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Pestalotiopsis pini sp. nov., an Emerging Pathogen on Stone Pine (*Pinus pinea* L.)

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Abstract: *Research Highlights*: *Pestalotiopsis pini* sp. nov. is an emerging pathogen on stone pine, *Pinus pinea* L., in Portugal. *Background and Objectives*: Stone pine is one of the most important forest tree species in Portugal and in the whole Mediterranean basin. *Pestalotiopsis* species are common endophytes, saprobes or pathogens in a variety of hosts and environments. The objective of the present study was to identify the *Pestalotiopsis* species associated with the symptomatic stone pine trees. *Materials and Methods*: Samples of stone pine trees showing shoot blight and stem necrosis were obtained from stone pine orchards and urban areas in Portugal, and the isolated *Pestalotiopsis* species were identified based on morphology and combined ITS, *TEF* and *TUB* DNA sequence data. Artificial inoculations on one-year-old stone pine seedlings were performed with the two species most frequently found in association with shoot blight disease. *Results*: Five *Pestalotiopsis* spp. were isolated. A taxonomic novelty, *Pestalotiopsis pini* is described, representing a new pathogen for stone pine. *Conclusions: Pestalotiopsis* species may represent a threat to the health of pine forests in the Mediterranean basin. Future research should be done in order to increase our knowledge about the potential impact of pestalotioid species in stone pine, in order to develop management strategies against these pathogens.

Keywords: dieback; Mediterranean forest; multi-locus phylogeny; pathogenicity; pestalotioid fungi

1. Introduction

Stone pine, *Pinus pinea* L., is one of the most important forestry species in Portugal and the Mediterranean basin. Stone pine forests play an important role in the economy of the areas where they are planted, especially due to the high value of edible pine nuts, which are the main resource of this industry [1]. *Pinus pinea* is broadly considered a robust species. In recent years, pine nut production has been decreasing due to several factors, including pests and diseases [1,2].

Pestalotiopsis is a widely distributed genus of appendage-bearing conidia belonging to the family Sporocadaceae [3]. Fungi within this genus are normally considered secondary pathogens that can be responsible for a variety of plant diseases, including cankers, dieback, leaf spots, needle blight, tip blight,

grey blight, severe chlorosis, fruit rots and various post-harvest diseases [4–11]. Species belonging to this genus are also commonly isolated as endophytes, and due to their ability to switch nutritional modes, many endophytic and plant pathogenic *Pestalotiopsis* species persist as saprobes [9,12].

Pestalotiopsis is distinguished from other pestalotioid genera in the family *Sporocadaceae* (*Heterotruncatella*, *Neopestalotiopsis*, *Pseudopestalotiopsis* and *Truncatella*) by the number of conidium cells and by the pigmentation of its median cells [9]. *Pestalotiopsis* can be easily identified based on its five-celled, fusoid conidia, with three brown concolourous median cells and hyaline end cells; *Neopestalotiopsis* can be distinguished from *Pestalotiopsis* by its five-celled, fusoid conidia, with versicolourous median cells; *Pseudopestalotiopsis* can be distinguished based on its five-celled, fusoid conidia, with three dark concolourous median cells; *Truncatella* and *Heterotruncatella* are easily identified based on their four-celled, fusoid conidia [3,9]. Nevertheless, identification to species level solely based on morphology is difficult, since the morphological characters used to differentiate species are limited, variable and may be influenced by different hosts and environments [10,13]. Combined phylogenetic analysis of the internal transcribed spacer of ribosomal DNA (ITS), partial β-tubulin (*TUB*) and partial translation elongation factor 1-alpha (*TEF*) DNA sequence data is often required for accurate species identification [3,7,9,10,12].

Few studies have been conducted regarding the pathogenicity of *Pestalotiopsis* species on pine tree species. Nonetheless, diverse studies obtained several *Pestalotiopsis* species as endophytes in *Pinus* and other conifers [9,14–18]. Hu et al. [16] reported the isolation of 19 different *Pestalotiopsis* species as endophytes from bark and needles of *Pinus armandii* Franch. in China. Botella and Diez [14] reported the isolation of a *Pestalotiopsis* sp. from *Pinus halepensis* Mill. in Spain, and Maharachchikumbura et al. [9] referred to a *Pestalotiopsis* sp. isolated from a *Pinus* sp. in China. *Pestalotiopsis* species have also been isolated as endophytes from pine seeds of *Pinus armandii* in China [17] and several other pine species across Europe and North America [15].

The objective of the present study was to identify the *Pestalotiopsis* species associated with stone pine diseases in pine orchards and urban areas across the mainland of Portugal, based on both morphological characters and multigene DNA phylogenetic inference.

2. Materials and Methods

2.1. Fungal Isolation

Isolates were obtained from samples of *Pinus pinea* showing shoot blight, trunk necrosis, needle blight and pine cone decay. A sample of *Pinus pinaster* Aiton with shoot blight was also analysed. After macro- and microscopic observation of the sampled material, small pieces from the leading edge of the lesions were surface sterilized for 1 min in 1% NaClO and plated onto potato dextrose agar (PDA) amended with 0.5 mg/mL of streptomycin sulphate in order to avoid bacterial growth. Materials were incubated for seven days with a 12 h light period at 23 ± 2 °C. The hyphal tips of fungi emerging from tissue pieces were transferred to PDA, and single-spore cultures were subsequently established. Fungal isolates were deposited in the culture collection of INIAV Institute (Micoteca da Estação Agronómica Nacional (MEAN)) (Table 1).

Table 1. Details of *Pestalotiopsis* isolates obtained in this study (bold) and of strains representing species of *Pestalotiopsis* and related genera retrieved from GenBank and used in phylogenetic analyses.

