned *Phaeomoniellaceae* sp.11 (CIMO 19DM172) / (N172)	Madural	Epiphytic
Unassigned Phaeomoniellales sp.1 (CIMO 19DM173) / (N173)	Madural	Epiphytic
Unassigned *Phaeomoniellaceae* sp.11 (CIMO 19DM226) / (N226)	Madural	Endophytic
Pseudophaeomoniella aff. oleícola (CIMO 19DM269) / (N269)	Cobrançosa	Epiphytic
Pseudophaeomoniella oleae (CIMO 19DM271) / (N271)	Cobrançosa	Epiphytic
Unassigned *Phaeomoniellaceae* sp.11 (CIMO 19DM294) / (N294)	Cobrançosa	Endophytic
Unassigned Phaeomoniellales sp.1 (CIMO 19DM299) / (N299)	Madural	Epiphytic
Pseudophaeomoniella oleae (CIMO 19DM300) / (N300)	Madural	Epiphytic

2.2. Molecular characterization and phylogenetic analysis of fungal strains

2.2.1. PCR amplification of the target genes

For the molecular characterization of the fungal isolates, the genomic DNA was first extracted from mycelium harvested from fully grown cultures in PDA medium. The DNA was extracted using the REDExtract-N-Amp™ Plant PCR Kit (Sigma-Aldrich, USA), following the supplier's instructions. After extraction, fungal isolates were molecularly identified by using a multi-gene phylogenetic approach. The genes used included the ITS of ribosomal nuclear DNA, in particular the region ITS1–5.8S–ITS2, but also the protein-encoding genes actin (*ACT*), chitin synthase 1 (*CHS-1*), beta tubulin (*β-tubulin*), translation elongation factor 1-alpha (*TEF-1α*) and histone H3 (*HIS*). Primers specific for the amplification of these genes are presented in Table 2.

Table 2. Primers used for the amplification of the different genes in the fungal isolates.

Gene region	Primers	Primers sequence (5'-3')	Optimal annealing temperature
TEF-1α	EF1-728F	CATCGAGAAGTTCGAGAAGG	50-54ºC
	EF1-986R	TACTTGAAGGAACCCTTACC	
CHS-1	CHS-79F	TGGGGCAAGGATGCTTGGAAGAAG	50-60ºC
	CHS-354R	TGGAAGAACCATCTGTGAGAGTTG	
ACT	ACT-512F	ATGTGCAAGGCCGGTTTCGC	50-60ºC
	ACT-783R	TACGAGTCCTTCTGGCCCAT	
HIS	CYLH3F	AGGTCCACTGGTGGCAAG	50-60ºC
	CYLH3R	AGCTGGATGTCCTTGGACTG	
β-tubulin	Bt2a	GGTAACCAAATCGGTGCTGCTTTC	50-54ºC
	Bt2b	ACCCTCAGTGTAGTGACCCTTGGC	
ITS	ITS_1	TCCGTAGGTGAACCTGCGG	52-56ºC
	ITS_4	TCCTCCGCTTATTGATATGC	

PCR amplification of the different genes was performed in a 20 µL reaction mixture as indicated in Table 3. The amplifications were performed in a BioRad T100™ Thermal Cycler (Hercules, CA, USA), For amplification of the ITS region a set program was used for an initial denaturation at 95°C for 5 minutes, followed by 34 cycles consisting of a denaturation step at 95°C for 40 seconds, an annealing step at variable temperature ranging from 50°C-60°C depending of the gene amplified (Table 2), and an extension step at 72°C for 45 seconds, followed by a final extension at 72 °C for 7 minutes. For the amplification of *TEF-1α* gene, the temperature cycling used involved a first denaturation step at 94°C for 5 minutes, followed by 35 cycles consisting of a denaturation step at 94°C for 45 seconds, an annealing step 50-54°C, and an extension step 72°C for 1 minute, followed by a final extension at 72ºC for 6 minutes. For the *HIS*, *ACT*, *β-tubulin* and *CHS-1* region, the temperature cycling used involved a first denaturation step at 95ºC for 5 minutes, followed by 35 cycles consisting of a denaturation step at 95ºC for 40 seconds, an annealing step 50-60ºC, and an extension step 72ºC for 45 seconds, followed by a final extension at 72ºC for 7 minutes. Sterile ultrapure water was included as negative control during the amplification of genomic DNA.

Table 3. Components and volumes used for the 20 µL PCR reactions.

Components	Volume (µL)	Final concentration
PCR reaction buffer (10X)	2	1X
Primer forward (10 µM)	0.4	0.2 µM
Primer reverse (10 µM)	0.4	0.2 µM
dNTPs (10 mM)	0.4	0.2 mM
DNA polymerase (5 U/µL) [1]	0.1	0.025 U/µL
ddH_2O [2]	14.1	-
DNA template (5-50 ng/µL)	1.6	0.4-4 ng/ µL

[1]DFS-Taq DNA polymerase (Bioron, Germany)
[2] Autoclaved distilled deionized water

All PCR products were analyzed by agarose gel electrophoresis (1.3%, w/v) in TBE buffer (1X) (89 mM Tris base, 89mM boric acid and 2 Mm EDTA, pH 7.6), in BioRad horizontal vials. PCR products (5µL) and the molecular marker 1Kb DNA Ladder (Bioron, Germany) were loaded onto the gel along with (2µL) of Loading Buffer (6X) (Bioron, Germany). Electrophoretic separation was carried out at a potential difference of 100 V for 30-45 minutes and, the separated bands were stained in an aqueous solution of GelRed™ Biotum (3X) for 20-30 minutes. The DNA amplified were visualized by fluorescence under ultraviolet radiation using a ChemiDoc™ XRS+with Image Lab™ Software from BioRad (Hercules, California). The amplified products were purified and sequenced by Macrogen Inc services (Madrid, Spain).

2.2.2. Sequence alignment and phylogenetic analyses

The contigs (formed from forward and reverse sequences) obtained were analyzed separately for each gene using the bioinformatics program Geneious (Biomatters, 2021). The fungal isolates were preliminary identified using the Nucleotide Basic Local Alignment Search Tool (BLASTn), with and without the 'Sequences from type material' selected, in the NCBI database (https://www.ncbi.nlm.nih.gov/) , that determine the degree of similarity between the sequences entered and those deposited in the database, by performing alignments. The results of these alignments were ranked by highest percentage of identity and lowest E-value.

The phylogenetic analysis of the isolates obtained, was performed using the software MEGA X software (Kumar et al., 2018). Accordingly, raw sequences of the reference strains with the best result in terms of % similarity (obtained in the BLAST) as

well as the sequences obtained in the present study were assembled, and further aligned. All multiple sequence alignments were done using ClustalW. Phylogenetic reconstructions of concatenated and individual gene trees were performed using the Maximum Likelihood (ML) method, based on the Kimura 2-parameter (Kumar et al., 2018), with 1000 bootstrap repetitions. According to the Corrected Akaike information criterion, the selected replacement models were K2+G for all phylogenies. The percentage taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. All sequences generated were deposited in GenBank (accession numbers not available yet). Detailed information about fungal sequences characterized in this study is summarized in Table S1 (Supporting information).

2.3. Evaluation of biocontrol traits on fungal isolates

From the fourteen fungal isolates, five (CIMO 19DM028, CIMO 19DM032, CIMO 19DM162, CIMO 19DM172 and CIMO 19DM299) were screened for their ability to suppress phytopathogenic growth and to produced metabolites with antagonistic proprieties. The selection of these five fungal strains was based on their ability to growth and produce spores on common artificial media. Indeed, for the utilization of these fungi as a commercial biocontrol agent, the fungus has to be mass-produced easily.

2.3.1. Antagonistic activity

The antagonist activity of fungal isolates was evaluated against the causal agent of anthracnose, *C. fioriniae*, by using the co-culture method. Accordingly, Petri dishes (9 cm diameter) containing 10 mL of PDA medium were inoculated with 10 µL of spore suspension (10^6 spores/mL) of each fungus, on opposite sides of the plate at a distance of 3 cm from each other. Cultures with two inocula of the same fungal species were used as controls. Five replicates of each combination were performed, and the plates were sealed with parafilm and incubated in the dark at 25 ± 2ºC. During interaction, the internal radial growth of the pathogen towards the interacting fungus was measured at two days-time intervals, by using a graduated ruler, until no further growth was observed, at least for one of the interacting species. The growth rate of the pathogen (cm/day) was determined by dividing the internal radius (in cm) by the number of incubation days at the time of measurement.

2.3.2. Production of metabolites with antagonistic proprieties

The metabolites surveyed on the five fungal isolates included lytic enzymes, namely cellulase, amylase and β-1,3-glucanase, antimicrobial compounds (hydrogen cyanide) and iron-chelating agents, like siderophores. The assessment of these compounds was performed using co-cultures established between the pathogen *C. fioriniae* and the fungal isolates, as described above. This procedure was used because the production of these metabolites by the fungal isolates can be only induced after exposure to the pathogen. As controls, fungal pairings of the same isolate were prepared.

For the assessment of lytic enzymes, the co-cultures were established in PDA media containing the enzyme substrates. Accordingly, carboxymethyl cellulose 0.5% (w/v) (Lobo et al., 2005), soluble starch 2% (w/v) (Lobo et al., 2005) and laminarin 1.5% (w/v) (Katatny et al., 2001) was used for the detection of cellulase, amylase and β-1,3-glucanase, respectively. The plates were incubated in the dark, at 25 ± 2ºC, for 4–7 days. After this period, the production of amylase production was detected by flooding the plates with 1% (w/v) iodine in 2% (w/v) potassium iodide solution and drained off to observe a clear halo zone around the colony. For both cellulase and β-1,3-glucanase detection, the plates were flooded with 0.2% (w/v) congo red dye for 15 min and washed with 1N NaCl and 1N NaOH for 15 min respectively till the appearance of a clear zone around the colony.

