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ORIGINAL ARTICLE

Rhizospheric *Penicillium* and *Talaromyces* strains in *Austrocedrus chilensis* native forests: identification and evaluation of biocontrol candidates against the pathogen *Phytophthora austrocedri*

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Abstract

The aims of this study were to prospect *Penicillium* and *Talaromyces* species associated with *Austrocedrus chilensis* in sites with and without Austrocedrus Root Disease (ARD), which is caused by the pathogen *Phytophthora austrocedri*; and to find *Penicillium* sp. strains with biocontrol activity against this disease. Isolations from sites with and without the prevalence of ARD were made, and the obtained isolates were phenotypically and molecularly identified to species level. Several *Penicillium* species were isolated from all sites; five of them were exclusively isolated from sites with ARD. *Penicillium* communities were compared through multivariate analyses; communities were more complex in sites with ARD. Biocontrol activity of *Penicillium* and *Talaromyces* isolates was evaluated using co-cultures with *Ph. austrocedri*. *Penicillium glabrum, T. amestolkiae, P. palitans,* and *P. crustosum* showed the highest biocontrol effect. Our results highlight that *Penicillium* species have high biocontrol activity, which also reinforces the hypothesis that some *Penicillium* species could be used in formulating a control strategy.

Keywords: *Austrocedrus chilensis*, biological conrol, fungal diversity, *Phytophthora* disease, rhizosphere

Introduction

Austrocedrus chilensis (Ciprés de la cordillera) is an endemic Cupressaceae species distributed in southern Argentina and Chile. This forest species is a key component of the Patagonian native forests, and also has an important impact on the local economy due to the high quality of its timber. Throughout all of its distribution area, *A. chilensis* suffers from a disease commonly known as "mal del ciprés" or "*Austrocedrus* root disease" (ARD), caused by *Phytophthora austrocedri* (Vélez *et al.* 2020). Mortality is grouped and occurs in sites with high predisposition to the disease (La Manna *et al.* 2012). Due to the severity of the disease, caused by factors unknown for more than 50 years, *A. chilensis* has been declared as a 'near threatened' species by The IUCN (International Union for the Conservation of Nature) since 2013.

"*Austrocedrus* root disease" also affects the economic exploitation of *A. chilensis* because the disease cannot be controlled efficiently, and this fact makes it impossible to carry out appropriate silvicultural management of diseased stands. The use of fungicides has been largely studied as a method to control *Phytophthora* diseases in agricultural productions and commercial nurseries. The mode of action, dosage, phytotoxicity, time, and mode of application, and the development of pathogen resistance, have been widely discussed in the literature (Erwin and Ribeiro 1996; Rosário *et al.* 2021). However, an effective control of *Ph. austrocedri* in *A. chilensis* has been poorly developed and there are only some successful assays involving preventive application of systemic fungicides such as Fosetyl-Aluminum and Metalaxyl-M (Silva *et al.* 2016).

Although *Phytophthora* diseases could be chemically managed, the use of fungicides in large natural areas is highly expensive and may have a negative impact on aquatic and terrestrial ecosystems, particularly on non-target organisms such as soil rhizospheric microorganisms. Fungicide applications cause a dramatic loss in microbial populations associated with forests, mainly because of a decrease of fungal diversity and richness (Wang *et al.* 2019). The modification of the funga causes a decrease in total nitrogen and carbon content in soil. Additionally, the good water solubility of Metalaxyl might cause its permeation into soil and could result in potential toxicity by rain-wash and irrigation (Marsala *et al.* 2020).

In this sense, biocontrol strategies are very useful alternatives for controlling diseases without having the ecological negative impacts that are caused by chemical products. Historically, microorganisms have been demonstrated to be effective as biocontrol agents against several species of *Phytophthora* (Loliam *et al.* 2012; Berger *et al.* 2015). One of the difficulties in the development of biocontrol strategies is the lack of information about rhizospheric microbiota and funga, and about their possible role in controlling ARD. Ecological information about rhizospheric funga associated with natural *A. chilensis* forests is very scarce. In a previous work we showed that *Penicillium spp.* are one of the dominant species there, and that some species of this genus could be involved in protection against ARD (Marfetán *et al.* 2020). Moreover, additional information about rhizospheric species of *Penicillium* and *Talaromyces* (a genus related to *Penicillium*) associated with *A. chilensis* could be used to select species and strains able to control ARD to be used in a biocontrol strategy. That is why the aims of this study were to describe rhizospheric *Penicillium* and *Talaromyces* species associated with living trees of Patagonian *A. chilensis* forests, and to evaluate their biocontrol activity against *Ph. austrocedri*.

Materials and Methods

Fungal sampling and isolations

Fungal sampling was conducted in the fall of 2019, in three sites in Los Alerces National Park, Chubut province, Argentina. To avoid sampling bias due to climatic conditions, all sites were surveyed the same day. Temperature and humidity were similar in all sites. Two of these sampling sites showed a high prevalence of ARD, with several affected trees (GPS: –42.773588, –71.728884 and –42.814343, –71.713545); the third one was a healthy site (without plants showing ARD symptoms) (GPS: –42.887074, –71.597458). Sites were separated by 21 km.

To isolate and evaluate rhizospheric microorganisms associated with roots, in each sampling site, six healthy seedlings less than 50 cm tall were taken (18 replicates in total, 12 replicates from sites that showed a high prevalence of ARD and 6 replicates from the healthy site). In all cases, plants were carefully dug up, and most of the soil was removed in order to obtain only the roots together with the thin layer of soil in direct contact with them (rhizospheric soil). Roots and rhizospheric soil were submerged 15 min in 250 ml of sterile water. Dilutions 1/10, 1/100 and 1/1000 were made from the original suspensions of rhizospheric soils. These dilutions were plated (100 µl from each dilution) in potato dextrose agar with chloramphenicol (PDAc) (broth from 200 g of potato per liter, 20 g · l^{-1} dextrose, 15 g · l^{-1} agar, 0.16 g · l^{-1} chloramphenicol) and Rose Bengal Agar (RBA)(10 g · l^{-1} dextrose, 10 g · l^{-1} meat peptone, 1 g · l^{-1} K_2HPO_4, 0.5 g · l^{-1} $MgSO_4.7H_2O$, 30 mg · l^{-1} Rose Bengal, 20 g · l^{-1} agar) and 15 replicates were made for each medium. Petri dishes were incubated 7 days at 24°C. All isolates were preliminarily separated into morphotypes, based on macro and micromorphology (Houbraken and Samson 2011; Houbraken *et al.* 2020; Visagie 2014b, 2020, 2021). For further identification, 10 isolates from each morphotype were maintained as pure culture in Petri dishes at 4°C, and in 80% glycerol at –80°C.

Phenotypic examination

Macroscopic characters of *Penicillium* and *Talaromyces* isolates were studied on Czapek Yeast Extract agar (CYA), Malt Extract agar (MEA), Creatine Sucrose agar (CREA), Oatmeal agar (OA), Yeast Extract Sucrose agar (YES) and Dicloran 18% Glycerol agar (DG18). With the exception of MEA (Oxoid, 0059) all media were prepared, as customary, compounding their analytical grade and raw ingredients according to Visagie *et al.* (2014). The different drugs used were

purchased from several commercial sources: agar (Oxoid, LP0011B), creatine monohydrate (Anedra), glucose (Oxoid, LP0071B), glycerol (Cicarelli, 708110), mineral salts (Merck, 1.07687.1000), all of analytical grade; meat peptone (Britania, B0100505), yeast extract (Oxoid, LP0021); dicloran (synthesis product from Facultad de Ciencias Exactas y Naturales, UBA), kitchen powdered oatmeal. Media preparations, inoculations, incubation conditions, and microscopic preparations were done following Visagie *et al.* (2014a). Czapek Yeast Extract agar with 5% NaCl (CYAS) was used for isolates from section Aspergilloides (Houbraken *et al.* 2011a, 2014). All media were poured into 90 mm plastic Petri dishes. Plates were inoculated in a three-point pattern using a micropipette and with an inoculum size of 1 µl per spot. In all cases, incubation was carried out in the dark, at 25ºC, for 7 d. After incubation, colony diameters on each agar medium were measured. The degree of sporulation, anverse and reverse colony colors, colony shape and texture, and the production of soluble pigments were determined. Acid production on CREA was indicated by a change from purple to yellow around growing colonies in pH sensitive bromocresol purple dye. To detect the presence of indole metabolites, Ehrlich reaction was used, with a filter paper method (Frisvad and Samson 2004). For micro-morphological observations, mounts from MEA colonies were made in 85% w/w lactic acid, and a drop of 70% v/v alcohol was added to remove air bubbles and an excess of conidia.