Species	Collection No. [1]	Host/Source	Country	Collection Year	GenBank Accession Number [2]		
					ITS	*TEF*	*TUB*
Neopestalotiopsis australis	CBS 114159	*Telopea* sp.	Australia	1999	KM199348	KM199537	KM199432
Neopestalotiopsis protearum	CBS 114178	*Leucospermum cuneiforme*	Zimbabwe	-	LT853103	KM199542	KM199463
Pestalotiopsis adusta	ICMP 6088	refrigerator door PVC gasket	Fiji	-	JX399006	JX399070	JX399037
Pestalotiopsis adusta	CBS 263.33	*Rhododendron ponticum*	Netherlands	1933	KM199316	KM199489	KM199414
Pestalotiopsis aggestorum	LC6301	*Camellia sinensis*	China	-	KX895015	KX895234	KX895348
Pestalotiopsis anacardiacearum	IFRDCC 2397	*Mangifera indica*	China	-	KC247154	KC247156	KC247155
Pestalotiopsis arceuthobii	CBS 433.65	*Arceuthobium campylopodum* f. *abietinum* shoot, on *Abies amabilis*	USA	-	MH554046	MH554481	MH554722
Pestalotiopsis arceuthobii	CBS 434.65	*Arceuthobium campylopodum* f. *tsugense* seed, on *Tsuga heterophylla*	USA	1965	KM199341	KM199516	KM199427
Pestalotiopsis arengae	CBS 331.92	*Arenga undulatifolia*	Singapore	1991	KM199340	KM199515	KM199426
Pestalotiopsis australasiae	CBS 114126	*Knightia* sp.	New Zealand	2002	KM199297	KM199499	KM199409
Pestalotiopsis australasiae	CBS 114141	*Protea* cv. 'Pink Ice'	Australia	1999	KM199298	KM199501	KM199410
Pestalotiopsis australis	CBS 114193	*Grevillea* sp.	Australia	1999	KM199332	KM199475	KM199383
Pestalotiopsis australis	CBS 119350	*Brabejum stellatifolium*	South Africa	2000	KM199333	KM199476	KM199384
Pestalotiopsis australis	**MEAN 1096 = CPC 36750 = CBS 146843**	***Pinus pinea*, blighted shoot**	**Portugal (Salvaterra de Magos)**	**2014**	**MT374679**	**MT374692**	**MT374704**
Pestalotiopsis australis	**MEAN 1109**	***Pinus pinea*, blighted shoot**	**Portugal (Tábua)**	**2017**	**MT374683**	**-**	**MT374708**
Pestalotiopsis australis	**MEAN 1110**	***Pinus pinea*, blighted shoot**	**Portugal (Salvaterra de Magos)**	**2017**	**MT374684**	**MT374696**	**MT374709**
Pestalotiopsis australis	**MEAN 1111**	***Pinus pinea*, blighted shoot**	**Portugal (Salvaterra de Magos**	**2017**	**MT374685**	**MT374697**	**MT374710**
Pestalotiopsis australis	**MEAN 1112**	***Pinus pinea*, blighted shoot**	**Portugal (Salvaterra de Magos)**	**2017**	**MT374686**	**MT374698**	**MT374711**
Pestalotiopsis biciliata	CBS 124463	*Platanus* × *hispanica*	Slovakia	-	KM199308	KM199505	KM199399
Pestalotiopsis biciliata	CBS 236.38	*Paeonia* sp.	Italy	1938	KM199309	KM199506	KM199401
Pestalotiopsis biciliata	**MEAN 1168**	***Pinus pinea*, dry 1st-year conelet**	**Portugal (Canha)**	**2019**	**MT374690**	**MT374702**	**MT374715**
Pestalotiopsis brachiata	LC2988	*Camellia* sp.	China	-	KX894933	KX895150	KX895265
Pestalotiopsis brassicae	CBS 170.26	*Brassica napus*	New Zealand	1926	KM199379	KM199558	-
Pestalotiopsis camelliae	CBS 443.62	*Camellia sinensis*	Turkey	-	KM199336	KM199512	KM199424
Pestalotiopsis camelliae	MFLUCC 12-0277	*Camellia japonica*	China	-	JX399010	JX399074	JX399041
Pestalotiopsis chamaeropis	CBS 113607	-	-	-	KM199325	KM199472	KM199390
Pestalotiopsis chamaeropis	CBS 186.71	*Chamaerops humilis*	Italy	1971	KM199326	KM199473	KM199391
Pestalotiopsis clavata	MFLUCC 12-0268	*Buxus* sp.	China	-	JX398990	JX399056	JX399025
Pestalotiopsis colombiensis	CBS 118553	*Eucalyptus eurograndis*	Colombia	2004	KM199307	KM199488	KM199421
Pestalotiopsis digitalis	ICMP 5434	*Digitalis purpurea*	New Zealand	1972	KP781879	-	KP781883
Pestalotiopsis dilucida	LC3232	*Camellia sinensis*	China	-	KX894961	KX895178	KX895293

Table 1. *Cont.*

Species	Collection No. [1]	Host/Source	Country	Collection Year	GenBank Accession Number [2]		
					ITS	*TEF*	*TUB*
Pestalotiopsis dilucida	LC8184	*Camellia sinensis*	China	-	KY464138	KY464148	KY464158
Pestalotiopsis diploclisiae	CBS 115587	*Diploclisia glaucescens*	Hong Kong	2001	KM199320	KM199486	KM199419
Pestalotiopsis disseminata	CBS 118552	*Eucalyptus botryoides*	New Zealand	-	MH553986	MH554410	MH554652
Pestalotiopsis disseminata	CBS 143904	*Persea americana*	New Zealand	-	MH554152	MH554587	MH554825
Pestalotiopsis disseminata	**MEAN 1165**	***Pinus pinea*, blighted shoot**	**Portugal (Cascais)**	**2018**	**MT374687**	**MT374699**	**MT374712**
Pestalotiopsis disseminata	**MEAN 1166**	***Pinus pinea*, blighted shoot**	**Portugal (Cascais)**	**2018**	**MT374688**	**MT374700**	**MT374713**
Pestalotiopsis diversiseta	MFLUCC 12-0287	*Rhododendron* sp.	China	-	JX399009	JX399073	JX399040
Pestalotiopsis dracontomelon	MFLUCC 10-0149	*Dracontomelon dao*	Thailand	2010	KP781877	KP781880	-
Pestalotiopsis ericacearum	IFRDCC 2439	*Rhododendron delavayi*	China	-	KC537807	KC537814	KC537821
Pestalotiopsis formosana	NTUCC 17-009	on dead grass	Taiwan	-	MH809381	MH809389	MH809385
Pestalotiopsis furcata	MFLUCC 12-0054	*Camellia sinensis*	Thailand	2010	JQ683724	JQ683740	JQ683708
Pestalotiopsis gaultheriae	IFRD 411-014	*Gaultheria forrestii*	China	-	KC537805	KC537812	KC537819
Pestalotiopsis gibbosa	NOF 3175	*Gaultheria shallon*	Canada	-	LC311589	LC311591	LC311590
Pestalotiopsis grevilleae	CBS 114127	*Grevillea* sp.	Australia	1999	KM199300	KM199504	KM199407
Pestalotiopsis hawaiiensis	CBS 114491	*Leucospermum* cv. 'Coral'	USA	1999	KM199339	KM199514	KM199428
Pestalotiopsis hispanica	CBS 115,391	*Protea* cv. 'Susara'	Spain	-	MH553981	MH554399	MH554640
Pestalotiopsis hollandica	CBS 265.33	*Sciadopitys verticillata*	Netherlands	1933	KM199328	KM199481	KM199388
Pestalotiopsis hollandica	**MEAN 1091 = CPC 36745 = CBS 146839**	***Pinus pinea*, blighted shoot**	**Portugal (Carregal do Sal)**	**2014**	**MT374678**	**MT374691**	**MT374703**
Pestalotiopsis humicola	CBS 115450	*Ilex cinerea*	Hong Kong	2002	KM199319	KM199487	KM199418
Pestalotiopsis humicola	CBS 336.97	soil in tropical forest	Papua New Guinea	1995	KM199317	KM199484	KM199420
Pestalotiopsis inflexa	MFLUCC 12-0270	unidentified tree	China	-	JX399008	JX399072	JX399039
Pestalotiopsis intermedia	MFLUCC 12-0259	unidentified tree	China	-	JX398993	JX399059	JX399028
Pestalotiopsis italiana	MFLUCC 12-0657	*Cupressus glabra*	Italy	2011	KP781878	KP781881	KP781882
Pestalotiopsis jesteri	CBS 109350	*Fragraea bodenii*	Papua New Guinea	-	KM199380	KM199554	KM199468
Pestalotiopsis jiangxiensis	LC4399	*Camellia* sp.	China	-	KX895009	KX895227	KX895341
Pestalotiopsis jinchanghensis	LC6636	*Camellia sinensis*	China	-	KX895028	KX895247	KX895361
Pestalotiopsis kenyana	CBS 442.67	*Coffea* sp.	Kenya	1967	KM199302	KM199502	KM199395
Pestalotiopsis knightiae	CBS 114138	*Knightia* sp.	New Zealand	-	KM199310	KM199497	KM199408
Pestalotiopsis leucadendri	CBS 121417	*Leucadendron* sp.	South Africa	-	MH553987	MH554412	MH554654
Pestalotiopsis licualicola	HGUP 4057	*Licuala grandis*	China	2012	KC492509	KC481684	KC481683
Pestalotiopsis linearis	MFLUCC 12-0271	*Trachelospermum* sp.	China	-	JX398992	JX399058	JX399027
Pestalotiopsis longiappendiculata	LC3013	*Camellia sinensis*	China	-	KX894939	KX895156	KX895271
Pestalotiopsis lushanensis	LC4344	*Camellia* sp.	China	-	KX895005	KX895223	KX895337
Pestalotiopsis macadamiae	BRIP 63738b	*Macadamia integrifolia*	Australia	-	KX186588	KX186621	KX186680
Pestalotiopsis malayana	CBS 102220	*Macaranga triloba*	Malaysia	1999	KM199306	KM199482	KM199411
Pestalotiopsis monochaeta	CBS 144.97	*Quercus robur*	Netherlands	1996	KM199327	KM199479	KM199386
Pestalotiopsis neolitseae	NTUCC 17-011	on leaf of *Neolitsea villosa*	Taiwan	-	MH809383	MH809391	MH809387