The hydrogen cyanide (HCN) production was estimated following a modified protocol of Miller (1970). Briefly, co-cultures were established in PDA medium supplemented with glycine (4.4 g per liter). A Whatman filter paper No. 1 soaked in picric acid (0.5%; w/v) and sodium carbonate (2%; w/v) was placed in the upper lid of the petri plate. The Petri plates were sealed with parafilm and further incubated in the dark, at 25 ± 2ºC, for 4–7 days. After this time changes in filter paper color from the original yellow /orange to red or brown were considered HCN positive. Scoring was done as weak (yellow to light red), moderate (brown), and strong (reddish brown).

Siderophores production was evaluated using Chrome azurol S (CAS) medium, prepared according to Pérez-Miranda et al. (2007). CAS medium (10 mL) was plated in 9 cm Petri plates, followed by the application of a PDA medium overlay (10 mL). Dual cultures were established in the surface of this overlay. After an incubation period at 25

± 2ºC in the dark, for 4–7 days, the orange zone formed around the fungal colonies was indicative of siderophore production.

For all these assays were performed five replicates for each fungus-pathogen combination. The level of enzyme activity and siderophore production was evaluated by subtracting the diameter of the fungal colony plus halo from the diameter of the fungal colony (in cm). For each fungal isolate, the percentage of increase on lytic enzyme or siderophore production in the presence of the pathogen was determined by using the equation [(ADC-AC)/AC]*100, where AC is the lytic enzyme / siderophore/production in control plates and ADC is the same in dual-culture assay.

2.4. Evaluation of plant growth promotion traits

Plant growth-promoting traits evaluated in the five fungal isolates included indolacetic acid (IAA) and phosphate solubilization. IAA production by fungal isolates was estimated in Potato Dextrose Broth (PDB) medium supplemented with L-tryptophan, which is the main precursor for the synthesis of IAA (Spaepen & Vanderleyden, 2011). Accordingly, 20 mL of PDB supplemented with 1g/L of L-tryptophan was inoculated with four mycelial plugs (5 mm diameter) removed from fungal cultures. After incubation at room temperature, for one week with shaking (100 rpm), the cultures were filtrated using a Whatman® filter paper. The production of IAA was measured by mixing 1mL of culture supernatant with 1mL of Salkowski's reagent (49mL 35% $HClO_4$, 1mL 0.5M $FeCl_3$) in 2 mL tubes. The tubes containing the mixture were incubated in the dark, at 30°C with agitation (250 rpm), for 30 min, for color development. The intensity of color was measured in a spectrophotometer at 536 nm using a standard curve for calibration by using authentic IAA in µg/mL (concentration ranging from 0 to 20 µg/mL; equation $y= 0.0324x – 0.023$, $R^2= 0.993$). Three replicates per fungal isolate were performed, being the results expressed in µg/mL.

The ability of the fungal isolates to solubilize phosphate was assessed by using the modified Pikovskaya agar (PVK) medium containing calcium triphosphate (5g/L) as insoluble phosphate source (Doilom et al., 2020). Accordingly, 10 µL of fungal inoculum, was placed onto the surface of PVK medium in the center of 9 cm Petri dishes. The plates were incubated at room temperature in the dark for 4–7 days. After this period, the halos appearing around the colonies were measured with a ruler (in cm). For each fungal isolate, five replicates were done.

2.5. Statistical analysis

The statistical analysis of the results was performed using the Past software version 4.03 (Hammer et al., 2001). First, the Shapiro-Wilk and Levene tests were used to analyze the normality and homogeneity of variance of the data obtained, respectively. In the case where normality and homogeneity of variance were not observed, non-parametric tests were applied, namely the Kruskal-Wallis Test. When the data followed a normal distribution and homogeneity of variances, the one-way ANOVA with Tukey's pairwise comparison as post hoc test for multiple comparisons ($p < 0.05$) was applied.

3. Results and discussion

3.1. Phylogenetic analysis based on single molecular marker

In this study, fourteen olive leaf fungal isolates from the CIMO microbial culture collection were molecularly characterized using a multi-locos PCR analysis. The different molecular markers used included the ribosomal marker ITS (ITS1, 5.8S and ITS2), and the protein-coding genes actin (*ACT*), chitin synthase 1 (*CHS-1*), *β-tubulin*, translation elongation factor 1-alpha (*TEF-1α*) and histone H-3 (*HIS*).

3.1.1. Internal transcribed spacer (ITS)

The nuclear ribosomal RNA internal transcribed spacer (ITS) region constitutes one of the most widely applied molecular markers in phylogenetic studies and species differentiation in fungi (Oliveira & Azevedo, 2022). These ribosomal RNA genes are present in clusters of tandem repeat units, being found several hundreds of copies per genome (Martin & Rygiewicz, 2005). In particular, the ITS region used as molecular marker comprises two non-coding regions (ITS1 and ITS2) and the coding region 5.8S. The 5.8S gene shows a slow rate of evolutionary change, being the level of sequence variation of the two ITS regions higher and thus preferably used to infer species fungal differentiation (Martin & Rygiewicz, 2005). All the fourteen fungal isolates analyzed in the present study were positive to PCR amplification of ITS regions, with amplicon sizes around the 600 base pairs (bp). Although, the ITS region is considered the universal DNA barcode for species-level identification of fungi (Schoch et al., 2012), it was not sufficient for identifying to species level all the fungal isolates analyzed. Indeed, some of the fungal isolates (N269) did not match with any ITS reference sequence in the databases, or it was only identified to class level (N163 - Dothideomycetes) (Table S1; Supporting information). An unexpected result was the detection of a number of fungal isolates that were misidentified in the CIMOCC. From the fourteen fungal isolates analyzed from this microbial collection, eight were identified as belonging to Phaeomoniellales order. The remaining six isolates, previously assigned to Phaeomoniellales order in the CIMOCC, were identified as belonging to different orders (five isolates belonged to Eurotiales order, *Aspergillaceae* family and *Penicillium* genus, and one isolated belonged to Dothideomycetes class).

The phylogenetic tree generated based on ITS sequences revealed that the fungal isolates were clustered in grouping patterns of close resemblance, being identified three

distinct subclusters (Fig. 3). The first comprised a larger subcluster with eight isolates belonging to *Celotheliaceae* and Incertae sedis families. The second subcluster encompasses five isolates belonging to *Aspergillaceae* family, and the third subcluster contained only one isolate belonging to Dothideomycetes class. *Trichoderma harzianum*, from the family *Hypocreaceae*, was used as an outgroup. Moreover, the phylogenetic tree generated showed that a number of fungal isolates could not be discriminated among species. Indeed, the ITS sequence (ITS1, 5.8S and ITS2) was not able to discriminate thee species (N028, N162, N172), belonging to the same genus (*Penicillium*). Previous studies have similarly reported insufficient variability on ITS sequences for some fungal genera, including *Penicillium* (Schoch et al., 2012; Skouboe et al., 1999; Visagie et al., 2014). Therefore, we opted to used additional molecular markers for accurate identification of fungal species and phylogenetic relationships, as previously suggested by other authors (Houbraken et al., 2020; Visagie et al., 2014). In this regard, protein-coding genes have been recommended to be used in combination to ITS for species identifications of certain fungal groups (Oliveira & Azevedo, 2022). It is likely that protein-coding genes are better than ITS region for species identification of certain fungal groups (Raja et al., 2017), because of the presence of intron regions in protein-coding genes, which sometimes evolve at a faster rate compared to ITS (Geiser, 2004). Moreover, these genes allow for an easy recognition of homology and convergence, as they are believed to occur as a single copy in fungi, are less variable in their length, and are easier to align over rRNA genes, as they contain less ambiguity due to codon constraints (Raja et al., 2017).