DNA extraction, sequencing, and phylogenetic analysis

DNA extraction was made using the ULTRACLEAN Microbial DNA extraction Kit (MoBio-Qiagen). *Penicillium* isolates were identified using Beta-tubulin (BenA) gene region (primers t10 (5'- ACGATAGGT TCACCTCCAGAC -3') and Bt2b (5'-ACCCTCAGT GTACTGACCCTTGGC-3').

Amplification was carried out in 50 µl with 1×GoTaq Buffer (Promega), 0.2 mM of each dNTP (PBL company); 1 U of Taq (Promega); 0.4 µM of each primer (PBL company) and 75–100 ng DNA, and purified H_2O to complete volume. PCR conditions for *Penicillium* isolates were: 5 min at 94ºC, 35 cycles with 45 s at 94ºC, 60 s at 55ºC and 60 s at 72ºC, and a final step of 7 min at 72ºC. Purification and sequencing of the PCR products were performed by Macrogen Corporation (Korea).

All sequences generated in this study were deposited in the European Nucleotide Archive under accession numbers OU070350 to OU070368. Sequences generated in this study and sequences available in GenBank were aligned using ClustalW, and edited manually using MEGA 10 software (Tamura *et al.* 2013). Phylogenies to identify isolates belonging to the genera *Penicillium* were separated based on their sections and series (Houbraken and Samson 2011; Houbraken *et al.* 2020; Visagie 2014b, 2020, 2021). Phylogenies were reconstructed using maximum likelihood (ML) and Bayesian inference (BI). For these analyses, gaps covering 85% of the positions or more were deleted. In addition, for both analyses, the DNA sequence evolution model was established based on the Akaike information criterion (AIC) implemented in MEGA 10. The Kimura 2-parameter model was selected for all sections with a Γ distribution to model evolutionary rate differences between sites [5 categories (+G, parameter = 0.017)].

The initial tree for the heuristic search was obtained by applying the Neighbor-joining method to a matrix of pairwise distances estimated by the maximum composite likelihood (MCL) approach. The tree was drawn to scale, with branch lengths measured in the number of substitutions per site. Heuristic ML bootstrap analysis consisted of 1000 pseudoreplicates.

The BI analysis was performed in MrBayes v. 3.1 (Ronquist and Huelsenbeck 2003). Posterior probabilities (PP) were calculated by a Metropolis-coupled Markov chain Monte Carlo analysis until the runs converged with a split frequency of 0.01. In all cases, the same sequence evolution model used in ML analysis was employed. A sample frequency of 1000 independent analyses, with 10 million generations each, was used. The first 25% generations were discarded as "burn-in". The consensus trees and the posterior probabilities were calculated from 15,000 trees. The program Figtree V1.4.2 (Yrew Rambaut, Institute of Evolutionary Biology, University of Edinburgh) was used to obtain the final tree and to analyse the Bayesian probability.

Isolation frequency of *Penicillium* and *Talaromyces* species

The fungal species obtained in each sample site from the rhizosphere of *A. chilensis* were compared by running a Two-Way Cluster Analysis. The variation in species composition between sites was evaluated using an absence/presence matrix of the *Penicillium* species/morphospecies and *Talaromyces* species in each site. Jaccard's Distance Coefficient was used as the distance method and Group average was used as the linkage method.

The way rhizospheric *Penicillium* species/morphospecies and *Talaromyces* species grouped in relation to sites was further described, using Correspondence Analysis calculated for absence/presence data of the fungal species. These analyses were carried out using PCOrd6 software (McCune and Mefford 2011).

Evaluation of biocontrol properties of *Penicillium* and *Talaromyces* isolates

To determine the ability of *Penicillium* and *Talaromyces* isolates to parasitize or degrade *Ph. austrocedri* mycelium, co-cultures were prepared using tomato agar medium (TA) as optimal medium for growing *Ph. austrocedri* (Marfetán *et al.* 2020). The isolates obtained in this study and *Ph. austrocedri* 195 (CIEFAP collection) were used for the co-culture assay. *Phytophthora* isolate was inoculated and grown alone until it reached an area of 6.5 cm^2. Then, *Penicillium* or *Talaromyces* isolates were inoculated on the opposite side of the Petri dishes (Marfetán *et al.* 2020). Ten replicates were used in all dual culture assay combinations. *Ph. austrocedri* 195 was grown alone, as a control (n = 10). The final area was measured after 14 days using the software ImageJ 1.4 (Wayne Rasband, National Institute of Health, USA). Final areas of *Ph. austrocedri* exposed to each *Penicillium* and *Talaromyces* spp. were compared with that of the control using non-parametric Kruskal Wallis test, with *a posteriori* contrast adjusted by Bonferroni correction to keep an alpha level of 0.05. Micro-cocultures using TA over slides were used to confirm the results, following Marfetán *et al.* (2020). The effect of *Penicillium* species on *Ph. austrocedri* hyphae growth and morphology was evaluated using an optical microscope (Leica, Model DM2500), every day for 10 days.

Results

Fungal survey

During the survey, 81 *Penicillium* spp. isolates were obtained from the rhizospheric soil. These isolates were separated into 20 different morphospecies, which were defined based on the morphology of each isolate. These isolates were included in seven *Penicillium* sections (Aspergilloides, Brevicompacta, Canescentia, Citrina, Exilicaulis, Fasciculata, and Ramosum) (Houbraken and Samson 2011; Houbraken *et al.* 2020).

Morphological and molecular identification

Phylogenies to identify *Penicillium* isolates were separated according to their sections and series also following Houbraken and Samson (2011) and Houbraken *et al.* (2020). The composition of the matrix for each section is described in Table 1.

The first three morphospecies were included in the Aspergilloides section. *Penicillium* sp. 1 and *Penicillium* sp. 2 were included in the series Glabra, and *Penicillium* sp. 3 in the series Pinetorum. Isolates of *Penicillium* sp. 1 were grouped in the *Penicillium glabrum* clades, reflecting the paraphyletic nature of this species in phylogenies with BenA gene (Houbraken *et al.* 2014). Isolates P2, P52, P51, and P42, were placed in *P. glabrum sensu stricto* clade, in close association with *P. glabrum* type material, with high support (ML 100%, BI 1). Isolates P18 and P54 were placed in the *P. glabrum* clade 2 (ML 85%, BI 1) (Fig. 1a), closely related to other strains isolated from geographically nearby sites.

The diagnostic characters of our isolates agreed with those of *P. glabrum* given by Houbraken *et al.* (2014). However, the growth rate of P18 and P54 in CYA and MEA (7 days, 25°C) was slower than the one reported for *P. glabrum* by these authors. Phenotypically, the colony diameters in CYA and MEA of isolates from clade 1 were bigger than the ones of isolates from clade 2 (30–45 and 32–45 mm for isolates from clade 1 *vs*. 23–25 and 23–30 mm for isolates from clade 2, in CYA and MEA, respectively). The phialides of isolates from clade 1 were slightly larger than those of isolates from clade 2, although the ranges overlapped in the different strains. On the other hand, crusts formation in the isolates of clade 1 in MEA (except for P2)

Table 1. Composition of Beta-tubulin (BenA) gene matrix for each section, describing the number of taxa and the number of nucleotide positions (characters) including gaps. Additionally, the number of conserved sites, variable sites and Parsimony informative sites are shown

Section	Series	Number of taxa	Characters	Conserved sites	Variable sites	Parsimony informative sites
Aspergilloides	Glabra	42	420	348	70	58
Aspergilloides	Pinetorum	23	419	350	66	24
Brevicompacta	Brevicompacta	23	319	204	114	48
Canescentia	Canescentia	38	384	292	90	39
Citrina	Westlingiorum	76	467	273	177	133
Exilicaulis	Corylophila	30	463	291	143	60
Fasciculata	Camberbetorium	50	340	271	69	47
Ramosa	Lanosa	17	432	348	74	28

was observed, while none of the isolates of clade 2 produced crusts.