Table 1. *Cont.*

Species	Collection No. [1]	Host/Source	Country	Collection Year	GenBank Accession Number [2]		
					ITS	*TEF*	*TUB*
Pestalotiopsis novae-hollandiae	CBS 130973	*Banksia grandis*	Australia	2010	KM199337	KM199511	KM199425
Pestalotiopsis oryzae	CBS 353.69	*Oryza sativa*	Denmark	-	KM199299	KM199496	KM199398
Pestalotiopsis pallidotheae	MAFF 240993	*Pieris japonica*	Japan	-	NR111022	LC311585	LC311584
Pestalotiopsis papuana	CBS 331.96	soil along the coast	Papua New Guinea	1995	KM199321	KM199491	KM199413
Pestalotiopsis parva	CBS 114972	Leaf	Hong Kong	-	MH553980	MH554397	MH704625
Pestalotiopsis parva	CBS 278.35	*Leucothoe fontanesiana*	-	1935	KM199313	KM199509	KM199405
Pestalotiopsis photinicola	GZCC 16-0028*	*Photinia serrulata*	China	2015	KY092404	KY047662	KY047663
***Pestalotiopsis pini*sp. nov.**	**MEAN 1092 = CPC 36746 = CBS 146840**	***Pinus pinea*, blighted shoot**	**Portugal (Salvaterra de Magos)**	**2016**	**MT374680**	**MT374693**	**MT374705**
***Pestalotiopsis pini*sp. nov.**	**MEAN 1094 = CPC 36748 = CBS 146841**	***Pinus pinea*, trunk of declining tree (necrosis and salmon- pinkish discoloration of wood)**	**Portugal (Lisbon)**	**2017**	**MT374681**	**MT374694**	**MT374706**
***Pestalotiopsis pini*sp. nov.**	**MEAN 1095 = CPC 36749 = CBS 146842**	***Pinus pinea*, blighted shoot**	**Portugal (Salvaterra de Magos)**	**2017**	**MT374682**	**MT374695**	**MT374707**
***Pestalotiopsis pini*sp. nov.**	**MEAN 1167**	***Pinus pinaster*, blighted shoot**	**Portugal**	**2018**	**MT374689**	**MT374701**	**MT374714**
Pestalotiopsis portugallica	CBS 684.85	*Camellia japonica*	New Zealand	-	MH554065	MH554501	MH554741
Pestalotiopsis portugallica	CBS 393.48	-	Portugal	1948	KM199335	KM199510	KM199422
Pestalotiopsis rhizophorae	MFLUCC 17-0416	*Rhizophora apiculata*	Thailand	-	MK764283	MK764327	MK764349
Pestalotiopsis rhododendri	IFRDCC 2399	*Rhododendron sinogrande*	China	-	KC537804	KC537811	KC537818
Pestalotiopsis rhododendri	CBS 144024	*Pinus* sp.	Zimbabwe	-	MH554109	MH554543	MH554782
Pestalotiopsis rhodomyrtus	HGUP 4230	*Rhodomyrtus tomentosa*	China	2011	KF412648	KF412645	KF412642
Pestalotiopsis rhodomyrtus	LC3413	*Camellia sinensis*	China	-	KX894981	KX895198	KX895313
Pestalotiopsis rosea	MFLUCC 12-0258	*Pinus* sp.	China	-	JX399005	JX399069	JX399036
Pestalotiopsis scoparia	CBS 176.25	*Chamaecyparis* sp.	-	1925	KM199330	KM199478	KM199393
Pestalotiopsis sequoiae	MFLUCC 13-0399	*Sequoia sempervirens*	Italy	2011	KX572339	-	-
Pestalotiopsis sp. 7 FL_2019	CBS 110326	*Pinus* sp.	USA	-	MH553957	MH554375	MH554616
Pestalotiopsis sp. 7 FL_2019	CBS 127.80	*Pinus radiata*	Chile	-	MH553995	MH554422	MH554664
Pestalotiopsis spathulata	CBS 356.86	*Guevina avellana*	Chile	1961	KM199338	KM199513	KM199423
Pestalotiopsis spathuliappendiculata	CBS 144035	*Phoenix canariensis*	Australia	-	MH554172	MH554607	MH554845
Pestalotiopsis telopeae	CBS 114137	*Protea* cv. 'Pink Ice'	Australia	1999	KM199301	KM199559	KM199469
Pestalotiopsis telopeae	CBS 114161	*Telopea* sp.	Australia	1999	KM199296	KM199500	KM199403
Pestalotiopsis terricola	CBS 141.69	Soil	Pacific Islands	-	MH554004	MH554438	MH554680
Pestalotiopsis thailandica	MFLUCC 17-1616	*Rhizophora apiculata*	Thailand	2016	MK764285	MK764329	MK764351
Pestalotiopsis trachicarpicola	IFRDCC 2440	*Trachycarpus fortunei*	China	-	JQ845947	JQ845946	JQ845945
Pestalotiopsis unicolor	MFLUCC 12-0275	unidentified tree	China	-	JX398998	JX399063	JX399029
Pestalotiopsis unicolor	MFLUCC 12-0276	*Rhododendron* sp.	China	-	JX398999	-	JX399030
Pestalotiopsis verruculosa	MFLUCC 12-0274	*Rhododendron* sp.	China	-	JX398996	JX399061	-
Pestalotiopsis cf. *verruculosa*	CBS 365.54	*Chamaecyparis lawsoniana*	Netherlands	-	MH554037	MH554472	MH554713

Table 1. *Cont.*

Species	Collection No. [1]	Host/Source	Country	Collection Year	GenBank Accession Number [2]		
					ITS	*TEF*	*TUB*
Pestalotiopsis yanglingensis	LC3412	*Camellia sinensis*	China	-	KX894980	KX895197	KX895312
Pestalotiopsis yanglingensis	LC4553	*Camellia sinensis*	China	-	KX895012	KX895231	KX895345

[1] Culture collections—BRIP: Queensland Plant Pathology Herbarium, Australia; CBS: Culture collection of the Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands; CPC: Working collection of Pedro W. Crous, housed at the Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands; GZCC: Guizhou Academy of Agricultural Sciences Culture Collection, GuiZhou, China; HGUP: Plant Pathology Herbarium of Guizhou University, GuiZhou, China; ICMP: International Collection of Micro-organisms from Plants, Landcare Research, Auckland, New Zealand; IFRDCC: International Fungal Research and Development Culture Collection, Yunnan, China; LC: working collection of Lei Cai, housed at the Institute of Microbiology, Chinese Academy of Sciences, Beijing, China; MEAN: culture collection of INIAV Institute, Oeiras, Portugal; MFLUCC—Mae Fah Luang University Culture Collection, Chiang Rai, Thailand; NOF: The Fungus Culture Collection of the Northern Forestry Centre, Alberta, Canada; NTUCC: National Taiwan University Culture Collection, Taiwan; [2] ITS: internal transcribed spacer-rDNA; *TEF*: translation elongation factor 1-α; *TUB*: β-tubulin.

