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Cylindrocladiella peruviana and *Pleiocarpon algeriense* causing stem and crown rot on avocado (*Persea americana*)

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Abstract During the winter of 2018, 3-years-old trees of avocado (*Persea americana*) cv "Hass" from Trapani province (Sicily, Italy) showed symptoms of stem and crown rot. Two different fungi were consistently isolated from infected tissues. Morphological characterization and multi-locus phylogenies using five genomic loci (ITS, *tef1*, *tub2*, *his3*, and *rpb2*) identified these fungi as *Cylindrocladiella peruviana* and *Pleiocarpon algeriense*. Pathogenicity tests on healthy 5-months-old seedlings and 3-year-old trees of avocado reproduced similar symptoms as those observed in nature, and Koch's postulates were fulfilled for both pathogens. Moreover, the tested fungal isolates revealed a different pathogenic behaviour among two species. Two isolates of *Pleiocarpon algeriense* resulted more aggressive than *Cylindrocladiella peruviana* isolates causing major lesions on young seedlings. This study is the first to report of stem and crown rot on avocado caused by *Cylindrocladiella peruviana* and *Pleiocarpon algeriense*.

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Introduction

Avocado (*Persea americana* Mill.) is native to Mexico but is spread around the world in tropical and subtropical regions. In southern Italy (Sicily), several farms of different extension coexist, directing their production to local market as well as European market (Migliore et al. 2018). Although the agronomic studies on this crop in Italy started more than 20 years ago, studies on phytopathological aspects have been limited. Recently, a wide study on branch cankers and stem-end rot conducted in the main avocado growing area in eastern Sicily revealed the presence of different species such as *Neofusicoccum parvum* (the highest virulent), *Diaporthe foeniculina* (=*D. foeniculaceae*), *D. sterilis*, *Colletotrichum gloeosporioides*, *C. fructicola*, and a novel species *Neocosmospora perseae* (Guarnaccia et al. 2016, 2018). These reports show how woody cankers and stem-end rot could be considered the most important threats for avocado production in Italy so far.

Avocado symptoms at the rhizosphere such as rot, discolouration, sunken lesions, and subsequent aspecific symptoms of the canopy such as wilt, leaves chlorosis/browning, and decline, have been studied worldwide. These studies showed how several species within the Nectriaceae (Hypocreales, Ascomycetes) are involved in what is defined "Black root rot disease" (Dann et al. 2011, 2012; Parkinson et al. 2017a, b). A recent research

conducted in Australia demonstrated that several *Dactylonectria* spp. and *C. ilicicola* are pathogenic to avocado, compared to other isolates of *Ilyonectria* sp., *Cylindrocladiella pseudoinfestans* and *Gliocladiopsis peggii* that did not result pathogenic (Parkinson et al. 2017b). Several reports also include the species *Ilyonectria destructans* (often with previous binomial denomination) associated with black root rot of avocado (Besoain and Piontelli 1999; Zilberstein et al. 2007; Ramírez-Gil and Morales-Osorio 2013) and *Ilyonectria macrodidyma* (Vitale et al. 2012). However, due to the numerous taxonomic changes and limited pathogenicity tests, *Ilyonectria* spp. were considered not pathogenic to this crop (Parkinson et al. 2017b), although species of this genus are well known as pathogen to many other plants (Úrbez-Torres et al. 2012; Lombard et al. 2013; Aiello et al. 2014, 2015). Among *Cylindrocladiella* species, *C. parva* has been found on roots and cuttings of avocado in South Africa (Crous et al. 1991) and associated with the dead 3-year-old avocado plants in Australia (Dann et al. 2012) and *C. pseudoinfestans* was collected from symptomatic avocado trees cultivated in nursery although theirs pathogenicity was not demonstrated (Parkinson et al. 2017b). *C. peruviana*, has been previously reported associated with root decay and cutting rot symptoms, but no data are available about its pathogenicity (Crous 2002; Van Coller et al. 2005). Root rot caused by the oomycete *Phytophthora cinnamomi*, and collar rot caused by *P. citricola* have been considered for decades the most important root diseases in avocado orchards (Erwin and Ribeiro 1996).

Recently in Italy, young avocado trees showing symptoms of stem and crown rot were observed and brought to our laboratory for further investigations. The aim of the present study is to identify the fungal species associated with those symptoms and to evaluate their ability to induce symptoms on seedlings and young trees of avocado.