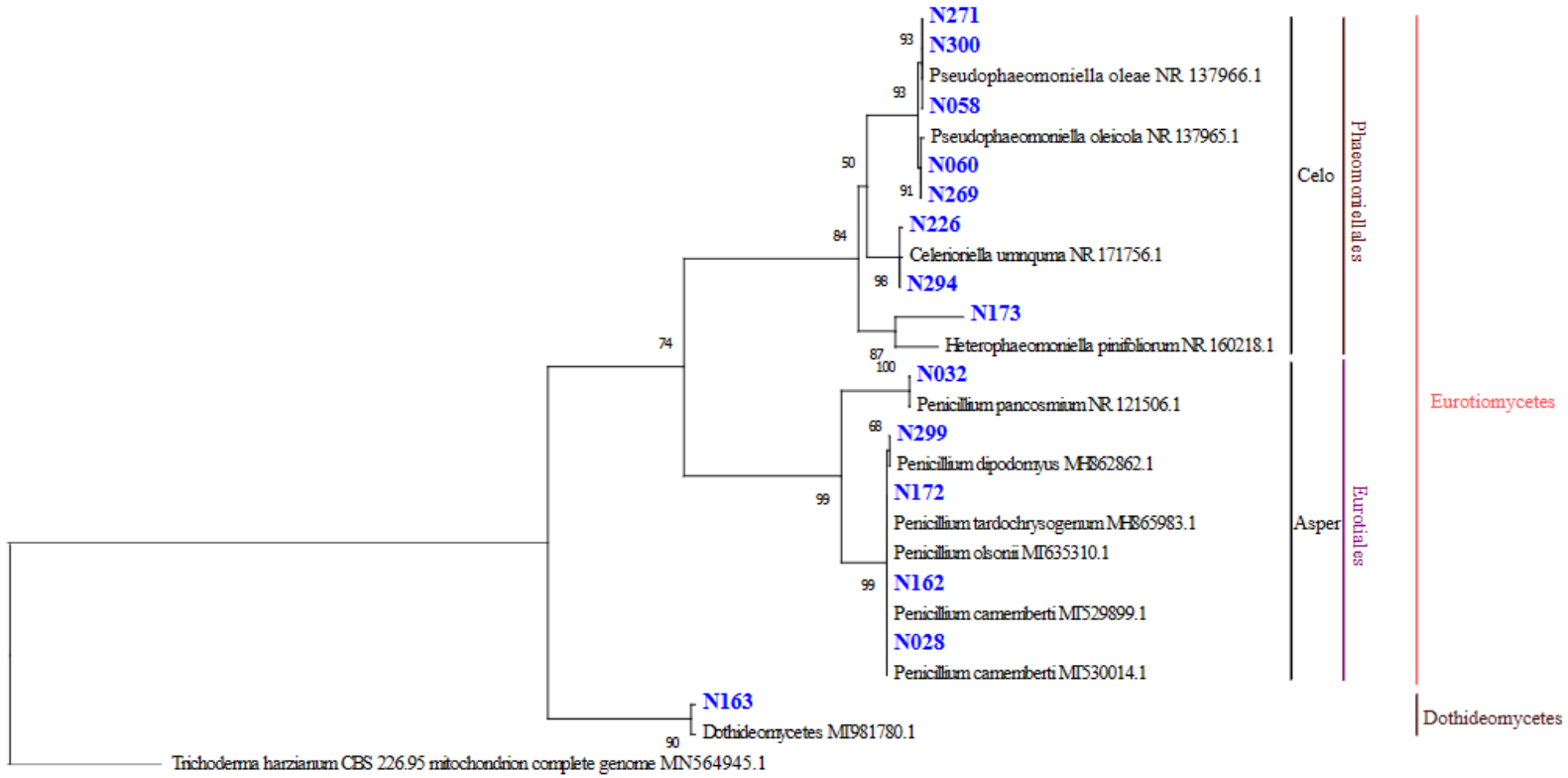

Figure 3. Maximum likelihood tree of internal transcribed spacer (ITS) sequences of fungal strains from CIMOCC. Numerical value presented in the node indicates bootstrap value with a 1000 bootstrap replicate analysis. Sequences from the CIMOCC are marked in blue and sequences from the database are marked in black. *Trichoderma harzianum* was used as an outgroup.

3.1.2. Actin (*ACT*)

Actins are proteins present in all eukaryotic cells that are involved in crucial cellular processes, including motility, structural stability, endocytosis, exocytosis, and regulation of cell growth and differentiation (Sun et al., 2022). The gene encoding actin comprises highly conserved sequences. But, the third codon positions of its DNA sequences shows substantial variation among closely related taxa (Daniel et al., 2001), which make actin gene sequences suitable for taxonomic and identification studies (Verkley et al., 2014). In our study, eleven fungal isolates out of the fourteen isolates were positive to PCR amplification of *ACT* sequence, with amplicon sizes around 300 bp. From these eleven positive isolates, four (N162, N163, N172 and N173) did not match with any *ACT* sequence in the databases (Table S1, Supporting information). Both GenBank and UNITE (User-friendly Nordic ITS Ectomycorrhiza Database), are the most comprehensive and widely used sequence repository in the field of fungal identification (Nilsson et al., 2019). The ITS region has been the most used molecular marker for fungal identification (Visagie et al., 2014), and therefore they are expected to be available in GenBank/UNITE in greater number when compared to *ACT* or other protein-coding genes. This can partly explain the lack of homology of our four *ACT* sequences with those available in the databases.

The phylogenetic tree constructed based on *ACT* sequences revealed that related fungal species are cluster together, which indicates a clear and well resolved classification (Fig. 4). In total, four subclades were identified. The first encompassed five isolates belonging to *Aspergillaceae* family, the second and third contained five fungal isolates from the *Celotheliaceae* family, and the four subclade is formed by a single isolate (N163) which identity is unknown. *Trichoderma harzianum*, from the family *Hypocreaceae*, was used as an outgroup. The first subclade comprises two of the unidentified fungal isolates (N162 and N172), suggesting that they are probably from the *Aspergillaceae* family. Similarly, the unidentified isolate N173 appear in the *Celotheliaceae* family cluster, and thus it is likely to belong to this family.

Moreover, the results showed that actin sequences were unable to identify closely related taxa of some fungal groups. This was observed particularly within members of the *Celotheliaceae* family. Thus, the actin gene alone seems to be insufficient for differentiating isolates within this family, as previously observed by Houbraken et al, (2020)

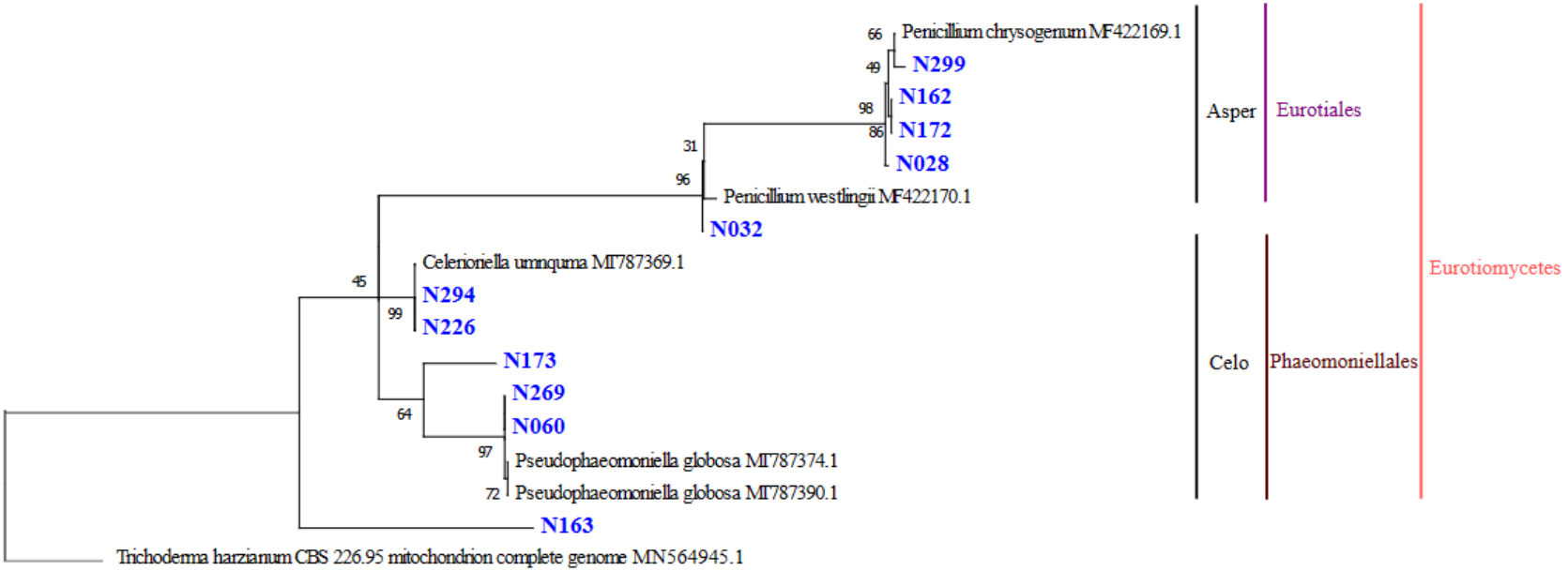

Figure 4. Maximum likelihood tree of actin sequences of fungal strains from CIMOCC. Numerical value presented in the node indicates bootstrap value with a 1000 bootstrap replicate analysis. Sequences from the CIMOCC are marked in blue and sequences from the database are marked in black. *Trichoderma harzianum* was used as an outgroup.

3.1.3. Chitin synthase 1 (*CHS-1*)

Chitin, is the second most abundant biopolymer in nature, and is found in various organisms, ranging from fungi to various invertebrates (Abo Elsoud & El Kady, 2019). In fungi, this compound is believed to be an essential component of the cell wall, at least under certain conditions, by providing sufficient rigidity to support fungal shape and structure (Patel & Free, 2019). Chitin is polymerized by large families of chitin synthase (*CHS*) enzymes, encoded by complex *CHS* genes families. For example, filamentous fungi usually contain seven to nine *CHS* genes (Qin et al., 2022). In the present study was used the *CHS-1* gene as a molecular marker, due to the higher number of available sequences of this gene in databases when compared to other *CHS* genes (Dzialo et al., 2017). From the fourteen fungal isolates analyzed, only seven were positive to PCR amplification of *CHS-1* sequence, yielding fragments of about 300 bp, consistent with the expected size of the *CHS-1* gene amplicon. From these seven positive isolates, only four were assigned to the genus *Penicillium* (Table S1, Supporting information). The remaining three isolates (N060, N058 and N271) did not match with any *CHS-1* sequence in the databases. The phylogenetic tree constructed based on *CHS-1* sequences clustered these seven fungal isolates into two groups, deriving from *Penicillium solitum* (Fig. 5). The unidentified isolates N060, N058 and N271 are in the same cluster of isolate N299 (identified as *Penicillium solitum*), but the genetic distance among them is very high. This suggest that these three isolates are probably not genetically related with *P. solitum*.