2.2. Morphology

Colony morphology was observed after 7 days of cultivation on PDA at 23 ± 2 °C at 12 h daylight. Conidiomatal development was observed on Synthetic Nutrient-poor Agar (SNA) by cultivating the isolates on autoclaved pine needles placed on the surface of SNA. Colony colour was determined on PDA using the colour charts of Rayner [19]. Conidia and conidiogenous cells were mounted in distilled water, and at least 30 measurements per structure were recorded at 400× magnification under a compound light microscope (Olympus BX51, Olympus Corporation, Tokyo, Japan) using the program Olympus DP-Soft, or under a Nikon Eclipse 80i compound microscope with differential interference contrast (DIC) illumination, equipped with a Nikon DS-Ri2 high definition colour digital camera.

2.3. DNA Extraction, PCR Amplification and Sequencing

Genomic DNA was extracted using the "DNA, RNA and Protein Purification—NucleoSpin Plant II" (Macherey-Nagel—MN) following the manufacturer's instructions. Fresh mycelium was disrupted by vortexing with approximately 200 µL glass beads (450–600 µm diameter) added to the extraction buffer [20].

Polymerase Chain Reactions (PCR) were performed to amplify three distinct DNA regions—the internal transcribed spacer of the ribosomal DNA (ITS), the partial translation elongation factor 1-alpha (*TEF*) and partial β-tubulin (*TUB*). The ITS, *TEF* and *TUB* genes were amplified using the primer pairs ITS5/ITS4 [21], EF1-728F/EF1-986R [22], and T1/Bt-2b [23,24].

All PCR reactions were performed in a 25 µL reaction containing DNA template (diluted 10×), 10× PCR reaction buffer, 3 mM $MgCL_2$, 0.5 mM of each deoxyribonucleotide triphosphate, 1 U of Taq DNA Polymerase, (BioTaqTM DNA Polymerase—Bioline, London, UK) and 2 µM of each primer, for ITS and TUB amplification, or 6 µM of each primer, for *TEF* amplification.

PCR reactions were performed in a Biometra TGradient thermo cycler (Biometra, Göttingen, Germany) with the following thermal cycling conditions, for ITS: initial denaturation at 94 °C for 3 min, followed by 30 cycles consisting of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 1 min, and a final extension at 72 °C for 10 min; for *TEF*: initial denaturation at 94 °C for 8 min, followed by 35 cycles consisting of denaturation at 94 °C for 15 s, annealing at 55 °C for 20 s and extension at 72 °C for 1 min, and a final extension at 72 °C for 5 min; and for *TUB*: initial denaturation at 94 °C for 1 min, followed by 30 cycles consisting of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min and extension at 72 °C for 1 min, and a final extension at 72 °C for 5 min.

PCR products were sequenced in both directions at STABVida Sequencing Laboratory (Caparica, Portugal) on an ABI PRISM 3730xl DNA analyser (Applied Bio systems) using the same primers as those used for the amplification reactions. The resulting nucleotide sequences were edited using the programs FinchTV version 1.4.0 (Geospisa Inc.) and BioEdit version 7.2.6 [25], and a consensus sequence was made from the forward and reverse sequences. Sequences obtained in this study were deposited in GenBank (see Table 1).

2.4. Phylogenetic Analyses

A BLAST engine search was used for sequence similarity searching on GenBank (NCBI—National Centre for Biotechnology Information). Based on blast search results and the literature, additional sequences were selected from GenBank and incorporated in the analyses (Table 1). Sequence alignments of the three individual loci (ITS, *TEF*, *TUB*) were made using MAFFT v. 7 (http://mafft.cbrc.jp/alignment/server/index.html), and were then manually edited using BioEdit version 7.2.6. Single gene datasets were combined using SequenceMatrix [26].

Phylogenetic analyses of the combined three-locus sequence dataset comprised Maximum Likelihood (ML), Maximum Parsimony (MP) and Bayesian Inference (BI).

ML were implemented on the CIPRES Science Gateway portal (https://www.phylo.org/) [27] using RAxML-HPC2 on XSEDE v. 8.2.12 [28]. For ML analyses, a GTR+CAT substitution model with 1000 bootstrap iterations was set.

MP analysis was performed using Phylogenetic Analysis Using Parsimony (PAUP) v. 4.0b10 [29]. Gaps were treated as missing data. Trees were inferred using heuristic search with random stepwise addition and tree-bisection reconnection (TBR). Maxtrees were set to 10,000 and branches of zero length were collapsed. Bootstrap support values with 1000 replications [30] were calculated for tree branches. Tree length (TL), consistency index (CI), retention index (RI), rescaled consistency index (RC) and homoplasy index (HI) were calculated for trees generated under different optimality criteria.

BI was performed by using the Markov Chain Monte Carlo method (MCMC) with MrBayes v. 3.2.6 [31]. JModelTest2 on XSEDE [32], implemented via the CIPRES portal, was used to determine the best-fit nucleotide substitution model for each partition using the Akaike Information Criterion (AIC) [33]. The GTR + I + G model was selected as the most suitable for ITS and *TUB* data partitions, and the GTR + G model was selected for *TEF* data partition. Four MCMC chains were run simultaneously, starting from random trees for 1,000,000 generations. Trees were sampled every 100 generations for a total of 10,000 trees. The burn-in fraction was set to 0.25, after which posterior probabilities were determined from a majority-rule consensus tree [34].

2.5. Pathogenicity Tests

Two isolates representing the most common *Pestalotiopsis* species isolated from stone pine trees with shoot blight disease in this study were selected to perform the pathogenicity tests: MEAN1095—*Pestalotiopsis pini* sp. nov. and MEAN1096—*Pestalotiopsis australis* Maharachch., K.D. Hyde & Crous.

To carry out the pathogenicity tests, 93 one-year-old stone pine seedlings were sourced from a nursery, where they were cultivated from seeds of a certified orchard. For each isolate and for the control treatment, 31 seedlings were randomly chosen and distributed along a plastic cell pack (6 × 11 plastic cells container). Each plastic cell pack with plants was randomly located in the greenhouse test area. The plants were then acclimatized during one month under greenhouse conditions, with temperatures varying from 18 to 28 °C, watered as needed (circa 2 L per plastic cell pack container, twice a week).

Spore suspensions of each isolate were prepared from cultures on PDA, grown at 25 ± 1 °C for 14 days (four plates/isolate). Sterile deionized water was added to the cultures and spores were dislodged by a sterile glass rod. The spore suspensions were resuspended in sterile deionized water and concentration adjusted to 1×10^5 conidia mL^{-1} with a haemocytometer.

The inoculations were performed by two combined methods. First, the stems were damaged by gently piercing them with a dissection needle that was previously dipped into the spore solution, while, in the control, the stems were pierced with a sterile needle. Five to six wounds were made per plant, approximately 3 cm apart from each other, in the upper third of the stem. Secondly, based on Talgø et al. [35], some needles were removed from plants, and the injured area subsequently brushed with the spore suspension. Sterile water was used in the control. Each container was covered with a plastic bag and maintained for one week to enhance fungal development.