Materials and methods

Field sampling, isolations and morphological characterization

During the autumn of 2018, 20 young 3-year-old trees of avocado cv "Hass" from Campobello di Mazara, Trapani province (Sicily, Italy) were sampled, and analyses have been conducted in the laboratory. All the samples showed symptoms of stem and crown rot. Occasionally, as a consequence of severe infection, root rot was also observed. Small sections of basal stem and crown tissue were surface disinfected for 1 min in 1.5% sodium hypochlorite (NaOCl), rinsed in sterile distilled water, dried on sterile absorbent paper, and placed on potato dextrose agar (PDA, Oxoid, Basingstoke, UK) amended with 100 mg/l of streptomycin sulfate (Sigma-Aldrich, USA) to prevent bacterial growth, and then incubated at 25 ± 1 °C for 5–7 days. Single-conidial cultures were transferred on synthetic nutrient-poor agar (SNA; Nirenburg 1981) for morphological characterization. The morphological characteristics were determined with 30 measurements at × 1000 magnification of conidia mounted in lactic acid.

Molecular characterization and phylogenetic analysis

Genomic DNA was extracted from fourteen isolates (Table 1) using the Wizard Genomic DNA Purification Kit (Promega Corporation, WI, USA). Species identification was achieved through DNA amplification and sequencing of a combined data set of loci: the nuclear ribosomal internal transcribed spacer (ITS) region, partial regions of the β-tubulin (*tub2*), the translation elongation factor-1α (*tef1*), the histone H3 (*his3*) and the RNA polymerase II second largest subunit (*rpb2*) genes. The primers used were ITS5 and ITS4 (White et al. 1990), T1 and Bt-2b (O'Donnell and Cigelnik 1997), EF1-728F and EF1-986R (Carbone and Kohn 1999), CYLH3F and CYLH3R (Crous et al. 2004), RPB2-5F2 and RPB2-7cR (O'Donnell et al. 2007), respectively. The PCR amplification mixtures and cycling conditions were adopted for the two loci were followed as described by Guarnaccia and Crous (2018) and Aigoun-Mouhous et al. (2019). Both strands of the PCR products were sequenced by Eurofins Genomics Service (Ebersberg, Germany). The generated DNA sequences were analyzed and consensus sequences were computed using the Geneious v. 11.1.5 software (Auckland, New Zealand).

Novel sequences obtained in this study were blasted using the NCBIs GenBank nucleotide database, to identify the most similar relatives for a taxonomic framework of the studied isolates. Alignments of different gene regions, including sequences obtained from this study and those downloaded from GenBank, were initially performed by using the MAFFT v. 7 online server

(http://mafft.cbrc.jp/alignment/server/index.html) (Katoh and Standley 2013), and then manually adjusted in MEGA v. 7 (Kumar et al. 2016).

Phylogenetic analyses were conducted for establishing the identity of the isolates at species level, first individually for each locus (data not shown) and then as combined analyses of five loci. Additional reference sequences were selected based on recent literature of Nectriaceae species (Polizzi et al. 2012, Aiello et al. 2014, Lombard et al. 2015, Aigoun-Mouhous et al. 2019, Marin-Felix et al. 2019a, b). Phylogenetic analyses were based on Maximum Parsimony (MP) for the individual loci and on MP and Bayesian Inference (BI) for the multi-locus analyses. Related with BI, the best evolutionary model for each partition was determined using MrModeltest v. 2.3 (Nylander 2004) and incorporated into the analysis. MrBayes v. 3.2.5 (Ronquist et al. 2012) was used to generate phylogenetic trees under optimal criteria per partition. The Markov Chain Monte Carlo (MCMC) analysis used four chains and started from a random tree topology. The heating parameter was set to 0.2, and trees were sampled every 1000 generations. Analyses stopped at the moment which the average standard deviation of split frequencies was below 0.01. The MP analyses were conducted using PAUP (Phylogenetic Analysis Using Parsimony, v. 4.0b10; Swofford 2003). Phylogenetic relationships were estimated by heuristic searches with 100 random addition sequences. Tree bisection-reconnection was used, with the branch swapping option set on 'best trees' only with all characters weighted equally and alignment gaps treated as missing data. Tree length (TL), consistency index (CI), retention index (RI) and rescaled consistence index (RC) were calculated for parsimony and bootstrap analyses (Hillis and Bull 1993), which were based on 1000 replications. Sequences generated in this study are deposited in GenBank (Table 1).