Penicillium sp. 2 was grouped with *Penicillium bussumense* (ML 100%, BI 1) (Fig. 1a). However, the features

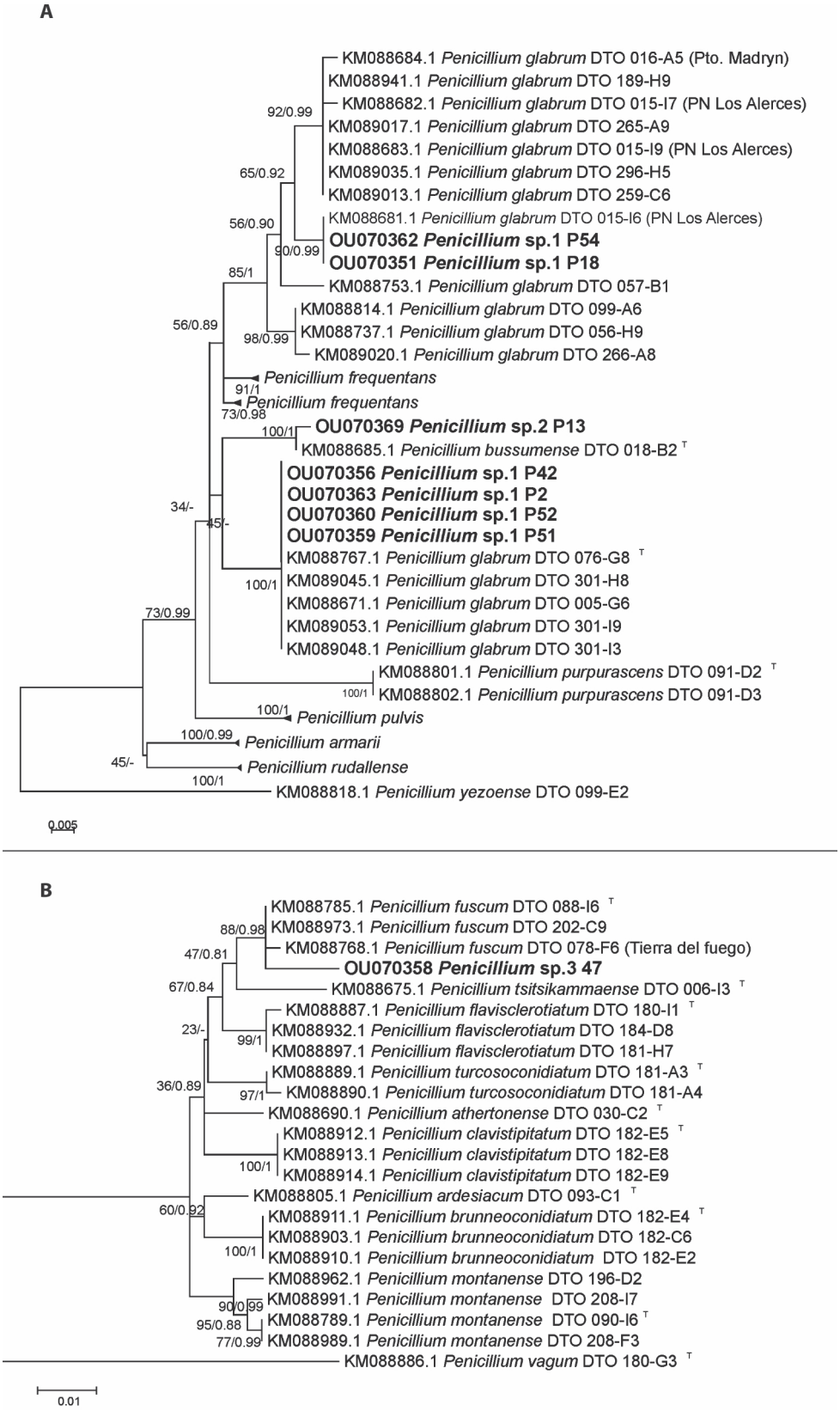

Fig. 1. Phylogenetic tree of *Penicillium* isolates belonging to *Aspergilloides* Section inferred from Beta-tubulin (BenA) gene, illustrating species relationships inferred from joint ML analysis of BenA gene analysis. Sequences generated in this study are shown in bold. The series of two values above internal branches correspond to ML and BI posterior probabilities, respectively. Nodes without support are indicated by a dash, and condensed clades with a black triangle; A – phylogenetic tree of isolates in series Glabra, B – phylogenetic tree of isolates in series Pinetorum. Type species have a superscript uppercase t (T)

of this morphospecies (isolate P13) did not agree with the description of *P. bussumense* (Houbraken *et al.* 2014). Diagnostic characters of this species are the CYAS : CYA ratio between 0.99–1.02, and the production of dark green conidia, floccose colonies and with the broad non-sporulating margin on MEA. Our strain had a CYAS : CYA ratio of 0.62 and green conidia. Additionally, our strain had a good sporulation in CYA, which was absent in *P. bussumense*. Although the observed characteristics coincided with members of the Aspergilloides section, other markers will be needed to identify this isolate.

Penicillium sp. 3 was related to *Penicillium fuscum* (ML88%, BI 0.98) (Fig. 1b). The morphology of the isolate P47 from this morphospecies did not agree with the morphological description of *P. fuscum* or any species in the section. Raper and Thom (1949) described conidiophores conspicuously septated, with walls comparatively heavy, light yellow-brown in colour and smooth or finely roughened; conidia globose, spinulose, became coarsely roughened with prominent dark tubercles when mature, mostly 3.5–4.0 µm or up to 4.5 µm in diameter. Our isolate had smooth to finely roughened, not pigmented conidiophores, sometimes septated. Conidia were smooth and subglobose, 2.5–3.5 × 2.4–2.5 µm. The strain DTO 078-F6 (formerly *A. lapatayae*), isolated from Tierra del Fuego (Argentina), and also included in the *P. fuscum* clade, produces conspicuously ornamented subglobose conidia and pink sclerotia (Houbraken *et al.* 2014). *Penicillium tsitsikammaense* is a species close to our isolate but, in contrast to it, sporulation was absent or poor on all media. Also, this species had sclerotia, while *Penicillium* sp. 3 isolate did not produce sclerotia after 60 days.

Penicillium sp. 4 was grouped, with a high support, in the *Penicillium bialowiezense* clade, in Brevicompacta Section (ML 98%, BI 1) (Fig. 2). The colony pattern and growth in the different media, and the micromorphology of these strains (P12 and P53), agree with the description given by Frisvad and Samson (2004).

One morphospecies, *Penicillium* sp. 5 (isolates P1 and P6), was grouped in the *Penicillium arizonense* clade, series Canescentia, in the Canescentia section (ML 62%, BI 0.94). Our data show that this clade was separated into two independent subclades (Fig. 3). Our isolate was grouped in a well-supported clade (ML 93%, BI 0.91), with an unidentified sequence (Genbank code KC175294), and separated from the material type. Isolates P1 and P6 are phenotypically closely related to *P. arizonense* (Grijseels *et al.* 2016). The macromorphology agreed with that of *P. arizonense*, except for the colonies on YES that present an orange reverse, whereas the protologue describes red-brown to violet-brown ones. As regards micromorphology, penicilli were observed irregularly biverticillate and strongly divaricate with smooth walled stipes (as described for *P. arizonense*), with slightly greater metulas than the ones in the original description (9.8–19 × 2–3 *vs.* 8–16 × 2–3 µm).

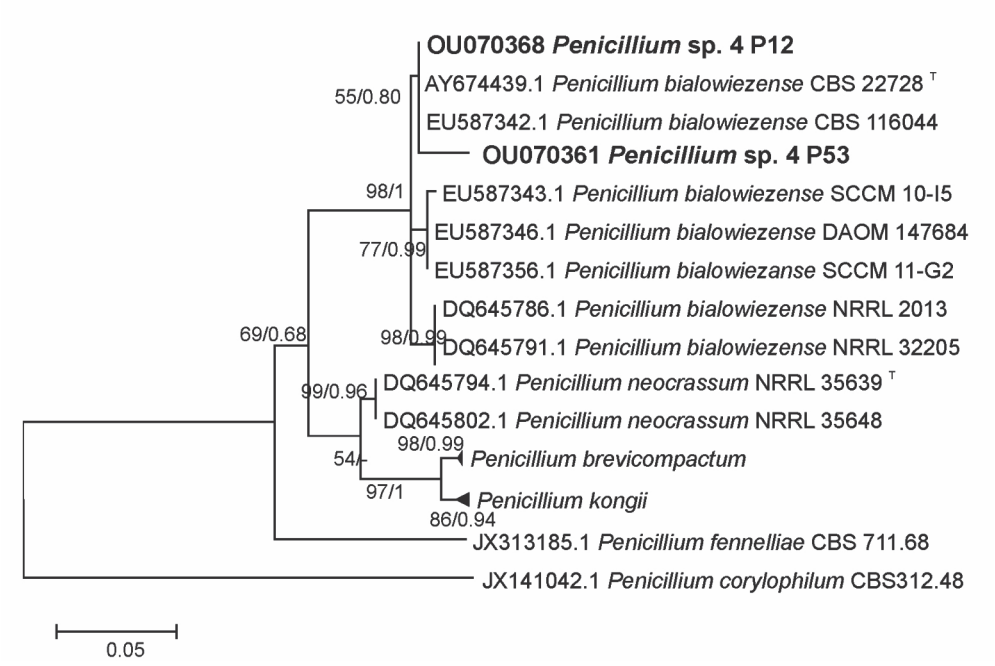

Fig. 2. Phylogenetic tree of series Brevicompacta (Section Brevicompacta) inferred from Beta-tubulin (BenA) gene, illustrating species relationships inferred from joint ML analysis of BenA gene analysis. Sequences generated in this study are represented in bold. The series of two values above internal branches correspond to ML and BI posterior probabilities, respectively. Nodes without support are indicated by a dash, and condensed clades with a black triangle. Type species have a superscript uppercase t (T)