The seedlings were kept in the greenhouse for four months (18 July to 17 November 2017).

At the end of the experiment, the number of affected plants was noted, and in order to attest Koch's postulates, re-isolations of fungi were carried out from the disease margins of three symptomatic seedlings, following the methodology described in Section 2.1.

3. Results

3.1. Fungal Isolation and Identification

Among other fungi, a total of 18 pestalotiopsis-like colonies were observed. After morphological observation and ITS sequence analyses, five isolates were identified as belonging to *Heterotruncatella*

and 13 to *Pestalotiopsis*. Further molecular studies were performed to identify the *Pestalotiopsis* species isolated.

3.2. Phylogenetic Analyses of Combined ITS, TEF and TUB Sequences

To determine the phylogenetic position of the *Pestalotiopsis* isolates, phylogenetic analyses were performed based on the combined ITS, *TEF*, and *TUB* sequence data. The combined alignment contained sequences from 104 strains (including two outgroups) with 1427 characters (including alignment gaps), divided in three partitions with 494 (ITS), 491 (*TEF*) and 442 (*TUB*) characters; 417 of these were parsimony-informative, 151 were variable and parsimony-uninformative, and 859 were constant. The combined *Pestalotiopsis* dataset was analysed using ML, MP and BI (Figure 1). The phylograms from the three analyses showed similar results in topology, and hence the best scoring tree resulting from ML analyses, with a final likelihood value of −10,646.254559, is shown in Figure 1. Maximum likelihood, MP bootstrap support values, and BI posterior probabilities (MLBS/MPBS/BIPP) are shown at common branches.

Isolates MEAN 1092, MEAN 1094, MEAN 1095 and MEAN 1167 were identical in our primary observations and formed a distinct clade, separate from previously described species within the genus. These isolates are well supported by all three phylogenetic analyses, and hence they are described as a new species of *Pestalotiopsis*.

Phylogenetic analyses allowed to identify the remaining isolates obtained in this study as belonging to four different species of *Pestalotiopsis*: *Pe. australis* (five isolates), *Pestalotiopsis disseminata* (Thüm.) Steyaert (two isolates), *Pestalotiopsis biciliata* Maharachch., K.D. Hyde & Crous (one isolate) and *Pestalotiopsis hollandica* Maharachch., K.D. Hyde & Crous (one isolate). Isolates MEAN 1109, MEAN 1110, MEAN 1096, MEAN 1111 and MEAN 1112 formed a clade along with reference strains of *Pe. australis*. MEAN 1165 and MEAN 1166 clustered with strains of *Pe. disseminata*. Isolate MEAN 1168 grouped with *Pe. biciliata*, while isolate MEAN 1091 was closely related to *Pe. hollandica*.

3.3. Morphology and Taxonomy

Pestalotiopsis pini A.C. Silva, E. Diogo & H. Bragança, sp. nov. (Figure 2)

MycoBank: MB 835952

Holotype: LISE 96316

Etymology: Named after the host genus from which it was isolated, *Pinus*.

Host/Distribution: On needles, shoots and trunks of *Pinus pinea* and on *Pinus pinaster* in Portugal (this study). Seen on *Pinus radiata* in Chile and on *Pinus* sp. in the USA also [3].

Description: Colonies on PDA attaining 82–85 mm diam after 7 d at 25 °C, with smooth edge, whitish to pale salmon coloured, with cottony aerial mycelium, forming abundant acervuli exuding black spore masses after two weeks. Reverse pale peach to salmon coloured. Conidiomata acervular on PDA, globose, aggregated or scattered, semi-immersed or partly erumpent, exuding black conidial masses. Conidiophores septate near base, simple or rarely branched at base, subcylindrical with a swollen base, hyaline, up to 28 µm long. Conidiogenous cells discrete, cylindrical, hyaline, smooth, 12–25 × 2–4 µm. Conidia fusoid to ellipsoid, straight to slightly curved, 4-septate, occasionally slightly constricted at septa (20.0–)23.3–24.6(–27.6) × (4.7–)7.4–7.8(–8.2) µm, av. ± S.D. = 24.0 ± 1.8 × 7.6 ± 0.6 µm; basal cell obconic, hyaline, smooth and thin-walled, 3.9–7.3 µm long; three median cells doliiform, (12.2–)14.8–15.6(–17.3) µm long, av. ± S.D. = 15.2 ± 1.3 µm, smooth and thin-walled, concolourous, but occasionally the two upper median cells are slightly darker than the lower median cell, olivaceous to brown, septa darker than the rest of the cell (second cell from the base 3.8–6.0 µm long; third cell 3.2–6.6 µm long; fourth cell 3.4–6.1 µm long); apical cell 2.4–4.8 µm long, hyaline, conical to subcylindrical, thin- and smooth-walled; with 3–4 tubular apical appendages (mostly 3), arising from the apical crest, unbranched, filiform, (9.7–)18.4–19.8(–27.8) µm long, av. ± S.D. = 19.1 ± 3.5 µm; basal appendage single, filiform, unbranched, centric, 1.4–7.6 µm long.