Pathogenicity and virulence on seedlings of avocado

Pathogenicity assays with two fungal species (*Pleiocarpon algeriense* and *Cylindrocladiella peruviana*) isolated from the avocado samples were performed to fulfil Koch's postulates. Simultaneously, the decay amounts (symptom severities on basal stem and crown as measure of relative virulence) associated to each fungal isolate were evaluated and compared using young seedlings and avocado plants. Four type isolates were selected from the sample subset of isolates comprising two strains of *Pleiocarpon algeriense*, Di3A-AP26 and Di3A-AP50, and two isolates of *Cylindrocladiella peruviana,* Di3A-AP41 and Di3A-AP39. Pathogenicity of these isolates was assayed on healthy, 5-months-old seedlings of avocado. In detail, 12 inoculated plants for each fungal isolate were arranged in a randomized complete block design (RCBD) with three replicates, each consisting of four inoculated seedlings. To this aim, a piece of bark was removed at the base of the stem with a 6 mm diameter cork-borer and 6-mm-diameter mycelial plugs taken from a 12-day-old fungal colony were applied as previously reported (Aiello et al. 2014, 2015, 2017). The wounds were covered with Parafilm to prevent desiccation. Controls consisted of sterile PDA plugs applied similarly to healthy young 12 seedlings. All avocado plants were kept in a growth chamber, with a 12 h photoperiod at 25 °C ± 1 °C and regularly watered. Disease incidence (DI, percentage of symptomatic plants), symptom severity (as lesion length - SSL) of the resulting lesions was recorded 2 months after inoculation. Re-isolations were performed for seedlings to confirm their pathogenicity. The experiments were performed twice.

Pathogenicity and virulence on young trees of avocado

Two isolates (Di3A-AP50 and Di3A-AP39) were inoculated onto 18 healthy 3-years-old avocado trees according to a randomized complete block design with three replicates, each consisting of three young trees. In detail, a piece of bark was removed at the base of the stem using a 7.5 mm diameter cork-borer and a mycelial plug of equal size taken from a 12-day-old fungal colony was applied. Controls consisted of sterile PDA plugs applied to nine healthy young trees. All avocado plants were kept in a growth chamber, with a 12 h photoperiod at 25 °C ± 1 °C and regularly watered. Disease incidence (DI, percentage of symptomatic plants) and symptom severity (as lesion length - SSL, and lesion depth - SSD expressed in mm) of the resulting lesions were recorded four months after the inoculations. Re-isolations were performed from artificially infected avocado trees to confirm their pathogenicity. The experiments were performed twice.

Data analysis

Data concerning virulence of *Pleiocarpon algeriense* and *Cylindrocladiella peruviana* isolates on avocado