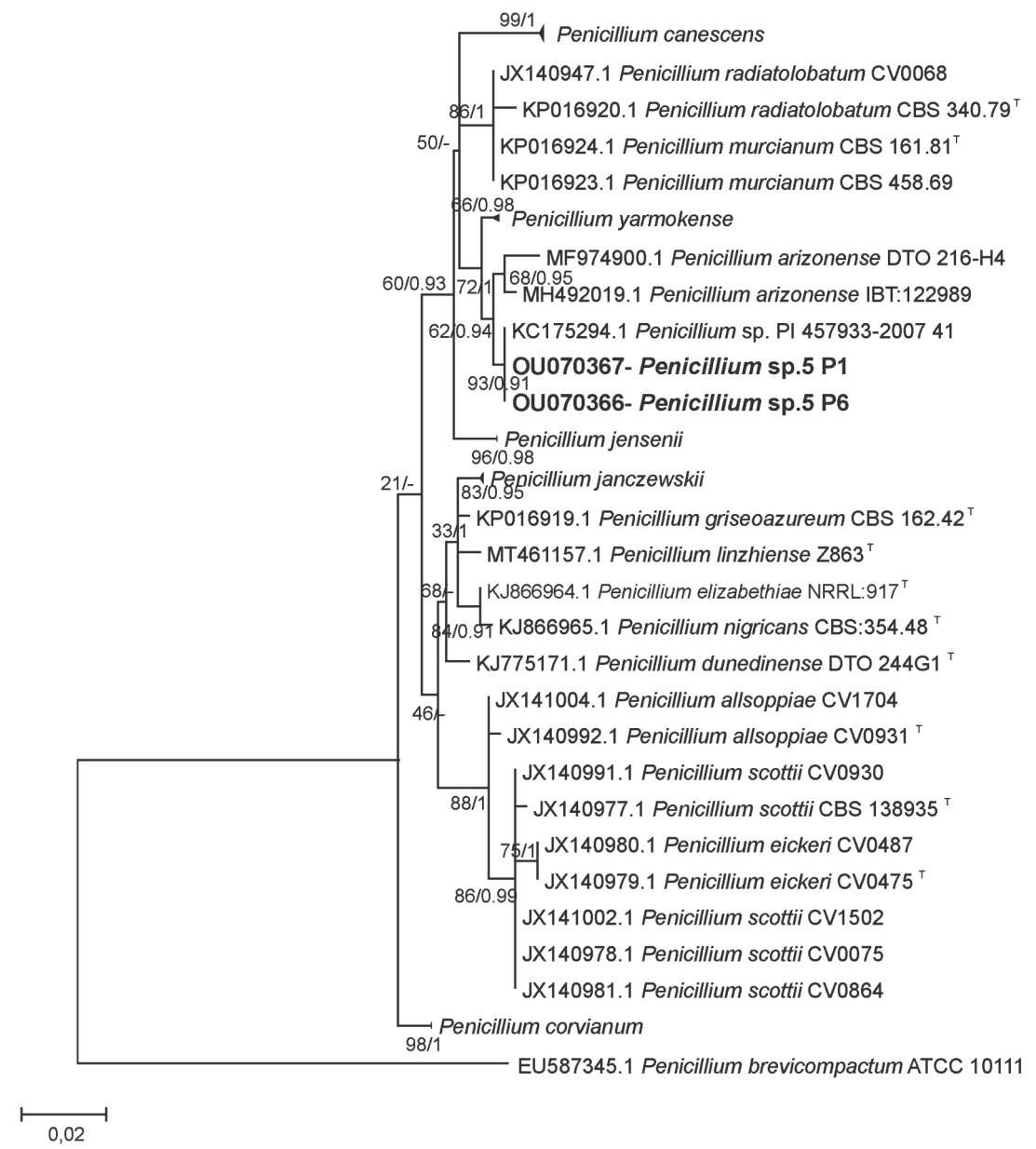

Fig. 3. Phylogenetic tree of *Penicillium* series Canescentia (Section Canescentia) inferred from Beta-tubulin (BenA) gene, illustrating species relationships inferred from joint ML analysis of BenA gene analysis. Sequences generated in this study are represented in bold. The series of two values above internal branches correspond to ML and BI posterior probabilities, respectively. Nodes without support are indicated by a dash and condensed clades with a black triangle. Type species have a superscript uppercase t (T)

Penicillium sp. 6 isolates (P10, P32, and P33) and *Penicillium* sp. 7 (isolate P38) were placed in the series Camembertiorum (Sect. Fasciculata). The former was grouped in the *P. palitans* clade (ML 98%, BI 1), while the latter was grouped in the *P. echinulatum* clade (ML 95%, BI 0.99) (Fig. 4). The culture and micromorphological features of the isolates P10, P32, and P33, agreed with the description of *P. palitans* (Frisvad and Samson 2004). The morphology of the isolate P38 agreed with the characteristics described for *P. echinulatum* (Frisvad and Samson 2004).

Penicillium sp. 8 was resolved in a well-supported lineage in the Westlingiorum series from the Citrina section. This morphospecies was grouped with *Penicillium manginii* strains, including type material for this species, forming a well-supported clade (ML 98%, BI 1) (Fig. 5). The phenotypic characters agreed with the description provided by Houbraken *et al.* (2011a). Nevertheless, the colony diameters obtained for MEA cultures (25°C, 7 days) were smaller than those described in the original description (17–19 vs. 25–37 mm, respectively). As described by Houbraken *et al.* (2011a), this species produced a strong red soluble pigment on YES, abundant orange sclerotia, and no ascospores were observed after a long incubation (MEA and OA for 60 days).