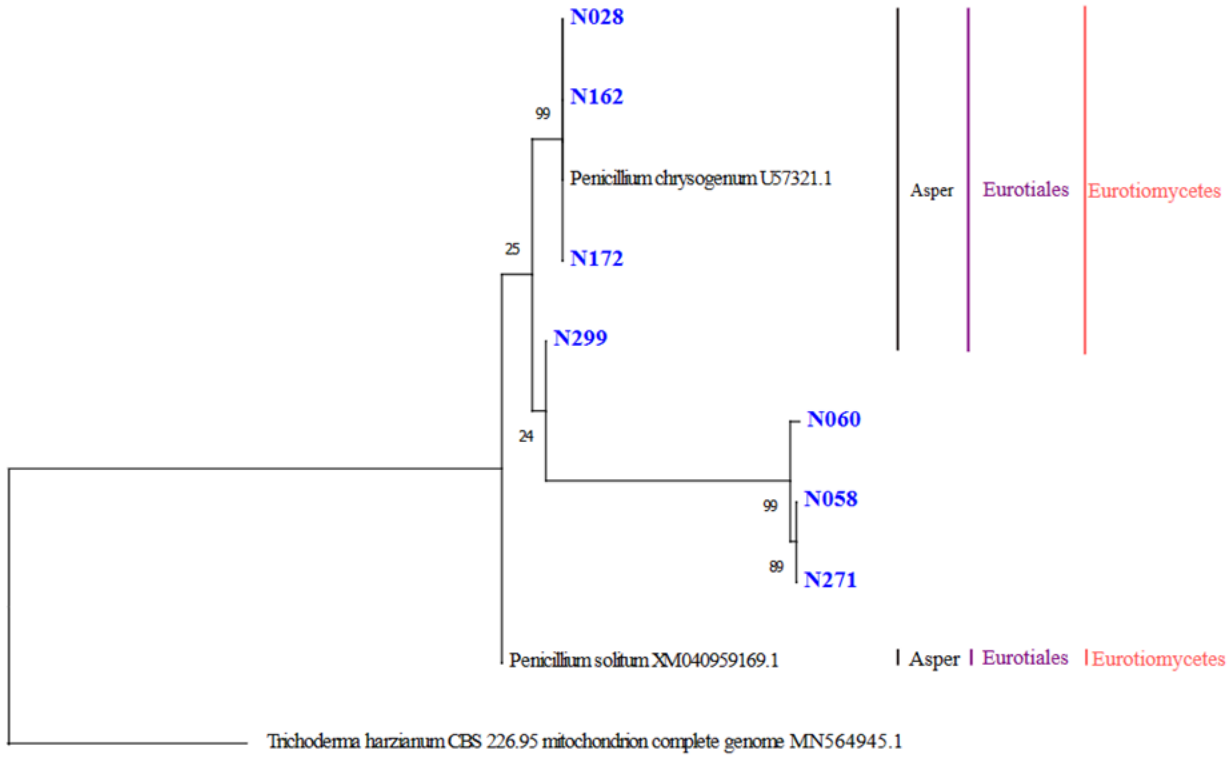

Figure 5. Maximum likelihood tree of chitin synthase 1 (*CHS-1*) sequences of fungal strains from CIMOCC. Numerical value presented in the node indicates bootstrap value with a 1000 bootstrap replicate analysis. Sequences from the CIMOCC are marked in blue and sequences from the databases are marked in black. *Trichoderma harzianum* was used as an outgroup.

3.1.4. Beta tubulin (*β-tubulin*)

Tubulin proteins are main components of the microtubules that are playing crucial roles in cellular processes, including cell division, intracellular transport, maintenance of shape and cell motility, in all eukaryotes (Wloga et al., 2017). They belong to a protein family involving seven different tubulins, being the α- and β-tubulins the most abundant tubulins in the eukaryotic cell and one of the main components of the microtubules (Binarová & Tuszynski, 2019). Therefore, the genes encoding α- and β-tubulins have been the most used to determine fungal phylogeny (Zhao et al., 2014). Especially, the *β-tubulin* gene is receiving increasing attention as a molecular marker for complex species groups, such as *Penicillium* (Kolanlarli et al., 2019; Visagie et al., 2016), and to resolved new species (Zhou & Cui, 2017). Therefore, in the present work the *β-tubulin* gene was selected as molecular marker to characterized the fourteen fungal isolates of the CIMOCC. All the fungal isolates analyzed were positive to PCR amplification of *β-tubulin* sequence, yielding amplicon sizes of around 600 bp. From these fourteen positive isolates, three (N173, N226 and N294) did not match with any *β-tubulin* sequence in the databases (Table S1, Supporting information).

The phylogenetic tree based on *β-tubulin* gene for the fourteen fungal isolates is depicted in figure 6. In this tree, two main subclades were derived from the two unidentified isolates N226 and N294. In the first subclade, there are two subclades, being one of them encompassing isolates from the *Penicillium* genus and *Aspergillaceae* family, and the second comprised a single isolate (N163) assigned to *Periconia macrospinosa*, belonging to *Periconiaceae* family. In the second subclade, the unidentified isolate N173 was clustered with fungal isolates belonging to *Pseudophaeomoniella* genus and *Celotheliaceae* family, suggesting to be genetically related. In contrast to previously reported (Kolanlarli et al., 2019; Visagie et al., 2016), in our study the *β-tubulin* gene when used alone was unable to distinguish different species (N028, N162, N172 and N299) of the genus *Penicillium*. However, this gene shows to provide resolution within the genus *Pseudophaeomoniella*, by allowing the distinction of the species *P. globosa* from *P. oleicola* (Abastabar et al., 2016; Mostert et al., 2006).

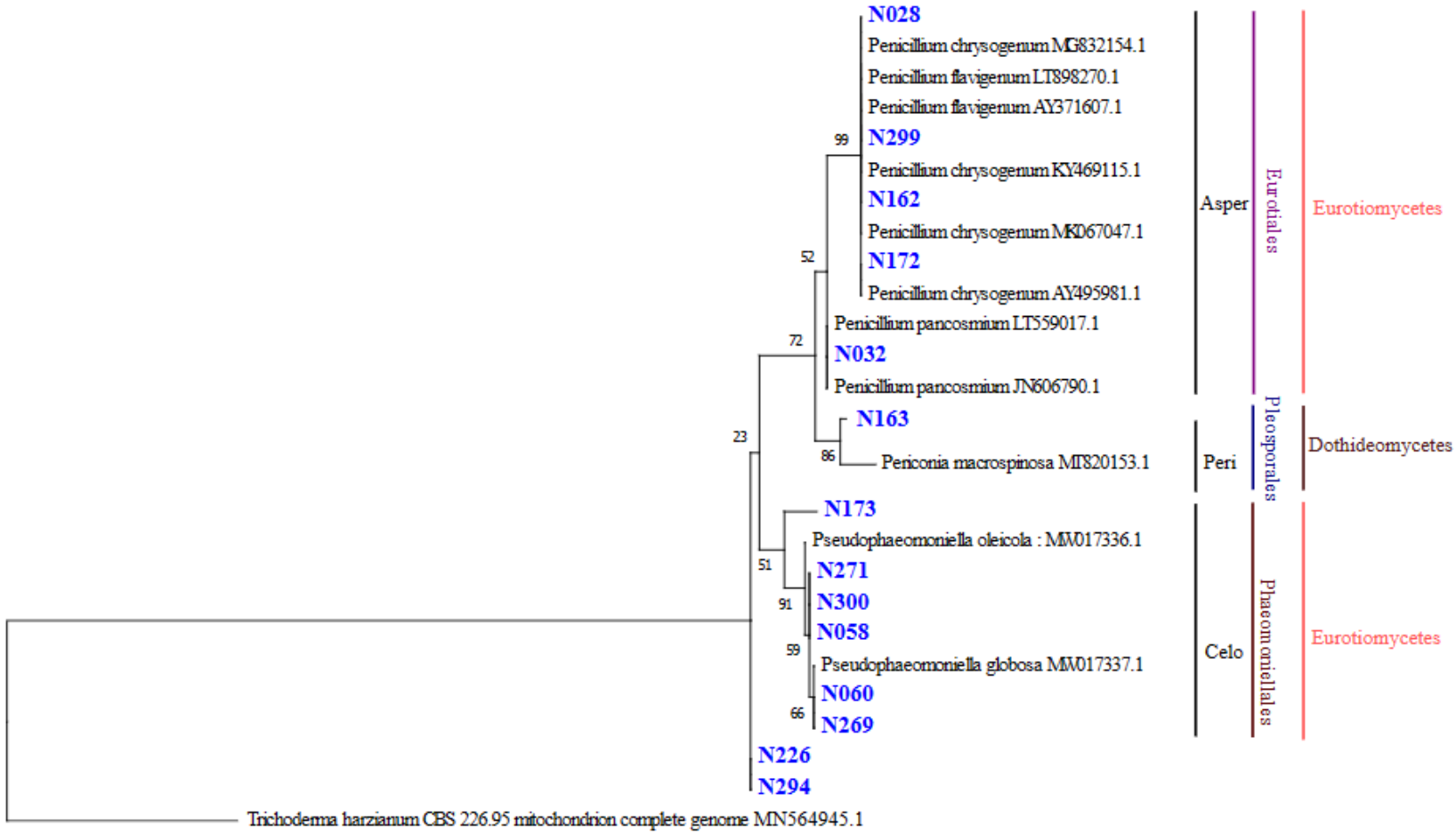

Figure 6. Maximum likelihood tree of *β-tubulin* sequences of fungal strains from CIMOCC. Numerical value presented in the node indicates bootstrap value with a 1000 bootstrap replicate analysis. Sequences from the CIMOCC are marked in blue and sequences from the database are marked in black. *Trichoderma harzianum* was used as an outgroup.

3.1.5. Translation elongation factor 1-alpha (*TEF-1α*)

The translational elongation factor 1-alpha (*TEF-1α*) gene encodes the expressed translation elongation factor EF1α protein, which is responsible for the binding reaction of aminoacyl-tRNA to ribosomes (Alves et al., 2015). In our study, seven fungal isolates out of the fourteen isolates were positive to PCR amplification of EF-1α sequence, with amplicon sizes of around 300 bp. From these seven positive isolates, two (N173 and N269) did not match with any *TEF-1α* sequence in the databases, being the remaining assigned to genera *Pseudophaeomoniella* and *Celerioriella*, both belonging to *Celotheliaceae* family (Table S1, Supporting information). Interestingly, none of the isolates previously identified as *Penicillium* gave positive amplification for *TEF-1α* gene.