Table 1 Codes and GenBank accession numbers for isolates included in this study

Species	Culture no.[1]	GenBank no.[2]				
		ITS	*tef1*	*tub2*	*his*	*rpb2*
Calonectria ilicicola	CBS 190.50^T	GQ280605	AY725726	AY725631	AY725676	KM232307
Cylindrocladiella addiensis	CBS 143794^T	MH111383	MH111393	MH111388	–	–
Cy. australiensis	CBS 129567^T	JN100624	JN099060	JN098747	JN098932	–
Cy. brevistipitata	CBS 142786^T	–	MF444940	MF444926	–	–
Cy. camelliae	IMI 346845	AF220952	JN099087	AY793471	AY793509	KM232304
Cy. clavata	CBS 129564^T	JN099095	JN098974	JN098752	JN098858	–
Cy. cymbiformis	CBS 129553^T	JN099103	JN098988	JN098753	JN098866	–
Cy. elegans	CBS 338.92^T	AY793444	JN099039	AY793474	AY793512	–
Cy. ellipsoidea	CBS 129573^T	JN099094	JN098973	JN098757	JN098857	–
Cy. hawaiiensis	CBS 129569^T	JN100621	JN099057	JN098761	JN098929	–
Cy. horticola	CBS 142784^T	MF444911	MF444938	MF444924	–	–
Cy. humicola	CBS 142779^T	MF444906	MF444933	MF444919	–	–
Cy. infestans	CBS 111795^T	AF220955	JN099037	AF320190	AY793513	–
Cy. kurandica	CBS 129577^T	JN100646	JN099083	JN098765	JN098953	–
Cy. lageniformis	CBS 340.92^T	AF220959	JN099003	AY793481	AY793520	KM232303
Cy. lanceolata	CBS 129566^T	JN099099	JN098978	JN098789	JN098862	–
Cy. lateralis	CBS 142788^T	MF444914	MF444942	MF444928	–	–
Cy. longiphialidica	CBS 129557^T	JN100585	JN098966	JN098790	JN098851	–
Cy. longistipitata	CBS 116075^T	AF220958	JN098993	AY793506	AY793546	–
Cy. microcylindrica	CBS 111794^T	AY793452	JN099041	AY793483	AY793523	–
Cy. natalensis	CBS 114943^T	JN100588	JN099016	JN098794	JN098895	–
Cy. nauliensis	CBS 143792^T	MH111387	MH111397	MH111392	–	–
Cy. nederlandica	CBS 152.91^T	JN100603	JN099033	JN098800	JN098910	–
Cy. novazelandica	CBS 486.77^T	AF220963	JN099050	AY793485	AY793525	–
Cy. parva	CBS 114524	AF220964	JN099009	AY793486	AY793526	
Cy. peruviana	CBS 114953	JN099123	JN099006	JN098805	JN098885	–
	IMUR 1843^T	AF220966	JN098968	AY793500	AY793540	–
	Di3A-AP39	*MT613323*	*MT510671*	*MT510677*	*MT635005*	*MT634991*
	Di3A-AP41	*MT613324*	*MT510672*	*MT510678*	*MT635006*	*MT634992*
	Di3A-AP44	*MT613325*	*MT510673*	*MT510679*	*MT635007*	*MT634993*
	Di3A-AP45	*MT613326*	*MT510674*	*MT510680*	*MT635008*	*MT634994*
	Di3A-AP47	*MT613327*	*MT510675*	*MT510681*	*MT635009*	*MT634995*
	Di3A-AP48	*MT613328*	*MT510676*	*MT510682*	*MT635010*	*MT634996*
Cy. pseudocamelliae	CBS 129555^T	JN100577	JN098958	JN098814	JN098843	–
Cy. pseudohawaiiensis	CBS 210.94^T	JN099128	JN099012	JN098819	JN098890	–
Cy. pseudoinfestans	CBS 114531^T	AF220957	JN099004	AY793508	AY793548	–
Cy. pseudoparva	CBS129560^T	JN100620	JN099056	JN098824	JN098927	–
Cy. queenslandica	CBS 129574^T	JN099098	JN098977	JN098826	JN098861	–
Cy. reginae	CBS 142782^T	MF444909	MF444936	MF444922	–	–
Cy. stellenboschensis	CBS 110668^T	JN100615	JN099051	JN098829	JN098922	–
Cy. terrestris	CBS 142789^T	MF444915	MF444943	MF444929	–	–
Cy. thailandica	CBS 129571^T	JN100582	JN098963	JN098834	JN098848	–
Cy. variabilis	CBS 129561^T	JN100643	JN099080	JN098719	JN098950	–

Table 1 (continued)

Species	Culture no.[1]	GenBank no.[2]				
		ITS	*tef1*	*tub2*	*his*	*rpb2*
Cy. viticola	CBS 112897^T	AY793468	JN099064	AY793504	AY793544	–
Cy. vitis	CBS 142517^T	KY979751	KY979891	KY979918	–	–
Dactylonectria macrodidyma	CBS 112615^T	AY677290	JF268750	AY677233	JF735647	JF268710
D. torresensis	CBS 129086^T	JF735362	JF735870	JF735492	JF735681	KM232347
Ilyonectria capensis	CBS 132815	JX231151	JX231119	JX231103	JX231135	KM232336
I. palmarum	CBS 135753	HF937432	HF922615	HF922609	HF922621	–
Neonectria ditissima	CBS 100316	KM515890	KM515944	DQ789858	–	DQ789787
Pleiocarpon algeriense	CBS 144964^T	MH587320	MH587323	MH587324	MH587296	MH587322
	Di3A-AP26	*MT613330*	*MT597130*	*MT597138*	*MT635011*	*MT634997*
	Di3A-AP27	*MT613331*	*MT597131*	*MT597139*	*MT635012*	*MT634998*
	Di3A-AP28	*MT613332*	*MT597132*	*MT597140*	*MT635013*	*MT634999*
	Di3A-AP29	*MT613333*	*MT597133*	*MT597141*	*MT635014*	*MT635000*
	Di3A-AP31	*MT613334*	*MT597134*	*MT597142*	*MT635015*	*MT635001*
	Di3A-AP50	*MT613335*	*MT597135*	*MT597143*	*MT635016*	*MT635002*
	Di3A-AP51	*MT613336*	*MT597136*	*MT597144*	*MT635017*	*MT635003*
	Di3A-AP52	*MT613337*	*MT597137*	*MT597145*	*MT635018*	*MT635004*
P. livistonae	CBS 145030^T	MK539963	MK540165	MK540179	MK540234	MK540095
P. strelitziae	CBS 142251^T	KY304644	KY304722	KY304750	KY304616	KY304697
	CBS 142252	KY304663	*KY304741*	*KY304769*	KY304635	KY304713
	CPC 27629	KY304645	*KY304723*	*KY304751*	KY304617	KY304698
Thyronectria quercicola	CBS 128976^T	JF832624	JF832581	JF832880	KM231595	KM232411
Xenogliocladiopsis cypellocarpa	CBS 133814^T	KM231760	KM231885	KM232017	KM231479	KM232332