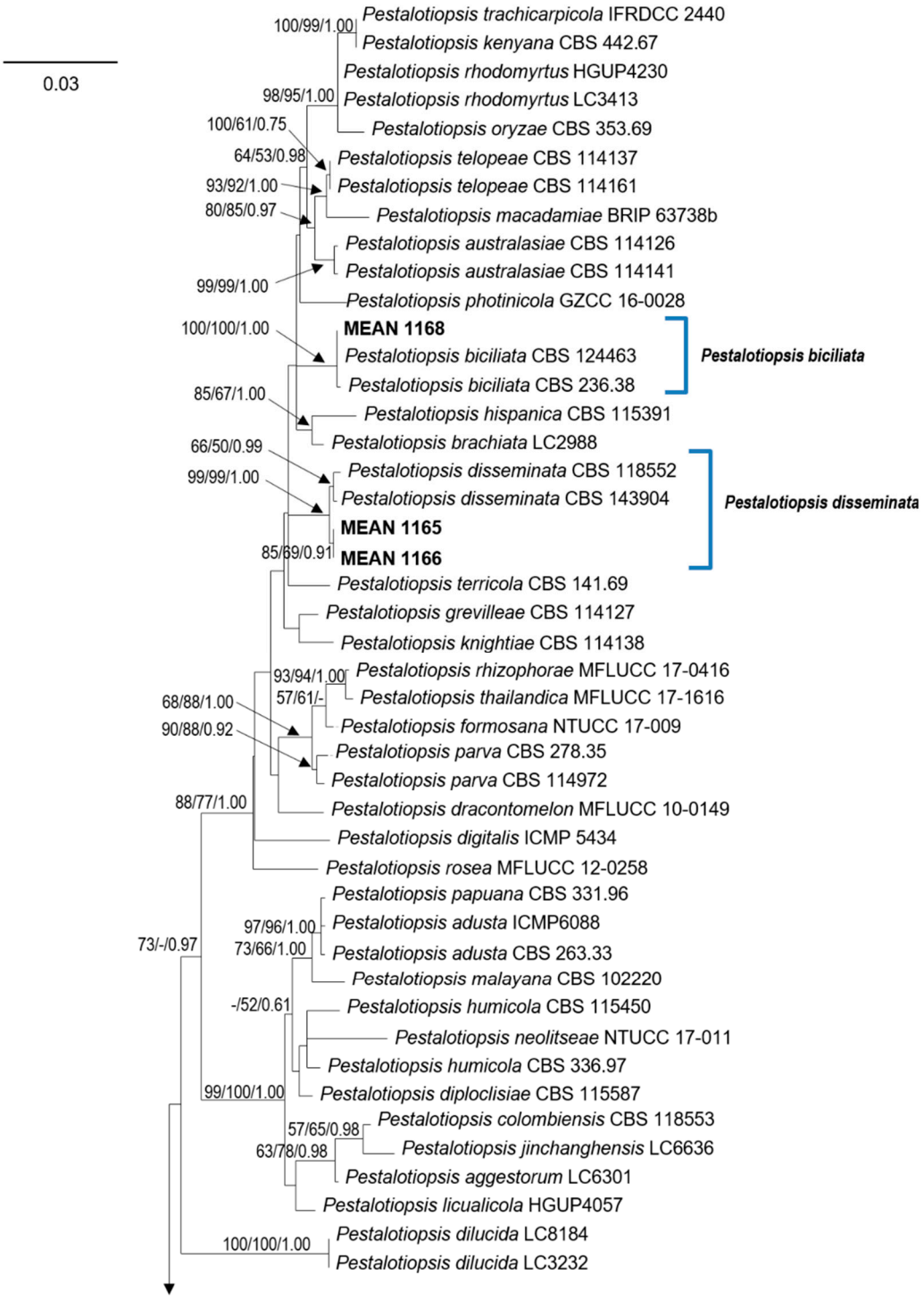

Figure 1. *Cont.*

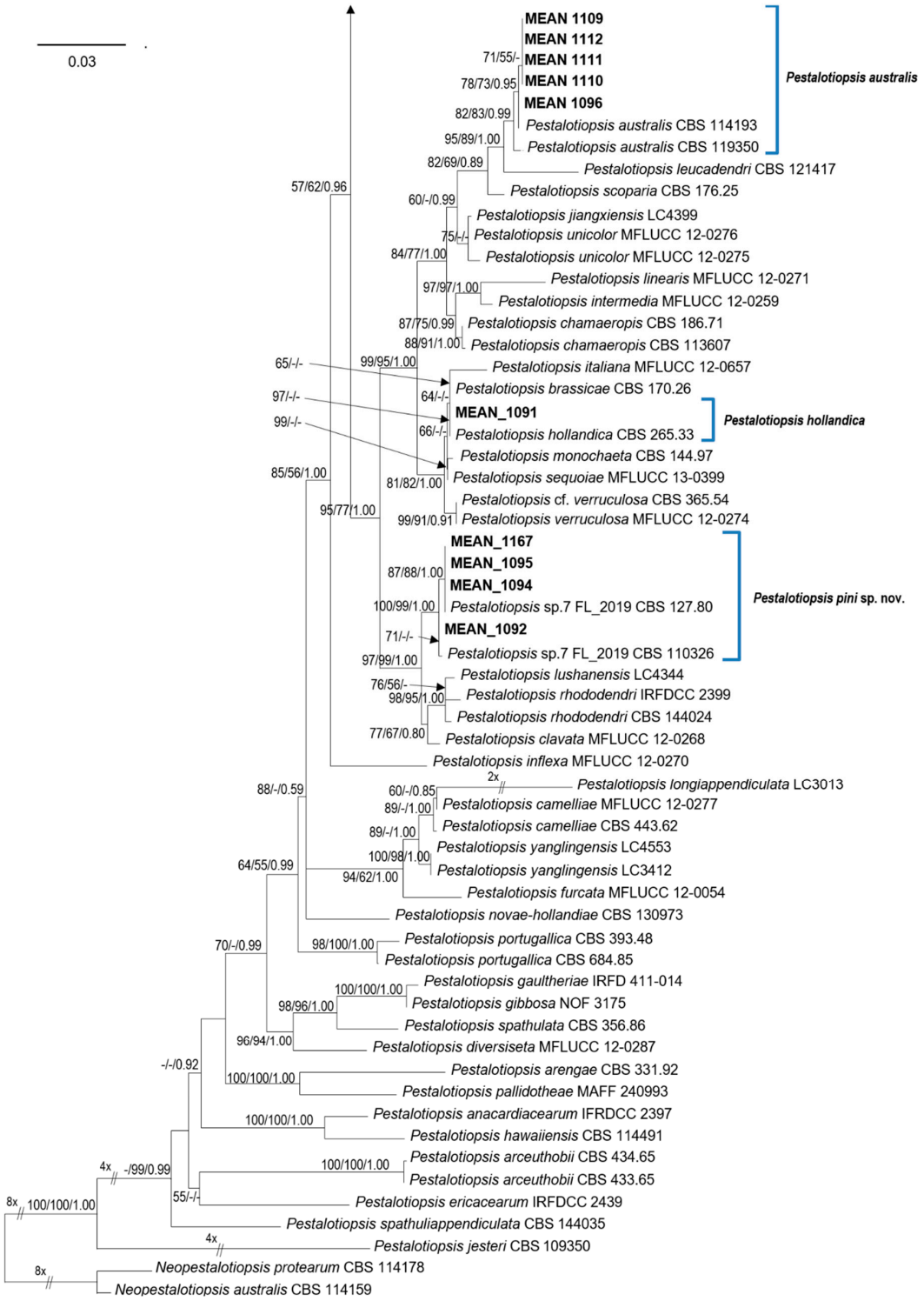

Figure 1. Phylogram generated from maximum likelihood (ML) analysis based on combined ITS, *TUB* and *TEF* sequence alignment for species of *Pestalotiopsis.* The best scoring ML tree with a final likelihood value of −10,646.254559 is presented. The tree was rooted to *Neopestalotiopsis australis* (CBS 114159) and *N. protearum* (CBS 114178). Maximum likelihood and maximum parsimony bootstrap support values ≥50% and Bayesian Inference posterior probabilities ≥0.90 (MLBS/MPBS/BIPP) are given at the nodes in common branches. The isolates obtained in this study are in bold. The scale bar represents the expected number of changes per site.