The phylogenetic tree generated based on *TEF-1α* sequences revealed that the fungal isolates were clustered in three distinct subclusters (Fig. 7). The first comprised four isolates, being the isolates N060 and N269 related to *Pseudophaeomoniella globosa*, and N300 and N271 isolates are maybe *Pseudophaeomoniella oleae*. The second subcluster comprises two isolates (N226 and N294) that are likely to be *Celerioriella umnquma*. Finally, the third subcluster comprises a single unidentified isolate (N173), that seems to be phylogenetically distant from the genera *Pseudophaeomoniella* and *Celerioriella*. Overall, the results showed that the *TEF-1α* gene when used alone, can provide resolution within the genus *Pseudophaeomoniella,* by allowing the distinction of the species *P. globosa* from *P. oleae.* This gene was previously reported to provide phylogenetic resolution, in other complex taxonomic groups of fungi, such as *Botryosphaeriaceae* and *Fusarium* (Úrbez-Torres et al., 2014).

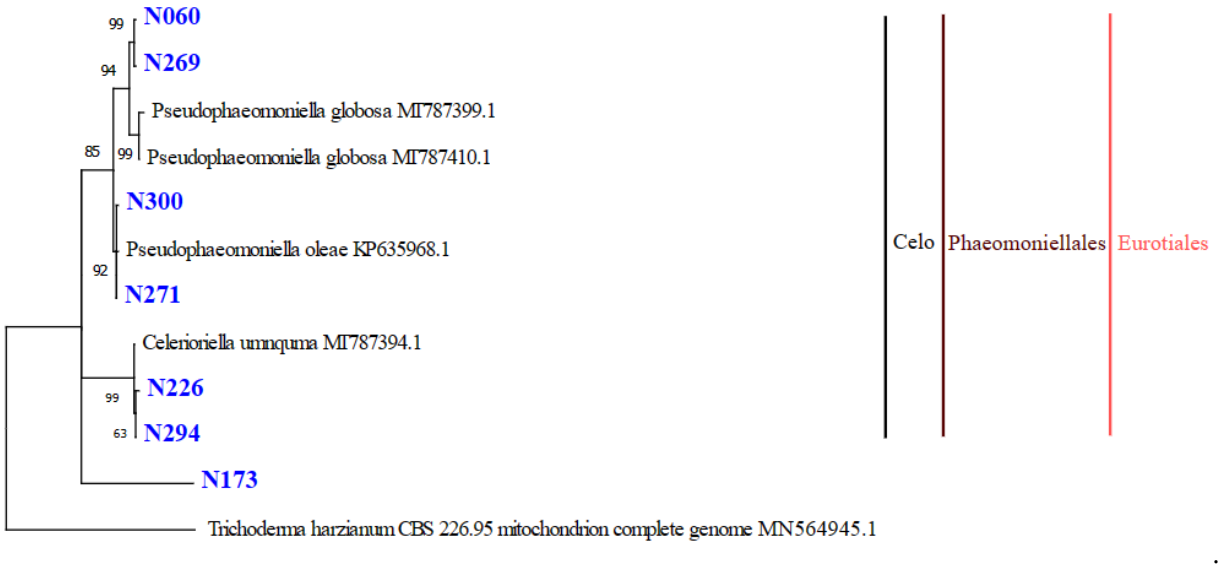

Figure 7. Maximum likelihood tree of translation elongation factor 1-alpha sequences of fungal strains from CIMOCC. Numerical value presented in the node indicates bootstrap value with a 1000 bootstrap replicate analysis. Sequences from the CIMOCC are marked in blue and sequences from the database are marked in black. *Trichoderma harzianum* was used as an outgroup.

3.1.6. Histone H-3 (*HIS*)

Histones are highly alkaline proteins found in nuclei acids of eukaryotic cells and play an important role in gene regulation. In total, there are four histones (H1, H2A, H2B, H3 and H4) that bind strongly to DNA, due to differences on their charge (histones are positively charged while DNA is negatively charge due to the presence of phosphate groups). In particular, the histone H-3 encoding gene comprises highly conserved sequences (Wichansawakun & Buttar, 2019). Of the fourteen fungal isolates analysed eight were positive to PCR amplification of the histone H3 sequence, yielding amplicon sizes of about 419 bp. Of these eight positive isolates, five (N032, N162, N172, N182 and N299) did not match with any *HIS* sequence in the databases (Table S1, Supporting Information).

The phylogenetic tree based on the *HIS* gene for the eight fungal isolates is depicted in figure 8. In this tree, five fungal isolates were derived from the isolates N250, N173, N182, that showed to be highly phylogenetic related. The unidentified isolates N032, N162, N172 and N299 grouped with isolate N028 that was assigned to *Mycosphaerellaceae* family in the blast. Therefore, it is likely that these five fungal isolates belong to this family. Overall, our study showed that the *HIS* gene, when used alone, was not able to distinguish different species from the family *Mycosphaerellaceae* as similarly reported previously by other authors (Breeijen et al., 2006; (Talbert et al., 2012).

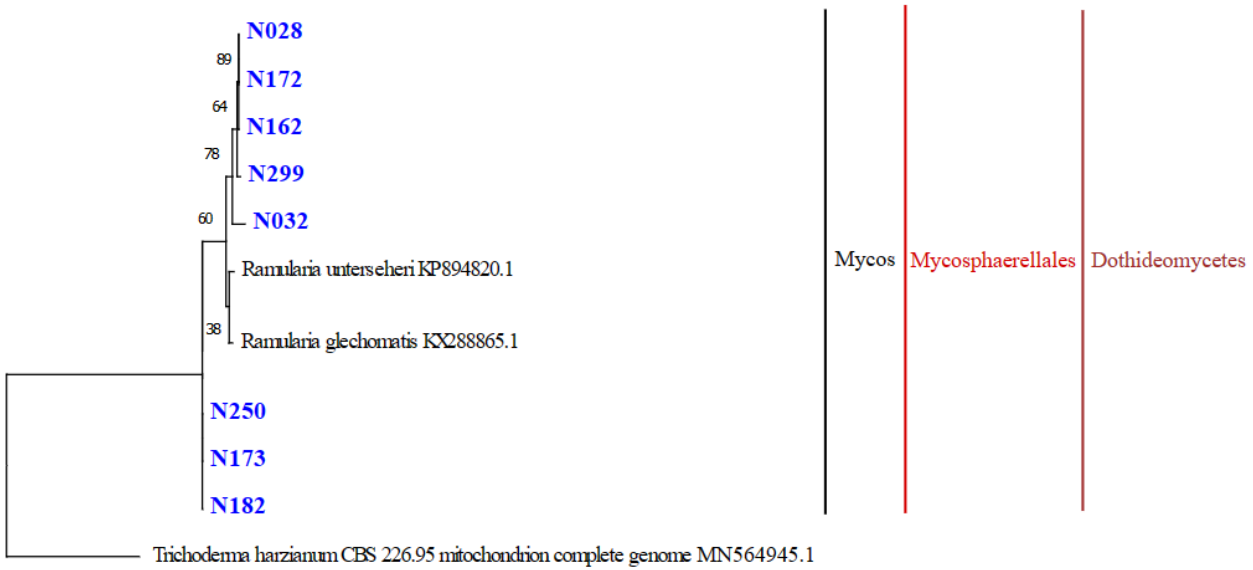

Figure 8. Maximum likelihood tree of histone H-3 sequences of fungal strains from CIMOCC. Numerical value presented in the node indicates bootstrap value with a 1000 bootstrap replicate analysis. Sequences from the CIMOCC are marked in blue and sequences from the database are marked in black. *Trichoderma harzianum* was used as an outgroup.

3.2. Concatenated ITS and beta-tubulin phylogenetic tree

Our results showed that none of the molecular marker tested were sufficient to provide an accurate identification of the fungal isolates until the species level, when used alone. Therefore, in order to obtain a more precise identification at the species level, a phylogenetic tree was constructed based on the aligned sequences of ITS + *β-tubulin* regions (Fig. 9). These two barcodes were selected, because they were the only regions that we have positive amplification for the fourteen fungal isolates. On the basis of the phylogenetic tree, two slightly separate subclades were formed. Suclade 1, comprises all the fungal isolates of the genus *Penicillium* and one isolate assigned to Dothideomycetes (N163). In subclade 2, there are eight fungal isolates phylogenetic related with *Heterophaeomoniella pinifoliorum* and *Pseudophaeomoniella globosa*. Contrary to our expectations, the fungal species did not separate better when the two loci (ITS and *β-tubulin*) are simultaneously used to build the phylogeny of the isolates. This lack of separation, can be due to the high number of species from different taxonomic groups used in the alignment. Indeed, there are several reports showing that an increase in the number of species in the alignment can lead to a decrease in the ability to separate them accurately (Rokas et al., 2003; Udayanga et al., 2011). An additional reason is related with the lack of sequences in the databases for the different loci analyzed in the surveyed fungal species, in particular those belonging to *Heterophaeomoniella* and *Pseudophaeomoniella* genera. Indeed, in the GenBank there are only nine sequences for *Heterophaeomoniella*, while for *Pseudophaeomoniella* there are a little more (116 sequences) (www.ncbi.nlm.nih.gov/genbank; accessed on 27 February 2023). Thus, creating phylogenies that include several loci is a very difficult task for these two genera.