[1] CPC: Culture collection of P.W. Crous, housed at Westerdijk Fungal Biodiversity Institute; CBS: Westerdijk Fungal Biodiversity Institute, Utrecht, the Netherlands; Di3A: Dipartimento di Agricoltura, Alimentazione e Ambiente, Catania, Italy. IMI: International Mycological Institute, CABI–Bioscence, Egham, Bakeham Lane, U.K.; IMUR: Institute of Mycology, University of Recife, Recife, Brazil. Ex–type and ex–epitype cultures are indicated with ^{T}

[2] ITS: the nuclear ribosomal internal transcribed spacer region; *tef1*: partial translation elongation factor 1–α gene; *tub2*: partial beta–tubulin gene; *his*: the histone H3 gene; *rpb2*: the RNA polymerase II second largest subunit gene. Sequences generated in this study are indicated in italics

plants cv 'Hass' from the repeated experiment were analysed by using the Statistica package software (version 10; Statsoft Inc., Tulsa, OK, USA). The arithmetic means of DI and SS values [expressed both as lesion length (= SS_L) and as lesion depth (= SS_D)] were calculated averaging the values determined for each replicates of each treatment. Percentage DI data were transformed into the arcsine (sin^{-1} square root x) prior to analysis of variance (ANOVA), whereas both SS values were not transformed. Initial analyses of DI and SS data were performed by calculating F and *P* values associated to evaluate whether the effects of single factor (fungal isolate) and isolate × trial interactions are significant on single disease parameters. In the post-hoc analyses, the corresponding mean values of DI and SS were subsequently separated by the Fisher's least significance difference test ($\alpha = 0.05$). Untransformed arithmetic means of DI and SS are presented in the Tables 2 and 3.

Results

Field sampling, isolation and morphological characterization

A widespread wilting of avocado plants was observed in one orchard located in Campobello di Mazara (Trapani province) where approximately 2,200 plants were

Table 2 Pathogenicity and compared virulence among *Pleiocarpon algeriense* and *Cylindrocladiella peruviana* isolates on avocado seedlings

Isolate	DI (%)[x]	SS (lesion length - mm)[x]
Pleiocarpon algeriense Di3A-AP50	100	35.44 ± 1.66 a
Pleiocarpon algeriense Di3-AP26	100	18.33 ± 0.36 b
Cylindrocladiella peruviana Di3A-AP41	100	12.18 ± 0.69 c
Cylindrocladiella peruviana Di3A-AP39	100	13.3 ± 0.99 c
	–	F (isolate) = *224.945; P = 0.000*[z] F (isol. × trial) = *0.012; P* = 0.998[ns]

[x] Data derived from two repeated experiments. Means are from three replicates (each consisting of four plants). ± standard error of the mean (SEM); Values followed by the same letters within the column are not significantly different according to the Fisher's least significance differences test ($\alpha = 0.05$)

[z] F test and associated P value of fixed effects; ns = not significant

cultivated. Disease incidence was approximately 40%. Symptomatic plants showed symptoms of stem and crown rot and subsequent stunted growth, general wilting and leaf chlorosis. Moreover, basal stem and crown were dry, cracked and showed external decay and internal discolouration of tissues, which sometimes resulted in the detachment of the bark and/or of roots from the stem (Fig. 1). Occasionally, in an advanced stage of infection root rot was also observed. More of 800 plants were dead. Nectriaceae-like fungi colonies were consistently obtained from isolation of symptomatic tissues obtained from 20 plants and divided in two typologies depending on their appearance in culture. A total of 34 isolates were obtained from a single conidium or hyphal tip of pure cultures on PDA at 25 °C ± 1 °C.