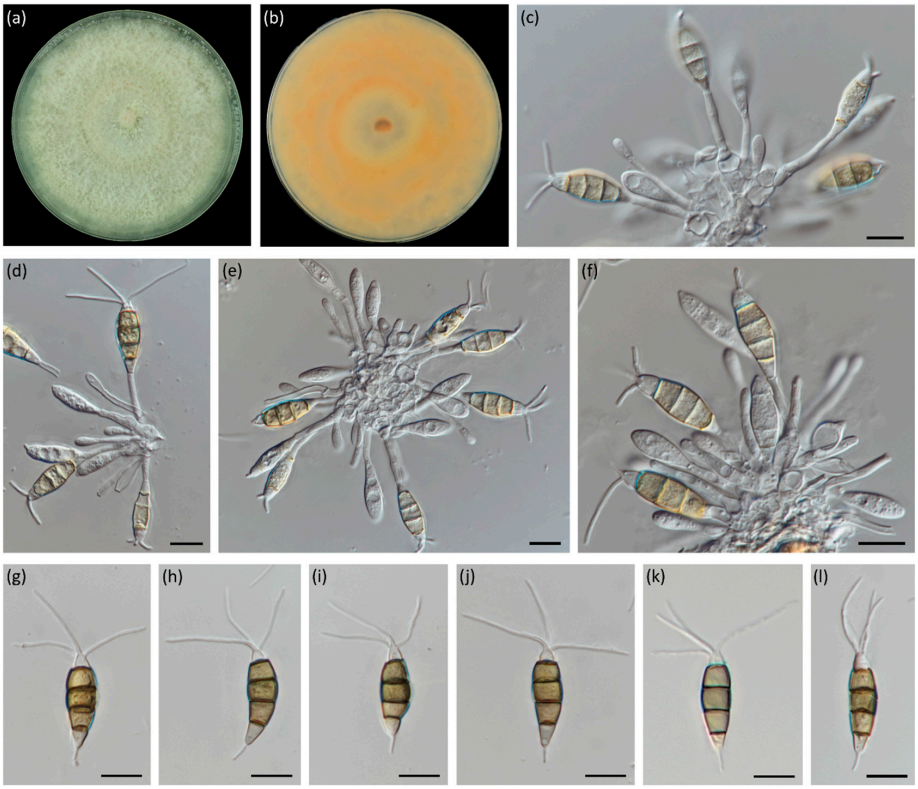

Figure 2. *Pestalotiopsis pini* (MEAN 1094). (**a**,**b**) Colony on PDA after 10 days at 23 ± 2 °C—surface view and reverse, respectively. (**c**–**f**) Conidiophores, conidiogenous cells and attached conidia. (**g**–**l**) Conidia. Scale bars: 10 µm.

Material examined: PORTUGAL, Lisbon, on rotten trunk of *Pinus pinea*, Ana C. Silva and Helena Bragança, March 2017 (LISE 96316 holotype; ex-type culture, MEAN 1094 = CPC 36748 = CBS 146841); PORTUGAL, Santarém, on blighted shoots of *Pinus pinea*, Ana C. Silva and Helena Bragança, March 2016 (living culture, MEAN 1092 = CPC 36746 = CBS 146840). PORTUGAL, Santarém, on blighted shoots of *Pinus pinea*, Ana C. Silva and Helena Bragança, March 2017 (living culture, MEAN 1095 = CPC 36749 = CBS 146842). PORTUGAL, unknown district, on blighted shoots of *Pinus pinaster*, Ana C. Silva, Eugénio Diogo and Helena Bragança, November 2018 (living culture, MEAN 1167).

Notes: *Pestalotiopsis pini* has similar-sized conidia to *Pestalotiopsis clavata* Maharachch., K.D. Hyde & Crous and *Pestalotiopsis lushanensis* F. Liu & L. Cai (20.0–27.6 × 4.7–8.2 µm in *Pe. pini* vs. 20–27 × 6.5–8 µm in *Pe. clavata* and 20–27 × 7.5–10 µm in *Pe. lushanensis*), but they are different in the number of appendages (*Pe. pini* has 3–4 appendages while *Pe. clavata* and *Pe. lushanensis* have 2–3 apical appendages) [12,36]. They are clearly separated in the phylogram based on combined ITS, *TEF*, and *TUB* sequence data, *Pe. pini* isolates formed a separate clade with strong support values on the three analyses performed (ML, MP and BI), (see Figure 1).

3.4. Pathogenicity

Two isolates, representing the most common *Pestalotiopsis* species isolated from pine trees with shoot blight disease in the present study, were submitted to pathogenicity tests by artificial inoculation on stone pine seedlings: MEAN1095—*Pestalotiopsis pini* sp. nov. and MEAN1096—*Pestalotiopsis australis*.

The development of disease symptoms was observed during a four-month period. Initial symptoms started after four weeks on seedlings inoculated with the *Pe. pini* isolate. Seedlings started to show yellowish and wilted needles in the apical third of the trunk. By the end of the experiment, symptomatic plants exhibited a dried apex in the inoculated branch/trunk (Figure 3). In total, 19.4% (6/31) of the plants inoculated with *Pe. pini* isolate MEAN 1095 were symptomatic. No symptoms were observed on the

control treatment, nor in plants inoculated with *Pe. australis* isolate MEAN 1096. *Pestalotiopsis pini* was successfully re-isolated from the three symptomatic plants sampled, thus fulfilling Koch's postulates and confirming its pathogenicity to stone pine.

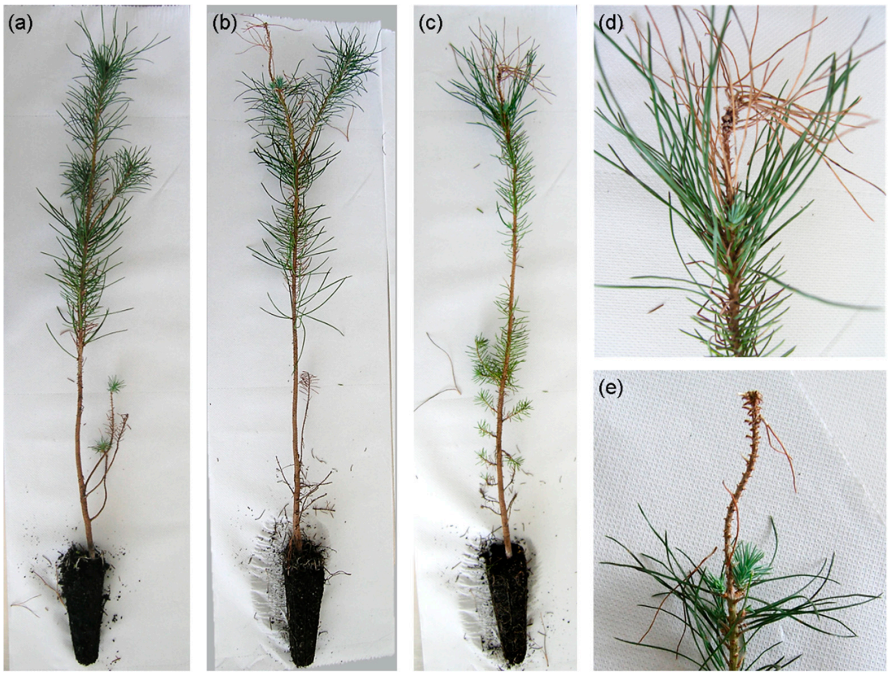

Figure 3. Aspect of inoculated seedlings four months after the inoculations. (**a**) Asymptomatic plant. (**b**,**c**) Symptomatic plants inoculated with *Pestalotiopsis pini* sp. nov. (**d**,**e**) Detail of dead apical shoots on symptomatic plants.

4. Discussion

In the present study *Pestalotiopsis pini* is described as a new species causing shoot blight and stem necrosis on *Pinus pinea*. Based on the morphology and molecular phylogenetic analyses of combined ITS, *TEF* and *TUB* sequence data, this taxon proved distinct from other species known from pine, or from DNA sequence data. Four other species of *Pestalotiopsis* were identified in association with symptomatic stone pines, namely, *Pe. australis*, *Pe. biciliata*, *Pe. disseminata* and *Pe. hollandica*.

Pestalotiopsis pini isolates obtained in this study (MEAN 1095, MEAN 1092, MEAN 1094, MEAN 1167) were grouped along with two unclassified *Pestalotiopsis* sp. strains included in the revision of Sporocadaceae, performed by Liu et al. [3], namely CBS 110326 and CBS 127.80. In the latter study, the authors retained these two isolates as an "informal species" "*Pestalotiopsis* sp.7 FL-2019", due to the lack of more isolates and limited phylogenetic support. In our phylogenetic analyses (Figure 1), these two strains were grouped with the four isolates obtained in this study, forming a separate clade with strong support values in all the phylogenetic analyses performed (MLBS = 100%, MPBS = 99%, BIPP = 1.00).