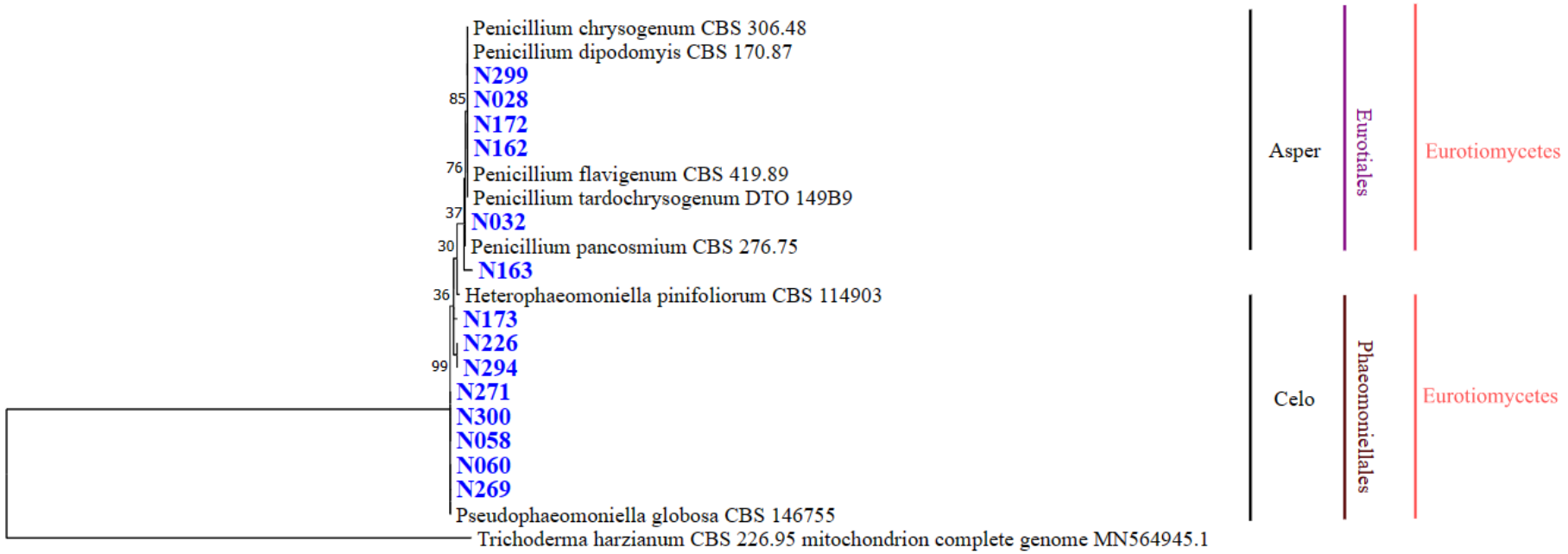

Figure 9. Maximum likelihood tree of ITS + *β-tubulin* gene sequences of fungal strains from CIMOCC. Numerical value presented in the node indicates bootstrap value with a 1000 bootstrap replicate analysis. Sequences from the CIMOCC are marked in blue and sequences from the database are marked in black. *Trichoderma harzianum* was used as an outgroup.

However, when analyzed altogether, the individual phylogenetic trees performed for the six loci showed to be useful for an accurate resolution of fungal isolates. For example, the ITS, *ACT* and *TEF-1α* were the most informative for the identification of *Celerioriella umnquma* species (*Celotheliaceae* family, Phaeomoniellales order) (Figs. 3, 4 and 7). According to these three loci, both N226 and N294 isolates are the same species (i.e., *Celerioriella umnquma*). Fungal isolates belonging to the genus *Pseudophaeomoniella* (*Celotheliaceae* family, Phaeomoniellales order) were well separated when using ITS, *β-tubulin* and *TEF-1α* (Figs. 3, 6 and 7). These three loci showed that fungal isolates N060 and N269 are the same species and closely related to *Pseudophaeomoniella globosa*. These two isolates were different from isolates N058, N271 and N300, that are the same species and relatedly with *Pseudophaeomoniella olea*. The locus *ACT*, and in less extent, the locus *HIS*, were the most informative of the six loci when it comes to separating *Penicillium* species (*Aspergillaceae* family, Eurotiales order) (Figs. 4 and 8). According to these two loci, the fungal isolates N028, N032 and N299 were different species. The same two loci also showed that the isolates N162 and N172 are the same species and different from the previous one. However, both *ACT* and *HIS* loci, were not able to determine the species through a BLAST, probably due to the lower number of these loci sequences in the GenBank in comparison to ITS and *β-tubulin*, Therefore, for species-level determination, the loci *ACT* must be use with other loci, being *β-tubulin* and/or ITS the best candidates. Accordingly, our results showed that isolate N028 is closely related to *P. chrysogenum*, while isolate N032 is closely related to *P. pancosmium* (based on ITS and *β-tubulin* loci) or to *P. westlingii* (based on *ACT* locus). The isolates N299, and the phylogenetic related isolates N162 and N172, were assigned to a number of different *Penicillium* species, thus being not possible at this stage their identification to an exact species name. Table 4 summarize these results and provide for each fungal isolate a putative identification to species level.

Table 4. Identity of the fungal species of the CIMOCC and their respective isolate codes.

CIMO code (Abbreviation)	**Fungal species**
CIMO 19DM028 (N028)	*Penicillium aff. chrysogenum*
CIMO 19DM032 (N032)	*Penicillium* sp. 1
CIMO 19DM162 (N162) CIMO 19DM172 (N172)	*Penicillium* sp. 2
CIMO 19DM299 (N299)	*Penicillium* sp. 3
CIMO 19DM226 (N226) CIMO 19DM294 (N294)	*Celerioriella umnquma*
CIMO 19DM060 (N060) CIMO 19DM269 (N269)	*Pseudophaeomoniella globosa*
CIMO 19DM058 (N058) CIMO 19DM271 (N271) CIMO 19DM300 (N300)	*Pseudophaeomoniella olea*
CIMO 19DM163 (N163)	Dothideomycetes
CIMO 19DM173 (N173)	*Ramularia glechomatis* *Heterophaeomoniella pinifoliorum*

Curiously, in this work was identified two species, namely *Celerioriella umnquma* and *Pseudophaeomoniella globosa*, that were recently reported as novel taxa in the Phaeomoniellales (Spies et al., 2020). These two species were reported for the first time in 2020, associated with olive dieback in South Africa (Spies et al., 2020). Similarly, *Pseudophaeomoniella oleae* is a pathogen of olive tree recently reported to cause wood streaking and decay on olive trees in Greece (Markakis et al., 2022). This fungus has been isolated previously from decayed olive trees showing vascular wilt in Italy (Crous et al., 2015). To our knowledge, this is the first report of these three fungal species in olive trees in Portugal. According to the CIMOCC databases, these fungi were isolated from asymptomatic olive tree leaves of cvs. Cobrançosa and Madural, collected in Mirandela. These fungi were associated to leaves either as endophytes or epiphytes.

3.3. Biocontrol traits on fungal isolates

Among the fourteen fungal isolates, all the five *Penicillium* fungal isolates (N028 – *Penicillium aff. chrysogenum*, N032 – *Penicillium* sp.1, N162 – *Penicillium* sp.2, N172 – *Penicillium* sp.2 and N299 – *Penicillium* sp.3) were screened for their ability to inhibited the growth of the causal agent of anthracnose, *C. fioriniae*. The results of the co-culture assay showed that none of the five fungal isolates significantly inhibited the growth of *C. fioriniae* when compared to control (co-culture established with the pathogen) (Fig. 10). Several species of *Penicillium* have been reported as efficient biocontrol agents against a number of pathogens, such as *Diaporthe citri*, *Phyllosticta citrichinaensis* and *Colletotrichum gloeosporioides*, either under *in vitro* conditions or in field/greenhouse assays (Liang et al., 2021). Despite the low ability of the five *Penicillium* isolates to reduce the *in vitro* growth *C. fioriniae*, their efficacy to control anthracnose disease should be tested under more realistic conditions. Indeed, the results get under *in vitro* conditions are often different from the ones performed under *in vivo* conditions (Adebanjo & Bankole, 2004).

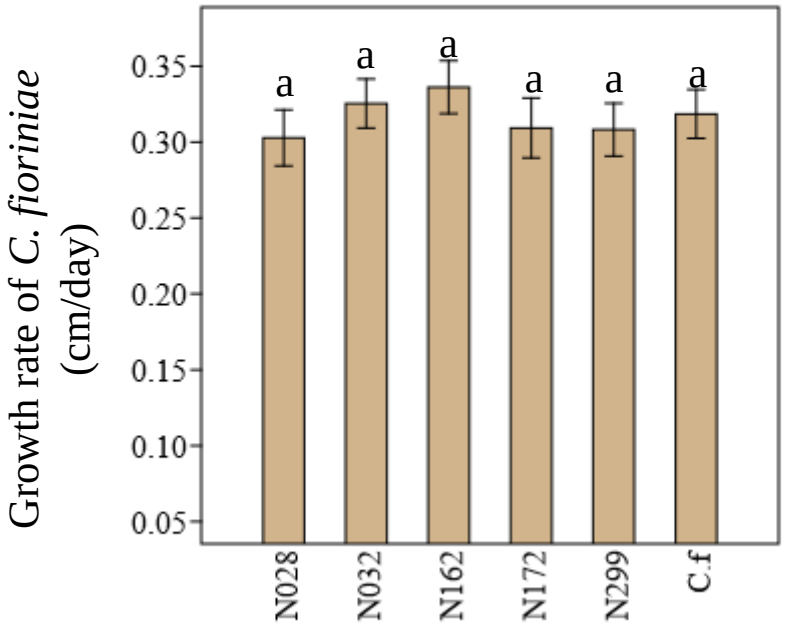

Figure 10. Growth rate of the pathogen *Colletotrichum fioriniae* in co culture with N028 (*Penicillium aff chrysogenum*, N032 – *Penicillium* sp.1, N162 – *Penicillium* sp.2, N172 – *Penicillium* sp.2, N299- *Penicillium* sp.3 and *C. fioriniae* (C.f.; control), in potato dextrose agar medium. Different superscript lowercase letters denote a statistically significant difference ($p<0.05$, N=5) between the several co-cultures.

The production of lytic enzymes (cellulase, amylase and β-1,3-glucanase), antimicrobial compounds (hydrogen cyanide) and siderophores by the five fungal isolates

when in co-culture with *C. fioriniae* were studied, as an attempt to identified metabolites with antagonistic proprieties.