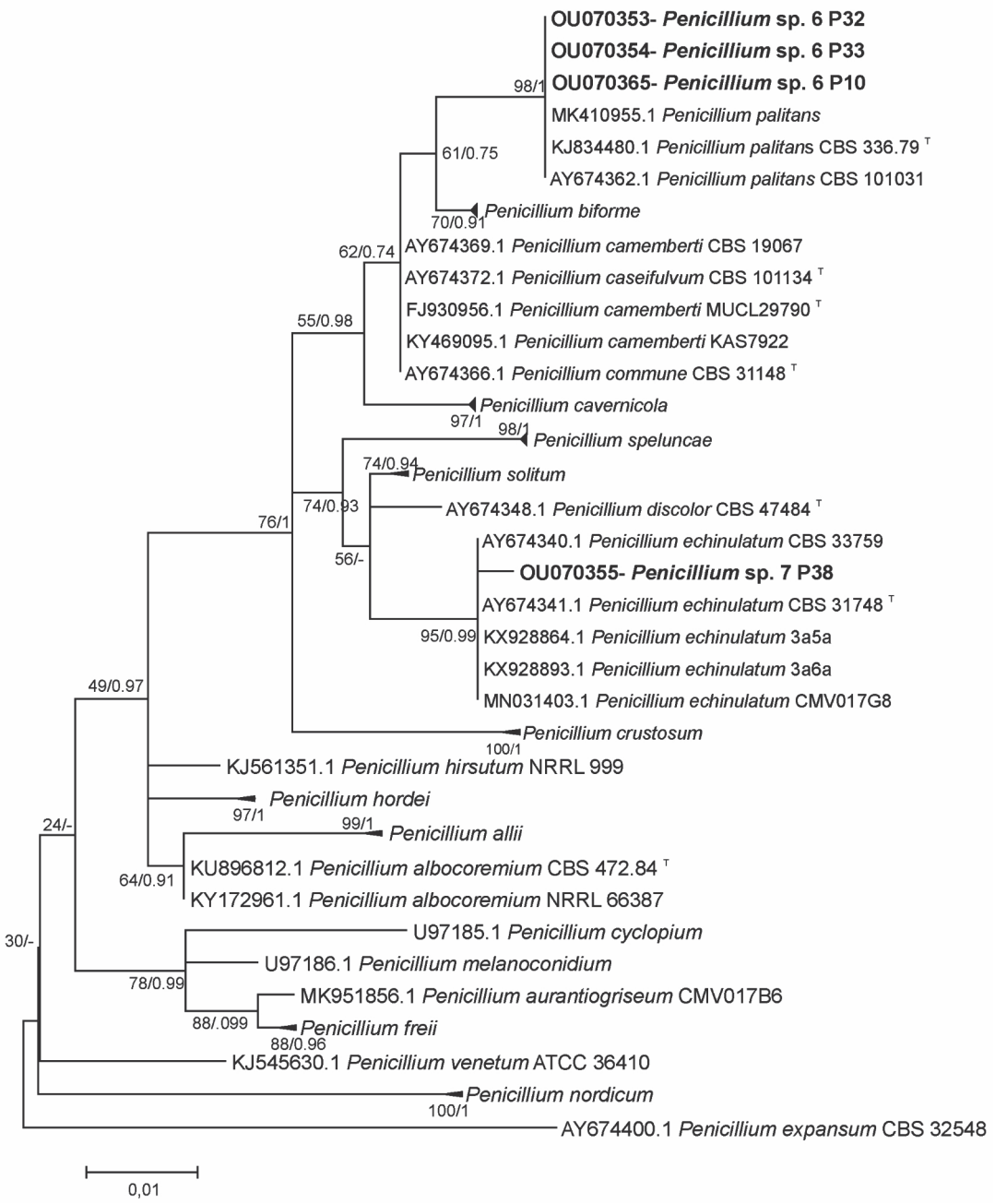

Fig. 4. Phylogenetic tree of *Penicillium* series Camembertiorum (Section Fasciculata) isolates inferred from Beta-tubulin (BenA) gene, illustrating species relationships inferred from joint ML analysis of BenA gene analysis. Sequences generated in this study are represented in bold. The series of two values above internal branches correspond to ML and BI posterior probabilities, respectively. Nodes without support are indicated by a dash and condensed clades with a black triangle. Type species have a superscript uppercase t (T)

Penicillium sp. 9 was identified as a member of the series Corylophila (Sect. Exilicaulis). The resulting tree showed that *Penicillium* sp. 9 was included in the *Penicillium corylophilum* clade with high support (ML 90%, BI 0.99) (Fig. 6). P45 strain agreed with most of the characteristics given in the descriptions of Pitt (1979) and of Samson *et al.* (2002). However, our isolate (P45) had slightly bigger conidia (3–3.5 × 2.5–3 µm) than the ones mentioned by these authors (up to 3.2 µm).

Finally, *Penicillium* sp. 10 and *Penicillium* sp. 11 were identified as members of the series Lanosa, in the Ramosum section. *Penicillium* sp. 10 was grouped in *Penicillium swiecickii* clade (ML 76%, BI 0.73), and *Penicillium* sp. 11 was grouped with good support in the *P. lanosum* clade (ML 68%, BI 0.98) (Fig. 7). In both cases, the morphological features agreed with the description given by Raper and Thom (1949).

Penicillium sp. 2 and *Penicillium* sp. 22 were morphologically identified as *Talaromyces amestolkiae*. The isolates showed morphological features in close agreement with those described in the protologue, even sclerotia which do not mature into cleistothecia, red reverses on CYA, MEA and YES, and acid production on CREA (Yilmaz *et al.* 2012).

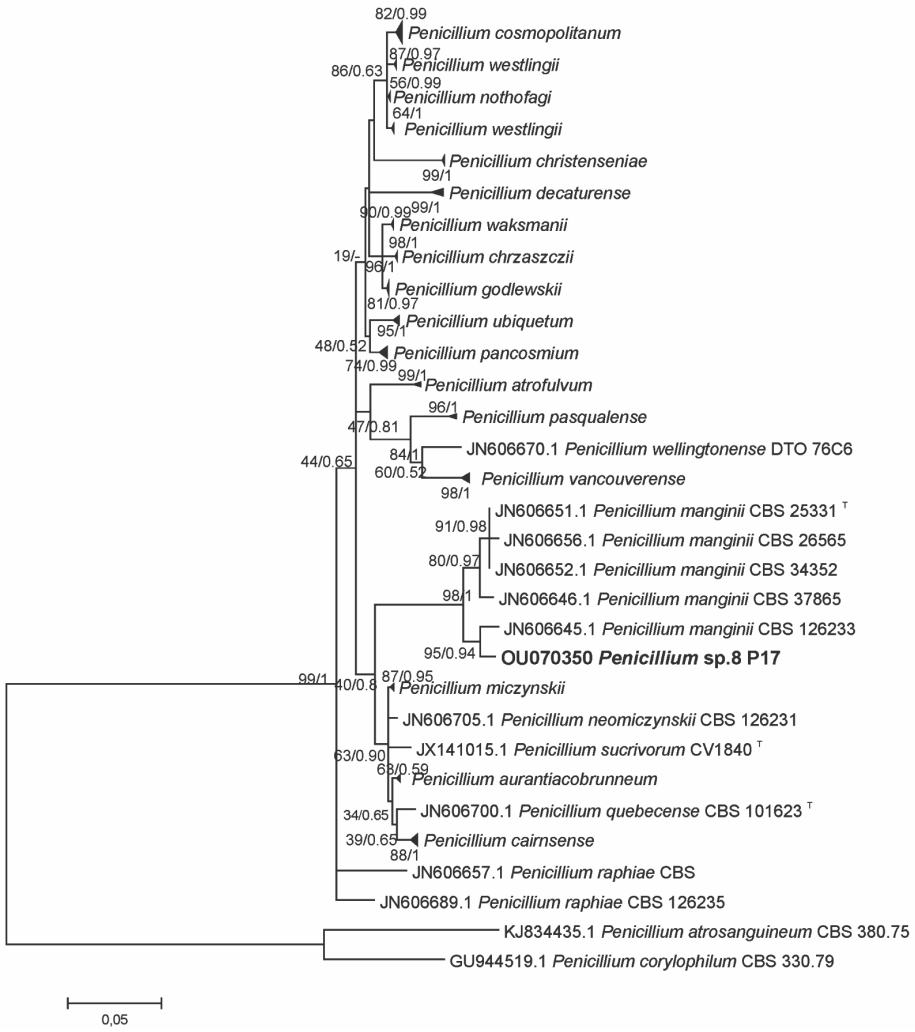

Fig. 5. Phylogenetic tree of *Penicillium* series Westlingiorum (Section Citrina) isolates inferred from Beta-tubulin (BenA) gene, illustrating species relationships inferred from joint ML analysis of BenA gene analysis. Sequences generated in this study are represented in bold. The series of two values above internal branches correspond to ML and BI posterior probabilities, respectively. Nodes without support are indicated by a dash and condensed clades with a black triangle. Type species have a superscript uppercase t (T)

The morphospecies *Penicillium* sp. A, *Penicillium* sp. 24, *Penicillium* sp. 26, *Penicillium* sp. 28, and *Penicillium* sp. B were not identified due to contaminations and loss of the cultures after the biocontrol assay. These isolates were considered as morphospecies in posterior analysis.

Isolation frequency of *Penicillium* and *Talaromyces* species

We found 81 isolates from the *Penicillium* genus, corresponding to 17 species. Differences in the *Penicillium* isolation pattern between sites were observed. Nineteen isolates corresponding to 9 different species were obtained from seedlings in sites without ARD, while 62 isolates corresponding to 16 different species were obtained from seedlings in sites with ARD. The species most frequently isolated, and in all sites, were *P. glabrum, P. swiecickii*, and *P. palitans* (frequency >0.105 in healthy sites, and >0.19 in sites with ARD); among them, *P. glabrum was* the dominant species in both types of sites (frequency 0.26 in healthy sites, and 0.30 in sites with ARD). *Penicillium arizonense* and *P. bialowiezense* isolates were present in both types of sites, but with a lower frequency than *P. glabrum, P. swiecickii* and *P. palitans*. In communities from healthy sites, *P. arizonense, P. manginii,* and *P. bialowiezense* were species with high frequency (0.105), while *P. corylophilum*, *T. amestolkiae* and *P. echinulatum* were species frequently isolated in communities from sites with ARD (frequency 0.08, 0.06, and 0.04, respectively) (Fig. 8).