Based on the microscopic observations, the isolates of *Cylindrocladiella peruviana* produced conidia cylindrical, rounded at both ends, straight, 1-septate, 8 to 13 × 2 to 3 μm in accordance with the description by Agustí-Brisach et al. (2012). *Pleiocarpon algeriense* produced microconidia and macroconidia straight to curved, 1–5-septate, predominantly 3, 4-septate. Macroconidia 3-septate were 41 to 48 × 5 to 7 μm while conidia 4-septate measured 52 to 64 × 7 to 8 μm in accordance with the description by Aigoun-Mouhous et al. (2019).

All the isolates were stored in the collection (label name Di3A-AP) of Dipartimento di Agricoltura, Alimentazione e Ambiente (Di3A), Catania, Italy. Moreover, two isolates of each typology were stored in the collection of Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands.

Molecular characterization and phylogenetic analysis

Six alignments were analysed representing single gene analyses of ITS, *tef1*, *tub2*, *his* and *rpb2*, and a combined alignment of the five genomic loci. The alignments provided topologically similar trees. The combined species phylogeny consisted of 65 sequences, including the sequences of *Xenogliocladiopsis cypellocarpa* (culture CBS 133814) as outgroup. A total of 3193 characters (ITS: 1–538, *tef1*: 545–1060, *tub2*: 1067–1782, *his*:

Table 3 Pathogenicity and compared virulence between *Pleiocarpon algeriense* and *Cylindrocladiella peruviana* isolates on avocado young trees

Isolate	DI (%)[x]	SS_L (lesion length, mm)[x]	SS_D (lesion depth, mm)[x]
Pleiocarpon algeriense Di3A-AP50	100	83.67 ± 1.6 a	1.92 ± 0.11 a
Cylindrocladiella peruviana Di3A-AP39	100	15.67 ± 0.09 b	1.0 ± 0.00 b
	–	*F (isolate) = 3435.4; P = 0.000*[z] F (isol×trial) = 2.05;*P* = 0.19[ns]	F (isolate) = *44.73; P = 0.000*[z] F (isol×trial) = 0.24*; P* = 0.64[ns]

[x] Data derived from two repeated experiments. Means are from three replicates (each consisting of three plants). ± standard error of the mean (SEM); Values followed by the same letters within the column are not significantly different according to the Fisher's least significance differences test ($\alpha = 0.05$)

[z] F test and associated *P* value of fixed effects; ns = not significant

Fig. 1 Symptoms caused by *Cylindrocladiella peruviana* and *Pleiocarpon algeriense* on avocado plants. **a** stunted growth, wilting and leaf chlorosis; **b**, **c**, **d** stem and crown rot; **e**, **f** symptoms on artificially inoculated young trees with *Cylindrocladiella peruviana* (**e**) and *Pleiocarpon algeriense* (**f**)

1789–2333, *rpb2*: 2340–3193) were included in the phylogenetic analysis; 1245 characters were parsimony-informative, 383 were variable and parsimony-uninformative, and 1541 were constant. A maximum of 1000 equally most parsimonious trees were saved (Tree length = 5390, CI = 0.559, RI = 0.836 and RC = 0.467). Bootstrap support values from the parsimony analysis are plotted on the Bayesian phylogenies in Fig. 2. For the Bayesian analyses, MrModeltest suggested that both the partitions should be analysed with dirichlet state frequency distributions. The following models were recommended by MrModeltest and used: GTR + I + G for ITS, *tef1* and *his*, HKY + I + G for *tub2*, SYM + G for *rpb2*. In the Bayesian analysis, the ITS *tef1*, *tub2*, *his* and *rpb2* partitions had 210, 377, 443, 279 and 350 unique site patterns, respectively. The analysis ran for 4,770,000 generations, resulting in 4771 trees of which 3579 were used to calculate the posterior probabilities.