The activity of lytic enzymes was detected by clearing zones in agar plates in the presence of appropriate substrates (Fig. 11). When compared to control (co-cultures of the same fungal isolate), only the N162 (*Penicillium* sp.2) and N299 (*Penicillium* sp.3 increased significantly the production of cellulase when challenged by *C. fioriniae* pathogen, up to 100% ($p<0.001$) and 23% ($p<0.05$), respectively (Fig. 11). Amylase production was increased significantly ($p<0.001$) only by N299 (*Penicillium* sp.3) up to 100% when compared to control. In contrast, no significant differences among fungal isolates in co-culture with *C. fioriniae* and control was observed to β-1,3-glucanase. Previous studies have similarly reported the capability of several species of *Penicillium* to produce lytic enzymes, inhibiting the growth of *Colletotrichum lindemuthianum* either *in vitro* and in the field/greenhouse assays (Adebanjo, 2004).

None of the fungal isolates tested were able to produce hydrogen cyanide (HCN) (data not shown). The siderophores production was detected by the changing of blue colour to orange of the CAS medium (Fig. 11). Among the five fungal isolates tested, only N032 (*Penicillium* sp.1) and N162 (*Penicillium* sp.2), were able to significantly increase the production of siderophores in the presence of *C. fioriniae* when compared to control, up to 100% ($p<0.001$) and up to 36% ($p<0.05$), respectively. Previous study reported that *Pennicillium chrysogenum* has the ability to produce siderophores and to inhibit pathogens, as similarly observed in the present work (Chowdappa et al., 2020).

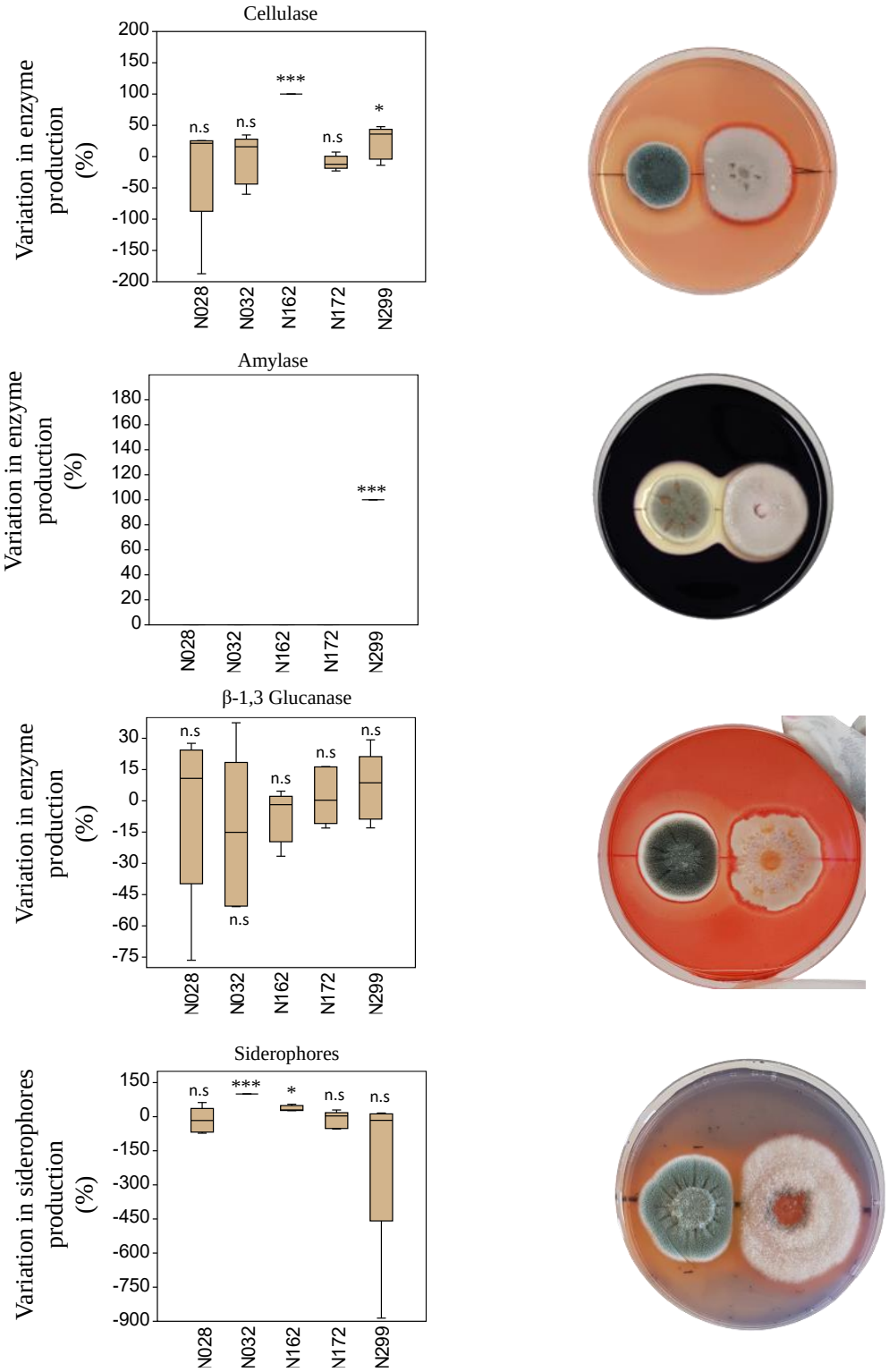

Figure 11. Lytic enzymes (cellulase, amylase and β-1,3-glucanase) and siderophores produced by N028 (*Penicillium aff chrysogenum*, N032 – *Penicillium* sp.1, N162 – *Penicillium* sp.2, N172 – *Penicillium* sp.2, N299- *Penicillium* sp.3 in co-culture with *Colletotrichum fioriniae*. All these antagonistic metabolites were expressed as the percentage of their increase in relation to the control plates (co-cultures of single fungal species). Box plots depict medians, the inter-quartile ranges (boxes), and 95% confidence intervals (whiskers). Statistically significant differences from control plates are indicated by an asterisk (*$p<0.05$; **$p<0.01$; ***$p<0.001$). Representative plates used to detect these metabolites are represented in the right side.

3.4. Plant growth promotion traits on fungal isolates

Plant growth-promoting fungi (PGPF) can be found as free-living microorganisms in bulk soil or in rhizosphere, with ability to improve the plant growth via several mechanisms (Motaher Hossain & Sultana, 2020). They can increase plant growth by participating in the solubilization of some elements, namely phosphate, and by producing phytostimulators or plant growth regulators, such as indole acetic acid (IAA) (Lebrazi et al., 2020). In this study, all the five *Penicillium* isolates were able to solubilized phosphate, with N172 – *Penicillium* sp.2 isolate having the highest production, followed by N162 (*Penicillium* sp.2) and N299 (*Penicillium* sp.3) (Fig. 12). Phosphate solubilization is a well-known phenomenon in *Penicillium*, being already reported the ability of some strains to increase the growth and P nutrition of herbaceous (Wakelin et al., 2007) and ligneous (Qiao et al., 2019) plants.

Similarly, all the five fungal isolates produced IAA ranging from 1.9 to 3.28 μg/mL, being N172 (*Penicillium* sp.2) the highest producer. Other high IAA-producers were N162 (*Penicillium* sp.2) and N032 (*Penicillium* sp.1). The stimulation of plants growth by *Penicillium* is often associated with their ability to produce IAA. Several species of *Penicillium* have been reported to be able to produce IAA in culture liquid medium, in concentrations reaching more than 133 μg/mL (Khalil et al., 2021).