The distribution of the *Penicillium* species changed between sites, as it was shown in the Two-Way Cluster analysis relating *Penicillium* species and different sites. The analysis showed five clusters. Cluster 1 grouped species present in all the sampled sites, cluster 2 grouped species only found in sites without ARD, and

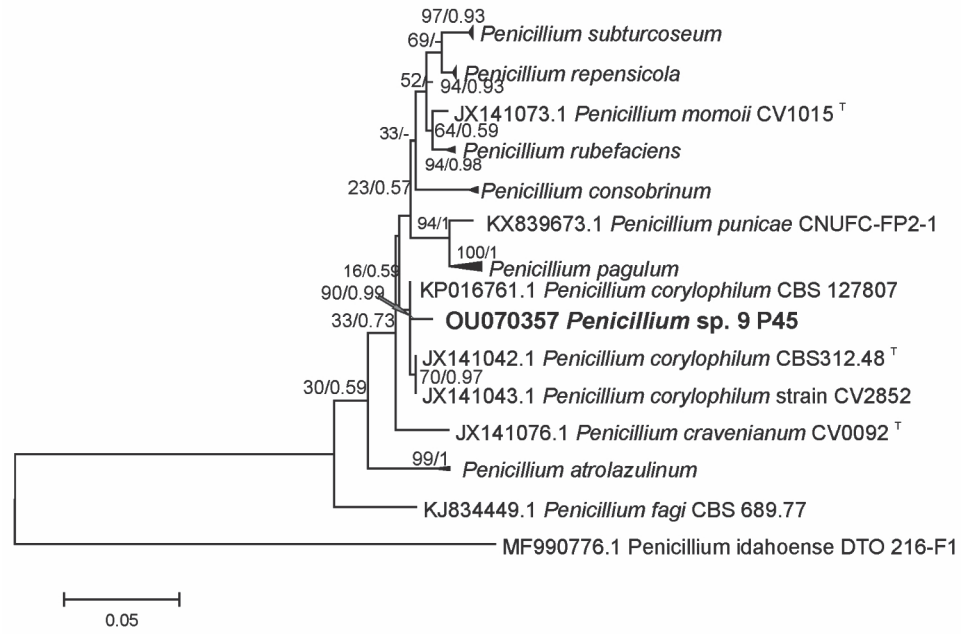

Fig. 6. Phylogenetic tree of *Penicillium* series Corylophila (Section Exilicaulis) isolates inferred from Beta-tubulin (BenA) gene, illustrating species relationships inferred from joint ML analysis of BenA gene analysis. Sequences generated in this study are represented in bold. The series of two values above internal branches correspond to ML and BI posterior probabilities, respectively. Nodes without support are indicated by a dash and condensed clades with a black triangle. Type species have a superscript uppercase t (T)

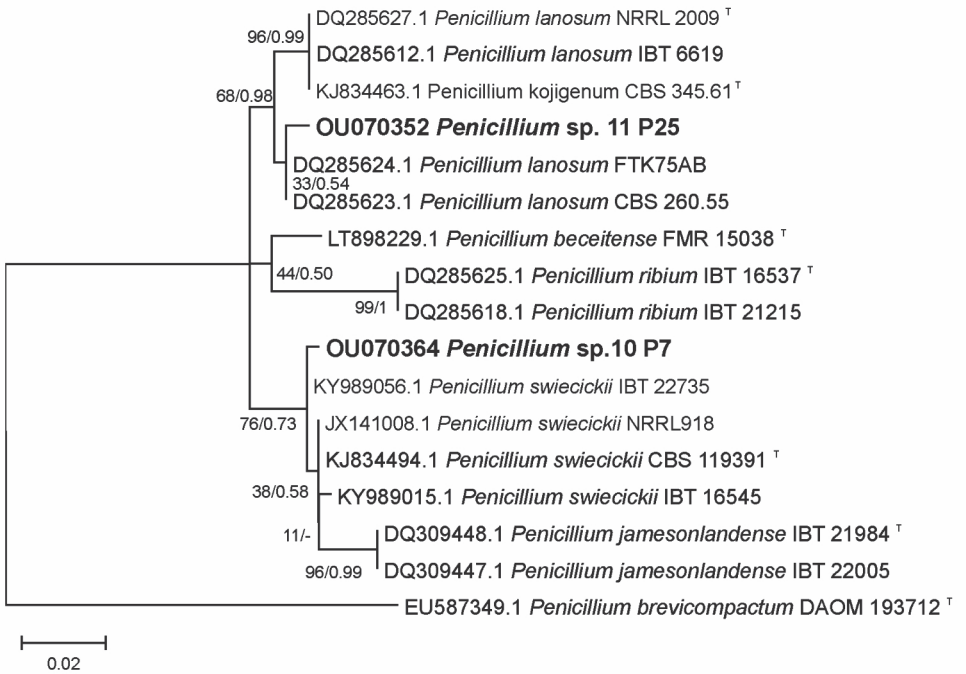

Fig. 7. Phylogenetic tree of *Penicillium* series Lanosa (Section Ramosum) isolates, inferred from Beta-tubulin (BenA) gene illustrating species relationships inferred from joint ML analysis of BenA gene analysis. Sequences generated in this study are represented in bold. The series of two values above internal branches correspond to ML and BI posterior probabilities, respectively. Nodes without support are indicated by a dash and condensed clades with a black triangle. Type species have a superscript uppercase t (T)

clade 4 grouped species only found in sites with ARD. Clades 3 and 5 grouped *Penicillium* species found in only one of the diseased sites (Fig. 9).

The correspondence analysis revealed the funga heterogeneity of the sites, determining three clusters, which confirmed the differences between sites

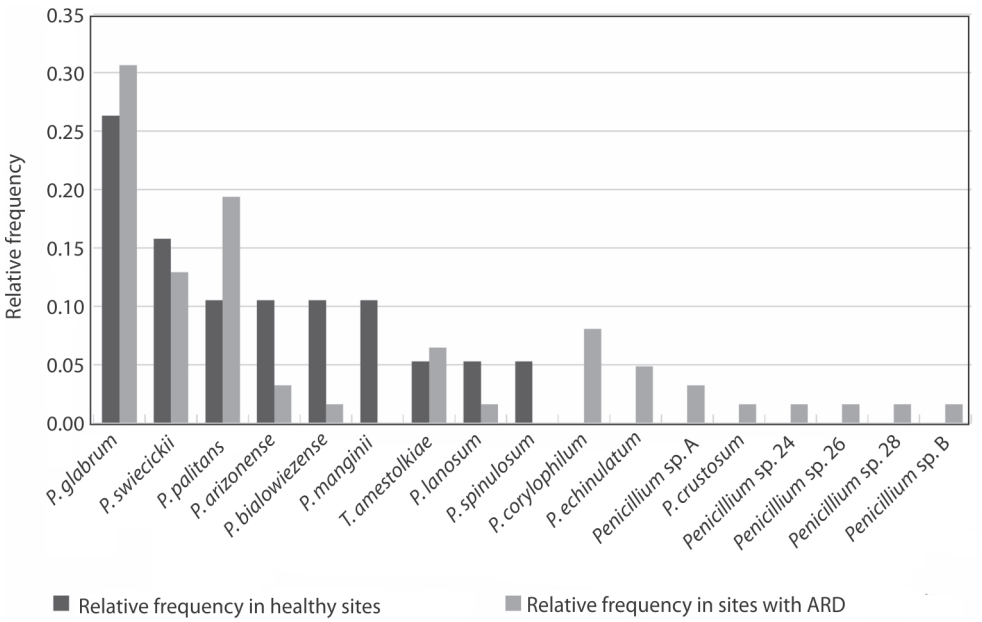

Fig. 8. Relative frequency of *Penicillium and Talaromyces* species in sites with symptoms of *Phytophthora austrocedri* (sites with *Austrocedrus* Root Disease) in comparison with disease-free sites (healthy sites)