In the present study, *Pe. pini* was isolated from blighted shoots of *P. pinea* and *P. pinaster* trees in pine plantations, and from the necrotic wood of a decayed stone pine trunk located in Monsanto Forest Park in Lisbon. Pathogenicity tests performed confirmed that *Pe. pini* is pathogenic to stone pine. Furthermore, in the Monsanto Forest Park, various stone pine trees exhibited the same symptoms, and no other potential pathogens were isolated along with *Pe. pini*, suggesting that this could be a primary pathogen for this host. Interestingly, despite *Pestalotiopsis* species generally not being regarded as host-specific and normally being found on a wide range of plants and substrates [9], the two *Pe. pini* strains included in the study of Liu et al. [3] were also isolated in pines—*Pinus* sp. in the USA (CBS 110326) and *Pinus radiata* D. Don. in Chile (CBS 127.80)—although no information about the health of these pine trees is available.

In this study, *Pestalotiopsis australis* was isolated from blighted stone pine shoots in *P. pinea* orchards. This is the first report of *Pe. australis* isolated from conifers and in Europe. Under the conditions of the trials, no symptom development occurred in any of the inoculated seedlings, suggesting that *Pe. australis* may behave as an endophyte on stone pine. *Pestalotiopsis australis* has been reported from *Proteaceae* hosts, it was isolated from *Grevillea* sp. in Australia and South Africa, and from *Protea neriifolia* × *susannae* cv. 'Pink Ice' and dead leaves of *Brabejum stellatifolium* L. in South Africa [3,9].

Pestalotiopsis hollandica was isolated from the blighted shoots of stone pine trees in stone pine orchards. *Pestalotiopsis hollandica* was first described from Sciadopityaceae (*Sciadopitys verticillata* (Thunb.) Siebold & Zucc.) in the Netherlands [9] and it has already been isolated from conifers in Spain, namely from *Cupressus sempervirens* L. (Cupressaceae) [37]. Isolate MEAN 1091 was closely related to the reference strain of *Pe. hollandica*. However, *Pe. hollandica* was not well resolved from *Pestalotiopsis brassicae* Maharachch., K.D. Hyde & Crous, *Pestalotiopsis Italiana* Maharachch., Camporesi & K.D. Hyde, *Pestalotiopsis Monochaeta* Maharachch., K.D. Hyde & Crous, *Pestalotiopsis sequoiae* W.J. Li, Camporesi & K.D. Hyde and *Pestalotiopsis Verruculosa* Maharachch. & K.D. Hyde, suggesting that these isolates may represent a single species, as suggested by Liu et al. [3]. Some of those species' names have also been associated with conifers in the past [9,38].

Pestalotiopsis biciliata was isolated from a dry conelet (1st year) from a stone pine orchard. This species was first described by Maharachchikumbura et al. [9], isolated from dry needles of *Taxus baccata* L. in the Netherlands, from *Paeonia* sp. in Italy and from *Platanus* × *hispanica* in Slovakia. *Pe. biciliata* was also isolated from dry needles of *Taxus baccata* in the UK [3]. The fungus was referred to as the causal agent of fruit rot on withered grapes in Italy [8], and is associated with grapevine trunk diseases in France [10]. Recently *Pe. biciliata* was also reported as a foliar pathogen of *Eucalyptus* spp. [11].

Pestalotiopsis disseminata was isolated from blighted shoots of stone pine trees in a stone pine orchard. *Pe. disseminata* was first described from *Eucalyptus botryoides* Sm. in Portugal [39], and has already been isolated from a wide range of hosts and locations worldwide [3,15,18,40], including the genus *Pinus* [15,16,18]. It was isolated as an endophyte from *Pinus armandii* in China, along with 18 other pestalotioid species [16]; from *Pinus parviflora* Siebold & Zucc. var. *pentaphylla* (Mayr) in Japan [18] and from seeds of *P. pinea* in Turkey, *Pinus elliottii* Engel., *Pinus patula* Schltdl & Cham, *P. radiata*, *Pinus taeda* L. in the USA and *P. pinaster* in Portugal [15].

Isolates identified in this study were associated with symptomatic stone pine trees with shoot blight, trunk necrosis and pinecone decay in Portugal. At least one of the five identified species, *Pestalotiopsis pini* sp. nov., is pathogenic to stone pine. In recent years, various species of *Pestalotiopsis* have been described [3,4,7,9,10], with many being associated with plant diseases and shown to be pathogenic to their host under certain biotic and abiotic conditions [4,5,8,11,41,42].

The symptoms observed in stone pine orchards in Portugal, in particular shoot blight disease, might be of special concern to the forest industry, since dry shoots in the tree canopy could lead to a decrease in pinecone development and pine nut production, which is the most profitable resource of this industry [1,2].

Shoot blight disease on stone pine and other pine species is normally associated with *Diplodia sapinea* (Fr.) Fuckel [43,44], and has recently also been associated with *Sydowia polyspora* (Bref. & Tavel) E. Müller [45]. In the present study, various *Pestalotiopsis* species were isolated from stone pine samples with similar symptoms, moreover, *Pe. pini* proved to be pathogenic on stone pine, causing dry shoots on artificially inoculated seedlings, thus suggesting that *Pe. pini* should also have an active role in the expression of shoot blight disease on stone pine. The fact that in the pathogenicity tests, *Pe. pini* only caused disease symptoms in approximately 20% of the inoculated seedlings may indicate relative host resistance due to genetic differences among the seedlings. Alternatively, the development of shoot blight disease is due to more than one factor, biotic or abiotic. In fact, *D. sapinea*, *S. polyspora* and other fungi were also present in some of the sampled symptomatic material (data not shown). Diverse authors also report more than one species involved in dieback and blight diseases, including pestalotioid species and other fungi [8,45–47] and observed that some abiotic factors also have a major

role in disease development, namely water stress and air temperature [41,42,47]. In this case, a synergic effect among *Pe. pini* and other pathogenic or endophytic fungi found in stone pine shoots may also trigger the development of shoot blight disease symptoms. Future research should be performed to evaluate shoot blight disease prevalence on *P. pinea* orchards in Portugal and other Mediterranean areas and the diverse biotic and abiotic agents that can be involved in disease development.

The present study represents a preliminary contribution of the *Pestalotiopsis* species diversity associated with shoot blight disease of stone pine in Portugal. Knowledge of *Pestalotiopsis* species associated with shoot blight and other pine diseases will provide a basis to better understand disease development and help to develop management strategies against these pathogens.

5. Conclusions

A novel fungal species, *Pestalotiopsis pini* was described. This study proves that *Pe. pini* is an emerging pathogen causing shoot blight and trunk necrosis on *Pinus pinea* in the Mediterranean area.

To our knowledge, this is also the first report of *Pe. australis* on conifers and in Europe, and of *Pe. hollandica* and *Pe. biciliata* on *Pinus* spp. and in Portugal. Information about *Pestalotiopsis* species associated with shoot blight and other diseases on pine species will help to provide a basis for a better understanding of disease development, and the development of management strategies against these pathogens.

Author Contributions: A.C.S. and H.B. designed the experiments and wrote the first draft of the manuscript; A.C.S., E.D. and H.B. performed morphologic and molecular analyses; J.H. contributed for molecular biology procedures and review of the manuscript; M.S.-D. gave support in laboratory work and review of the manuscript; A.P.R. and P.W.C. gave support during the development of the study and discussion of the manuscript. H.B. supervised the study. All authors contributed to the final version of the manuscript. All authors have read and agreed to the published version of the manuscript.

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