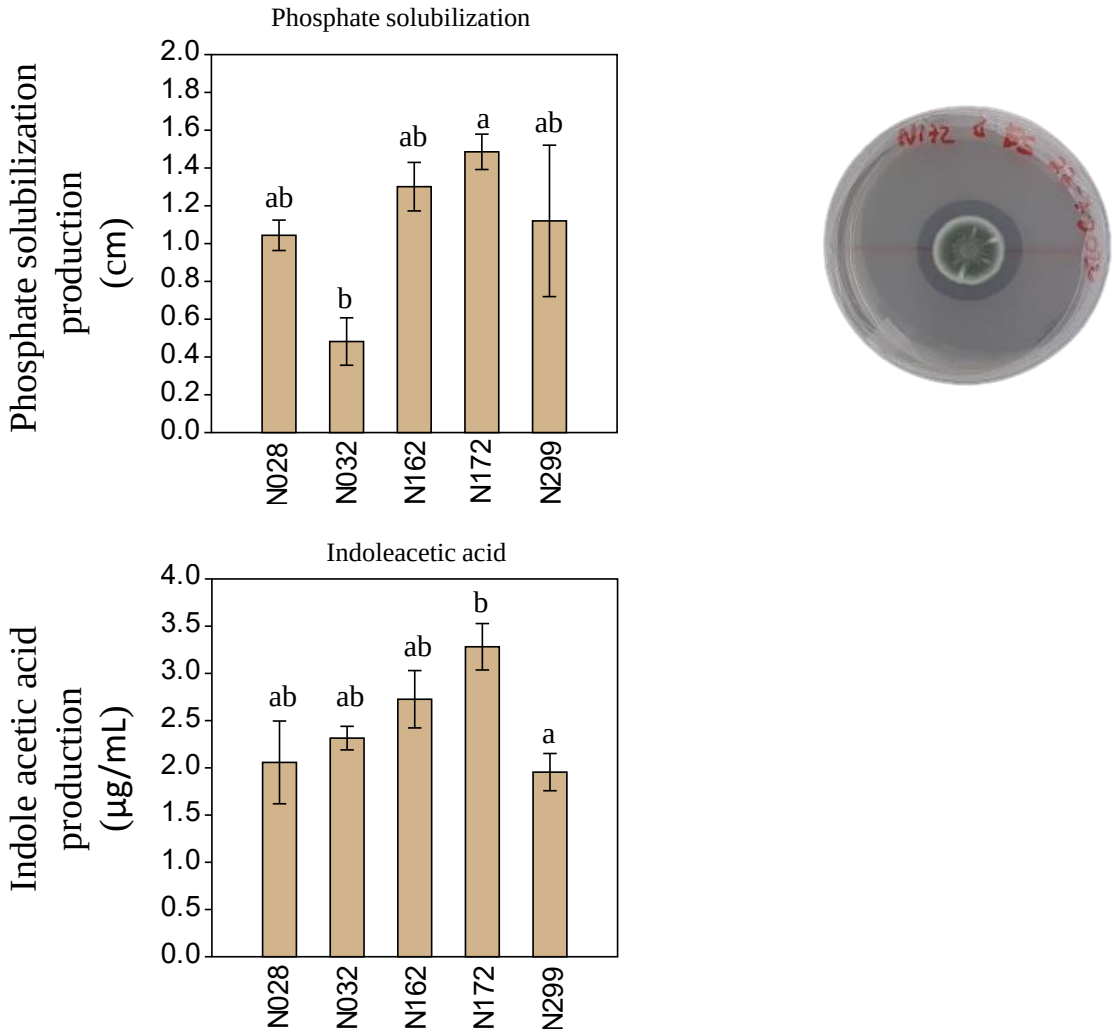

Figure 12. Solubilization of phosphate (cm) and production of indole acetic acid (µg/mL), (IAA) by N028 *Penicillium* aff *chrysogenum*, N032 – *Penicillium* sp.1, N162 – *Penicillium* sp.2, N172 – *Penicillium* sp.2, N299- *Penicillium* sp.3. Different superscript lowercase letters denote a statistically significant difference ($p<0.05$, N=5) between the fungal isolate. Representative plates used to detect solubilization of phosphate are represented in the right side.

4. Conclusions and future perspectives

In this work was characterized fourteen fungal isolates deposited on CIMO-CC, previously isolated from olive tree leaves, and putatively identified as belonging to Phaeomoniellales order. Accordingly, a multi-locus phylogenetic analysis, based on six loci (ITS, *ACT*, *CHS-1*, *β-tubulin*, *TEF-1α* and *HIS*), were first used to molecularly characterized these fungal strains. The results obtain showed that:

1) Contrary to our expectations, from the fourteen isolates, only eight N058, N060, N173, N226, N269, N271, N294 and N300 were assigned to the Phaeomoniellales order (*Celotheliaceae* family, and genera *Celerioriella*, *Pseudophaeomoniella* and *Heterophaeomoniella*). Five were from Eurotiales order (*Aspergillaceae* family, *Penicillium* genus) and one was identified only to the class level (Dothideomycetes);
2) When used alone, the ITS locus (which is the official DNA barcode region in fungi) was not sufficient for separate and identify to species level all the fungal isolates analysed. Multi-loci DNA barcoding showed to provide a more accurate species separation, being necessary to use at least three loci;
3) The best set of loci for identifying and resolving *Penicillium* species was *ACT*, *β-tubulin* and/or ITS; while the three loci ITS, *TEF-1α* and *β-tubulin* was the best set of loci for resolving *Pseudophaeomoniella* species. The ITS, *ACT* and *TEF-1α* were the most informative for the identification of *Celerioriella umnquma* species;
4) Overall, the *TEF-1α* locus seems to be a better candidate for single locus DNA barcoding in the family *Celotheliaceae* than the ITS locus.

Among the fourteen fungal isolates molecularly characterized, five *Penicillium* isolates were selected and screened for their ability to produced compounds frequently associated to biocontrol and plant growth promoting traits. The results obtain showed that:

1) None of the *Penicillium* isolates were able to reduce significantly the *in vitro* growth of the causal agent of olive anthracnose, *Colletotrichum fioriniae*. However, some of the tested isolates showed to increase significantly the production of lytic enzymes, particularly cellulase (isolates N162 and N299)

and amylase (isolate N299), as well as of siderophores (isolates N032 and N162), when challenged by the pathogen. Overall, the results indicate that both isolates N162 (*Penicillium* sp. 2) and N299 (*Penicillium* sp. 3) were the most promising as biocontrol agents against pathogens;

2) All the tested *Penicillium* isolates were very promising as plant growth promotors, due to their ability in solubilizing phosphate and to produce IAA. The isolates N172 and N162 (*Penicillium* sp. 2) were the strongest producers of these compounds.

Overall, the results highly the importance on characterizing the fungal isolates of the CIMOCC in terms of their identification and biotechnological potential. Indeed, in this study, three fungal isolates were assigned to species (*Celerioriella umnquma*, *Pseudophaeomoniella globosa* and *Pseudophaeomoniella oleae*) that have never been reported in Portugal. Other isolates (CIMO 19DM163) were only possible to identify to the class level, suggesting that probably they have never been characterized/describe before. Moreover, the screening of the *Penicillium* isolates showed that there are a number of potentially interesting strains for a comprehensive study of their metabolome for future biotechnological application. Therefore, future works are needed to better characterized not only the isolates of the present study but others deposited in the CIMOCC.

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Supporting information

Table S1. Blast results showing the final taxonomic classification of the fungal isolates based on different molecular markers, namely ribosomal marker ITS (ITS1, 5.8S and ITS2), and the protein-encoding genes actin (*ACT*), chitin synthase 1 (*CHS-1*), β-tubulin, translation elongation factor 1-alpha (*TEF-1α*) and histone H-3 (*HIS*).

CIMO code (Abbreviation)	**Blast classification* / accession number (% of identity)**					
	ITS	***ACT***	***CHS-1***	***β-tubulin***	***TEF-1α***	***HIS***
CIMO 19DM028 (N028)	*Penicillium camemberti* MT530014.1 (100 %)	*Penicillium chrysogenum* MF422169.1(96.92 %)	*Penicillium chrysogenum* U57321.1 (98,01%)	*Penicillium chrysogenum* MG832154.1(99.55%)	No homology	*Ramularia unterseheri* KP894820.1(96.50 %)
CIMO 19DM032 (N032)	*Penicillium pancosmium* NR_121506.1(99.40 %)	*Penicillium westlingii* MF422170.1(96.30 %)	No homology	*Penicillium pancosmium* JN606790.1(100%)	No homology	No homology
CIMO 19DM058 (N058)	*Pseudophaeomoniella oleae* NR_137966.1 (100%)	No homology	No homology	*Pseudophaeomoniella globosa* MW017337.1; (96.51%)*Pseudophaeomoniella oleicola* : MW017336.1(97.72%)	No homology	No homology
CIMO 19DM060 (N060)	*Pseudophaeomoniella oleicola* NR_137965.1(99.82 %)	Pseudophaeomoniella globosa MT787390.1 (99.4%)	No homology	*Pseudophaeomoniella globosa* MW017337.1; (97.42%)*Pseudophaeomoniella oleicola* : MW017336.1(96.50%)	*Pseudophaeomoniella globosa* MT787399.1(96.40 %)	No homology
CIMO 19DM162 (N162)	*Penicillium camemberti* MT529899.1(100%)	No homology	*Penicillium chrysogenum* U57321.1 (98.87%)	*Penicillium chrysogenum* KY469115.1(100%)	No homology	No homology

CIMO 19DM163 (N163)	Dothideomycetes MT981780.1(99.42 %)	No homology	No homology	Periconia macrospinosa MT820153.1 (96.45%)	No homology	No homology
CIMO 19DM172 (N172)	*Penicillium tardochrysogenum* MH865983.1(100%)	No homology	*Penicillium chrysogenum* U57321.1 (97.71%)	*Penicillium chrysogenum* AY495981.1(99.05%)	No homology	No homology
CIMO 19DM173 (N173)	*Heterophaeomoniella pinifoliorum* NR_160218.1(96.01 %)	No homology	No homology	No homology	No homology	*Ramularia glechomatis* KX288865.1(97.05 %)
CIMO 19DM226 (N226)	*Celerioriella umnquma* NR_171756.1(99.60 %)	*Pseudophaeomoniella globosa* MT787390.1 (99.07%)	No homology	No homology	Celerioriella umnquma MT787394.1(98.71 %)	No homology
CIMO 19DM269 (N269)	No homology	*Pseudophaeomoniella globosa* MT787374.1(97.40 %)	No homology	*Pseudophaeomoniella globosa* MW017337.1;(97.46%) *Pseudophaeomoniella oleicola* : MW017336.1(97.01%)	No homology	No homology
CIMO 19DM271 (N271)	*Pseudophaeomoniella oleae* NR_137966.1(100%)			*Pseudophaeomoniella globosa* MW017337.1; (96.55%)*Pseudophaeomoniella* oleicola : MW017336.1(97.78%)	*Pseudophaeomoniella oleae* KP635968.1(99.19 %)	
CIMO 19DM294 (N294)	*Celerioriella umnquma* NR_171756.1(99.80 %)	*Celerioriella umnquma* MT787369.1(99.51 %)			*Celerioriella umnquma* MT787394.1(99.60 %)	

CIMO 19DM299 (N299)	*Penicillium dipodomyus* MH862862.1(100%)	*Penicillium chrysogenum* MF422169.1(96.36 %)	*Penicillium solitum* XM_040959169.1(96.08 %)	*Penicillium flavigenum* AY371607.1(99.77%)	
CIMO 19DM300 (N300)	*Pseudophaeomoniell a oleae* NR_137966.1(100%)			*Pseudophaeomoniella globosa* MW017337.1; (96.69%)*Pseudophaeomonie lla oleicola* : MW017336.1(97.93%)	*Pseudophaeomoniel la oleae* KP635968.1(97.61 %)

* The fungal isolates were identified using the Nucleotide Basic Local Alignment Search Tool (BLASTn) in the NCBI database (www.ncbi.nlm.nih.gov), and the results were arranged based on a higher identity score and the lowest E-value. Species names were accepted only for identity core >96%.