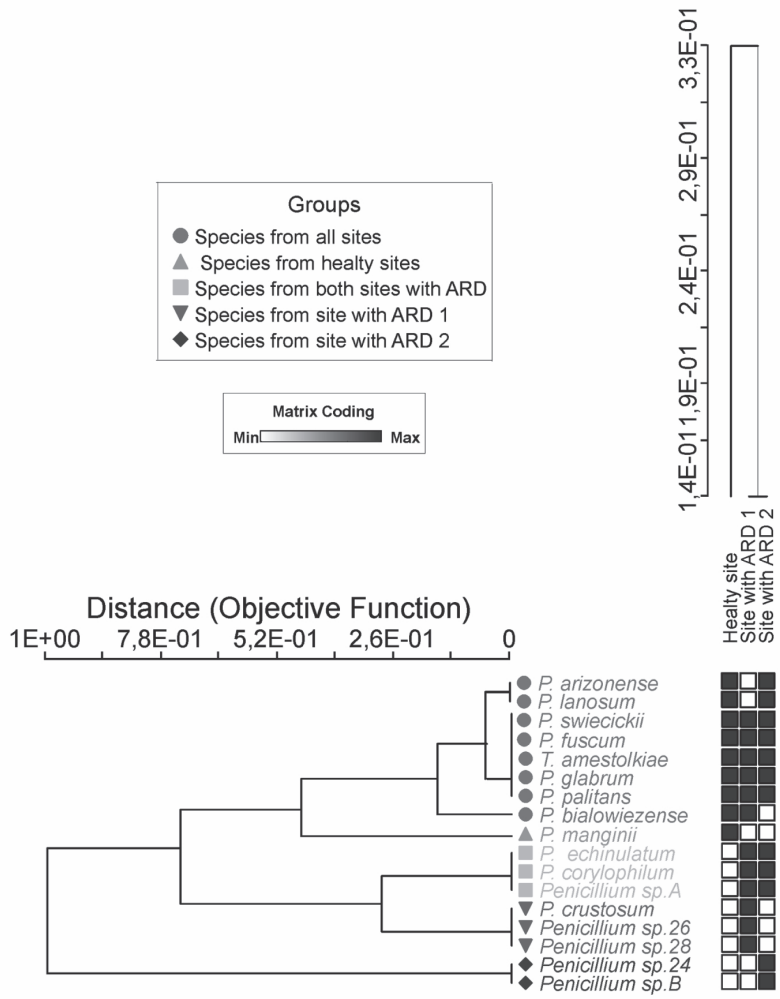

Fig. 9. Two-way cluster analysis evaluating differences in the community composition among different sites with different health conditions (one site without symptoms and two sites with high number of trees with *Austrocedrus* Root Disease)

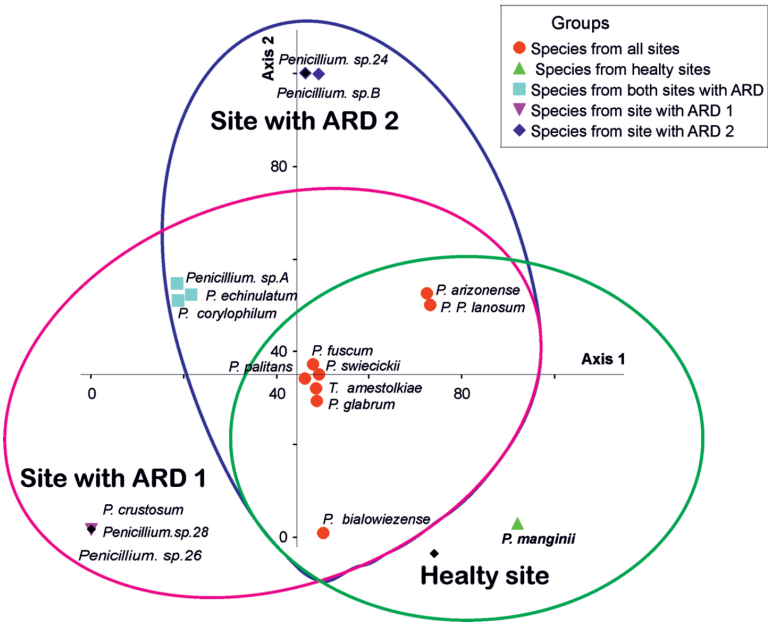

Fig. 10. Correspondence analysis ordering the different sampling sites using *Penicillium* and *Talaromyces* species present in each site

(Fig. 10). All clusters shared a group of species that included *P. glabrum, P. swiecickii, P. palitans, P. fuscum, P. arizonense, P. bialowiezense, T. amestolkiae,* and *P. lanosum*, showing that these species were present in all sites, regardless of the health condition. *Penicillium* sp. B and *Penicillium* sp. 24 were obtained from one ARD site, while *P. crustosum, Penicillium* sp. 26, and *Penicillium* sp. 28 were obtained from the other ARD site. *Penicillium* sp. A, *P. corylophilum,* and *P. echinulatum* were present in both sites with ARD (Fig. 10). One species, *P. manginii,* was exclusively isolated from the site without ARD symptoms (healthy site).

Biocontrol activity of *Penicillium* and *Talaromyces* isolates against *Ph. austrocedri*

The control group (*Ph. austrocedri* growing alone) showed normal growth, with abundant mycelium, and a final area of 60.32 cm^2. In contrast, in co-cultures (*Ph. austrocedri* and a penicilate strains) all *Penicillium*-like isolates showed a negative effect on *Ph. austrocedri* growth. The co-cultivated *Phytophthora* isolate showed a lower growth rate, with a smaller final area (area < 49.49 cm^2, H = 92.63, p < 0,0001) in comparison to the control group (Fig. 11a). All the isolates of the species *P. arizonense, P. swiecickii, P. fuscum, P crustosum, P. palitans, P. glabrum,* and the isolate *T. amestolkiae* sp. 22 showed a significantly smaller area than the control (p < 0.003). However, *Penicillium glabrum* sp. 21, *P. crustosum*, *P. palitans*, *T. amestolkiae* sp. 22 and *P. glabrum* sp. 16 were the strains with the higher inhibitory effect on *Phytophthora* growth (final area from 26.52 to 4.48 cm^2, respectively) (Fig. 11a). As an example, the growth of *Ph. austrocedri* in culture, alone or in the presence of the isolates *P. Glabrum* sp. 21 or *P. lanosum*, is shown (Fig. 11b–d). Micro-cocultures confirmed the inhibitory effect of *P. arizonense, P. swiecickii, P. fuscum, P crustosum, P. palitans, P. glabrum,* and *T. amestolkiae* sp. 22 against *Ph. austrocedri*. Microscopic images showed a growth restriction of *Ph. austrocedri* to the initial inoculum, and *Phytophthora* hyphae showed necrotic symptoms such as reduction of the cell wall thickness and cell collapse (Fig. 12c, d).

Discussion

This study reports *Penicillium*-like species isolated from the rhizosphere of *A. chilensis,* and the possible application of species belonging to the genus *Penicillium* and *Talaromyces* as biocontrol agents against *Ph. austrocedri*. Although information about rhizospheric funga associated with natural forests is very scarce, our results agreed with the results obtained in studies about virgin forests, in soils of Caatinga vegetation and Atlantic Forest region from Brazil (Cruz *et al.* 2013), and leaf litter and soil in Colombian Amazon Forest (Houbraken *et al.* 2011b). In all cases, species of the genus *Penicillium* were the dominant species and the richness of *Penicillium* species was particularly high. This study describes several *Penicillium* and one *Talaromyces* species obtained from *A. chilensis* forests, and shows that isolation frequency of each species changed between sites. Fungal diversity was

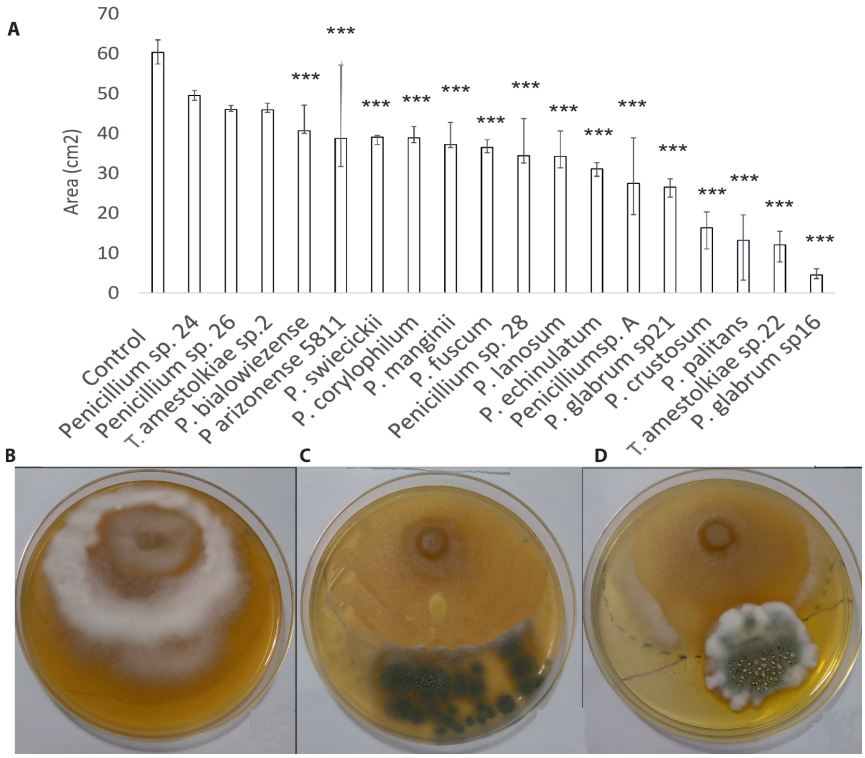

Fig. 11. Co-culture assay analysing the effect of different *Penicillium* and *Talaromyces* isolates on *Phytophthora austrocedri* growth. A – final area of *Ph. austrocedri* (cm^2) colony in the control group (*Ph. austrocedri* growing alone) and in the dual cultures, expressed as median and quartiles. Asterisks refer to significant differences with the control group ($p < 0.001$); B – control group: *Ph. austrocedri* growing alone; C – dual culture of *Ph. austrocedri* and *P. Glabrum* sp 21 isolate; D – dual culture of *Ph. austrocedri* and *P. lanosum* isolate