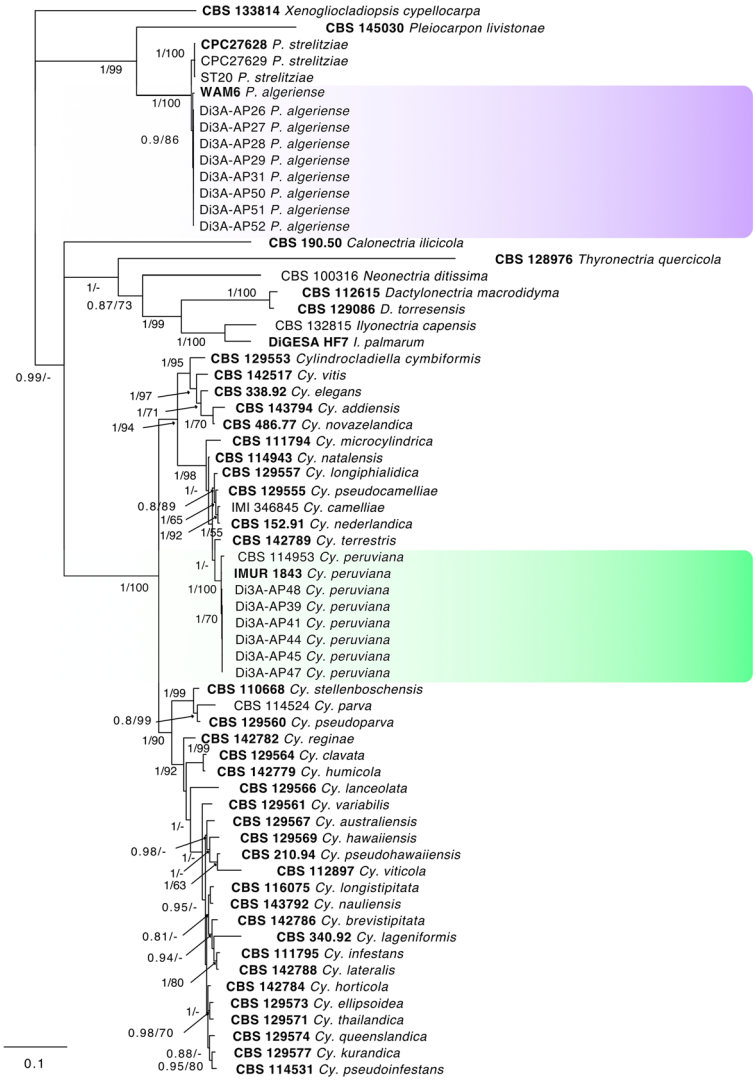

Fig. 2 Consensus phylogram of 4771 trees resulting from a Bayesian analysis of the combined ITS, *tef1*, *tub2*, *his* and *rpb2* sequences. Bayesian posterior probability and bootstrap support values are indicated at the nodes. Bold indicates ex–type strains. The tree was rooted to *Xenogliocladiopsis cypellocarpa* (CBS 133814)

In the combined analysis, eight representative isolates from avocado roots clustered with the ex-type of *P. algeriense*. A further six isolates identified as *C. peruviana*, formed with two reference strains, a highly supported subclade (1.00/100) in the broad group of *Cylindrocladiella* spp. The individual alignments and trees of the five single loci used in the analyses were compared with respect to their performance in species recognition.

Pathogenicity and virulence on avocado

In in vivo experiments (both on seedlings and trees of avocado) a significant effect of fungal isolate was always detected on SS parameters (P value<0.0001) except for DI values since they were always 100%. Since isolate × trial interactions were always not significant (P value >0.6) for all detected SS parameters (Tables 2 and 3) the two trials regarding both avocado seedlings and young trees were combined and statistically analysed.

Pathogenicity and virulence on seedlings of avocado 2 months after artificial inoculation of both fungi, symptoms produced in seedlings were similar to those present in the orchard. These consisted of stem and crown rot. In correspondence to the inoculated site, above the bark was visible necrotic lesion which also extended under the bark (Fig. 3). No symptoms were observed on control plants. The pathogens were re-isolated from symptomatic tissues, thus fulfilling Koch's postulates.

Fig. 3 Symptoms on artificially inoculated seedlings with *Pleiocarpon algeriense* and *Cylindrocladiella peruviana*. Internal necrotic lesions caused by *Pleiocarpon algeriense* (**a**, **b**) and internal necrotic lesions and stem and crown rot caused by *Cylindrocladiella peruviana* (**c**, **d**)

Regarding disease severities in the *post-hoc* analyses, the tested fungal isolates revealed a different pathogenic behaviour, resulting *P. algeriense* species more virulent than *C. peruviana* species. Moreover, *P. algeriense* Di3A-AP50 resulted the most aggressive isolate in inducing symptoms on avocado seedlings among all tested isolates (Table 2).