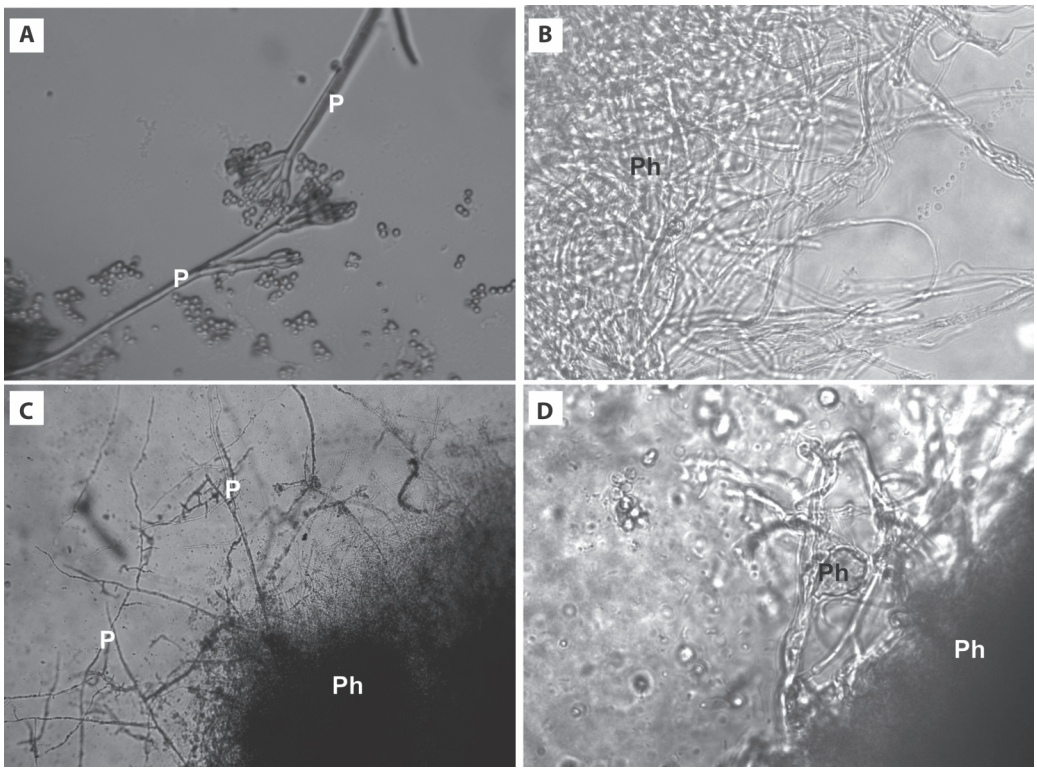

Fig. 12. Microscopic interactions between *Ph. austrocedri* (Ph) and the *Penicillium* (P) isolates. A – control group (*Penicillium* spp. growing alone); B – control group (*Ph. austrocedri* growing alone); C, D – fungicide effect of *Penicillium* strains on *Ph. austrocedri*, showing hyphal degradation (arrow) at 100X and 400X, respectively

significantly greater in sites with ARD than in healthy sites. Some species, such as *P. glabrum, P. swiecickii, P. palitans, P. fuscum, P. arizonense, P. bialowiezense, T. amestolkiae*, and *P. lanosum*, were isolated from both types of sites, but other species were only found in one site. Particularly, *Penicillium* sp. B and *Penicillium* sp. 24 were associated with just one of the sites with ARD; and *P. crustosum, Penicillium* sp. 26 and *Penicillium* sp. 28 with the other site with ARD. Changes in *Penicillium* communities among different habitats, sites and plant communities were previously reported (Cruz *et al.* 2013).

Although this was a small-scale study, many morphological differences with the species previously described for this genus were found, leading to a potentially new species. We observed slight differences in some isolates of *P. glabrum* and *P. manginii*. Isolates *P. glabrum* P18 and P54 showed a slower growth rate in CYA and MEA than the description of *P. glabrum* (Houbraken *et al.* 2014). For *Penicillium manginii* strains, the colony diameters obtained for MEA cultures were smaller than those given in the original description (Houbraken *et al.* 2011a). These data could be considered as normal morphological variation within the species and may contribute to the correct identification of the Argentinean isolates from these species.

On the other hand, some isolates from species such as *P. bussumense* and *P. arizonense* showed significant differences in morphology or phylogeny when compared to the original descriptions (Houbraken *et al.* 2014; Grijseels *et al.* 2016). The diagnostic characters for the identification of *P. bussumense* are the CYAS:CYA ratio between 0.99–1.02, and the production of dark green conidia, floccose colonies and with the broad non-sporulating margin on MEA. Our *P. bussumense* strain had a CYAS:CYA ratio of 0.62, green conidia, and velutinous colonies with floccose centres (CYA and MEA). It is possible that BenA molecular markers were not adequate to satisfactorily resolve phylogenetic relationships at the species level. The implementation of additional markers could give a precise identity of this isolate. Additionally, isolates from *P. arizonense* were clustered in two independent clades (Fig. 3). Isolates P1 and P6 were grouped in the second clade, separated from the type strain, possibly suggesting a new species. Supporting this separation, the colonies on YES showed an orange reverse, but the protologue describes red-brown to violet-brown ones. Moreover, metulae were slightly bigger than the ones reported in the original description. Despite the necessity of further studies using additional molecular markers to accurately identify these isolates, our results showed the importance of a taxonomic study of rhizospheric *Penicillium* species of Patagonian forests.

Although the role of *Penicillium* species in the rhizospheric soil is unclear, it might have important effects on plants. Some *Penicillium* species produce solubilized phosphorus, siderophores, and phytohormones such as indole acetic acid and gibberellic acid, which are important for plant health and for growth promotion. Some species also have been reported to have a biocontrol effect against fungal plant pathogens (Fang and Tsao 1995; Marfetán *et al.* 2020). Accordingly, we focus on the role of *Penicillium*-like species in the biocontrol activity against *Ph. austrocedri*. Species with higher inhibitory effect on *Phytophthora* growth were *P. glabrum*, *P. crustosum*, *P. palitans*, and *T. amestolkiae*. In this sense, our results also showed that *Penicillium glabrum*, *P. palitans,* and *T. amestolkiae* are species present in trees from all the sampled sites. This result suggests that these species can be great candidates for a biocontrol strategy to protect *A. chilensis*. In a previous study, we showed that *P. glabrum* has a fungistatic effect, decreasing the growth rate of *Ph. austrocedri* mycelium, and that *P. corylophilum* and *P. palitans* isolates were able to degrade the pathogen hyphae through a parasitic process (Marfetán *et al.* 2020).

Conclusions

Our results highlight that *Penicillium* and *Talaromyces* species have a high biocontrol activity, which also reinforces the hypothesis that some *Penicillium*-like species could be used in the formulation of a control strategy. This study lays the foundation for more in-depth studies on the application of *Penicillium* and *Talaromyces* isolates in biological control strategies. Moreover, as *P. glabrum*, *P. crustosum* and *T. amestolkiae* do not produce important mycotoxins (Samson *et al.* 2019), their inoculation to increase their abundance in soil could be a useful tool to control ARD in native forests. Further studies about the mechanisms of induction of pathogen resistance are needed to understand the modes of action of *Penicillium* species in ARD control.

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