Four months after artificial inoculation of both fungi, symptoms produced in trees were similar to those present in the orchard. These consisted of necrotic lesions above the bark in correspondence to the inoculated site (Fig. 1). No symptoms were observed on control plants. The pathogens were re-isolated from symptomatic tissues, thus fulfilling Koch's postulates. As shown in Table 3, *P. algeriense* Di3A-AP50 caused always SS_L and SS_D values significantly higher than those recorded for *C. peruviana* Di3A-AP39 (Table 3).

Discussion

This study reveals for the first time the presence and pathogenicity of *C. peruviana* and *P. algeriense* on avocado. The polyphasic approach based on morphological and molecular analyses permitted to distinguish the collected strains at the species level within the broad genera of *Cylindocladiella* and *Pleiocarpon*.

Cylindrocladiella (Hypocreales, Nectriaceae) genus includes soil-borne fungi, often reported in several studies with the name of *Cylindrocladium,* lately separated in two distinct genera, having *Calonectria* and *Nectricladiella* teleomorphs respectively (Boesewinkel 1982; Crous and Wingfield 1993; Crous et al. 1994; Schoch et al. 2000; Crous 2002). *Cylindrocladiella* spp. are frequently associated with root rot diseases of many plant species, e.g. on woody plants like *Eucalyptus* spp. and *Pinus* sp. (Boesewinkel 1986; Mohanan and Sharma 1985; Lombard et al. 2012). In Sicily, several studies have been conducted on crown and root rot of various plant hosts, revealing how species of the close genus *Cylindrocladium* (*Calonectria*) are widely spread across different hosts (Polizzi et al. 2007, 2012; Vitale et al. 2009, 2013). Different species of *Cylindrocladiella* including *Cy. peruviana* have been found on roots and cuttings of avocado. However, theirs pathogenicity has not been demonstrated on this crop (Darvas 1978; Crous et al. 1991; Van Coller et al. 2005; Dann et al. 2012; Parkinson et al. 2017b). Agustí-Brisach et al. (2012) reported *Cy. peruviana* for the first time in Spain associated with black-foot disease of grapevine, referring symptoms of reduced vigor, necrotic root lesions, and occasionally mortality. Similarly, Álvarez et al. (2012) in Perù and Koike et al. (2016) in California reported *Cy. peruviana* and other Nectriaceae involved in black-foot disease of grapevine. These recent reports, including the present study, confirm that this fungus plays a role in causing diseases, although *Cylindrocladiella* spp. are not typically considered

primary pathogens (Lombard et al. 2012; Jayawardena et al. 2018).

Regarding the other nectriaceous species reported in this study, Aiello et al. (2017) described the new genus *Pleiocarpon* and *P. strelitziae* sp. nov. in Italy causing basal stem and root rot of the ornamental *Strelitzia reginae*. Pathogenicity tests demonstrated the aggressive pathogenic nature of *P. strelitziae*, resulting in mortality of all inoculated test plants within 2 months (Aiello et al. 2017). Recently, two new species of *Pleiocarpon* have been described; in particular *P. livistonae* from *Livistona rotundifolia* (Arecaceae) in Sri Lanka, causing root and corm rot (Marin-Felix et al. 2019a), and *P. algeriense* isolated in Algeria from grapevine cuttings with black-foot disease (Aigoun-Mouhous et al. 2019). Results of this studies demonstrated the pathogenicity of *C. peruviana* and *P. algeriense* on avocado. Moreover, the tested fungal isolates revealed a different pathogenic behaviour, resulting *P. algeriense* species more virulent than *C. peruviana* species. However, the symptoms observed in orchard could be the result of the interactions between different Nectriaceae species with different virulence levels as reported by some studies (Tewoldemedhin et al. 2011; Whitelaw-Weckert et al. 2013). Further evidences will be required to investigate the role of the single species involved in the disease and their interactions in pathogenesis. Prevention is the first strategy to manage these diseases. Affected plants produced in nursery could represent the primary way for nectriaceous spread through commercial orchards. Thus, the use of healthy plants and rapid fungi detection are crucial steps in prevention of stem and crown rot disease (Dann et al. 2012).

This study reports the presence and pathogenicity of two nectriaceous species on avocado in Italy for the first time, thereby the high risk of stem and crown rot in avocado commercial orchards. As described in this paper, *Cy. peruviana* and *P. algeriense* isolates recovered from infected stem and crown tissue, were able to cause different symptom severity levels among them. Further studies should be addressed to establish the most effective strategies to prevent their spread from the nursery to the open field.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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