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RESEARCH PAPERS

Isolation and pathogenicity of *Phytophthora* species and *Phytopythium vexans* recovered from avocado orchards in the Canary Islands, including *Phytophthora niederhauserii* as a new pathogen of avocado

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Summary. Root rot, caused by *Phytophthora cinnamomi*, is the most important disease of avocado, but few studies have determined whether other *Phytophthora* or oomycete species are involved in crop decline. Avocado orchards in the Canary Islands were surveyed for the presence of *Phytophthora* and *Phytophthora*-like oomycetes. Isolates obtained were identified morphologically and by sequence analysis of their internal transcribed spacer (ITS) regions, and their pathogenicity was tested by root and stem inoculation of avocado seedlings. *Phytophthora* species were isolated in 41 of 99 orchards sampled, and 10% of orchards were infected with more than one species. The species most frequently isolated was *P. cinnamomi*, which was detected in 26 orchards. In addition, *P. multivora* (ten orchards), *P. niederhauserii* (four orchards), *P. nicotianae* (four orchards), *P. palmivora* (one orchard) and *Phytopythium vexans* (20 orchards) were isolated. *Phytophthora nicotianae* and *P. palmivora* have been previously reported as pathogens of avocado, but *P. niederhauserii*, *P. multivora* and *Pp. vexans* are reported for the first time to be associated with this host. *Phytophthora niederhauserii* was the most virulent of these species. It was isolated from declining trees, and root rot severity was comparable to that caused by *P. cinnamomi* in two independent pathogenicity tests. In addition, *P. niederhauserii* caused cankers after stem inoculation. The pathogenicity results for *P. multivora* and *Pp. vexans* varied depending on isolates and pathogenicity tests. This study increases the knowledge of oomycetes associated with avocado, highlighting the potential threat posed by *P. niederhauserii* to this important fruit crop.

Key words: *Persea americana*, *Phytophthora cinnamomi*, *Phytophthora multivora*, *Phytopythium*, avocado root rot.

Introduction

Avocado (*Persea americana* Mill.) is the most important subtropical fruit crop in the Canary Islands (Spain), with annual production of approx. 10,000 t representing 13% of Spanish avocado production (Ministerio de Agricultura y Pesca, Alimentación y Medio Ambiente. Spanish Government. http://www.mapama.gob.es/es/estadistica/temas/estadisticas-agrarias/agricultura/superficies-producciones-anuales-cultivos/). Avocado production has increased in recent years in the Canary Islands, with the production area increasing by 32% between 2010 and 2015 (Instituto Canario de Estadística. Canary Islands Government. http://www.gobiernodecanarias.org/

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istac/temas_estadisticos/sectorprimario/agricultura/agricultura). Avocado fruit has been produced commercially in the Canary Islands since the 1950s; however, it was probably introduced as an ornamental plant during the Spanish colonization of the New World. Many of the rootstocks that are currently used in avocado orchards originate from seeds collected from old trees, which are adapted to the local climate and soil conditions, and mostly belong to the West Indian race. Avocados are mainly grown in the Canary Islands in small plots of approx. 1 ha or less at approx. 200 m above sea level. The climatic diversity (microclimates) of the Canary Islands permits avocado to be commercially produced throughout the year.

Avocado root rot, caused by *Phytophthora cinnamomi*, is the main disease that affects this crop in most avocado-producing areas of the world (Zentmyer, 1980; Erwin and Ribeiro, 1996). In the Canary Islands, *P. cinnamomi* was first isolated in 1975 (Gallo-Llobet *et al.*, 1978) and has been described as a limiting factor for many avocado orchards on the islands of Tenerife, La Palma and Gran Canaria. Several other species of *Phytophthora*, including *P. cactorum*, *P. citricola*, *P. citrophthora*, *P. heveae*, *P. nicotianae* and *P. palmivora*, have been described worldwide from *P. americana*, causing different symptoms (Erwin and Ribeiro, 1996). More recently, the avocado subgroup of *P. citricola,* recognized as the causal agent of avocado trunk canker, was renamed by Hong *et al.* in 2009 as *P. mengei*, based on morphological studies and sequence analyses of its ITS region. This *Phytophthora* species is considered the second most important in avocado groves throughout California (Hong *et al.*, 2009). Some of the *Phytophthora* species that have been previously described as pathogens of avocado have been detected in the Canary Islands on other crops. *Phytophthora cactorum* has been found on apple and strawberry, *P. citrophthora* on *Citrus* spp., *P. palmivora* on papaya, and *P. nicotianae* on tomato, pepper, and pineapple (Gallo *et al.*, 1988), but their presence in avocado crops in the Canary Islands is unknown.

The genus *Phytophthora* has received increased attention in the last decade in response to the involvement of several new invasive species that are causing forest decline worldwide (Hansen *et al.*, 2012; Jung *et al.*, 2013); however, less attention has been given to its impact on commercial crops. The introduction of molecular tools has provided a significant advance for studying *Phytophthora* taxonomy, which has traditionally been based on the concept of morphological groups (Waterhouse *et al.*, 1983), resulting in many described species (Brasier, 2009; Martin *et al.*, 2012). Therefore, identification of *Phytophthora* at the species level currently requires the use of reliable molecular tools, especially within species complexes that include new, recently described *Phytophthora* species without sufficiently discriminating morphological features. Although there is enough information on effects of *P. cinnamomi* on avocado, there are few studies of other possible *Phytophthora* species that affect this important fruit crop. Accordingly, the aims of this study were (i) to isolate *Phytophthora* or oomycete species that are associated with commercial avocado orchards in the Canary Islands, (ii) to identify them using morphological characteristics and sequence analysis of the internal transcribed spacer (ITS) region of their ribosomal DNA (rDNA), and (iii) to evaluate their pathogenicity to avocado plants.

Materials and methods

Sampling and *Phytophthora* isolation

Ninety-nine avocado orchards, located in the five islands of the Canary Islands where avocado is grown (Tenerife, La Palma, Gran Canaria, La Gomera and El Hierro: Figure 1), were investigated for the presence of *Phytophthora* over two years (2011–2012). Most orchards were selected based on tree health information provided by growers and technical advisors from agricultural cooperatives, who noticed early symptoms of tree decline (reduction in growth and vigour, foliar chlorosis) or other more obvious decline symptoms such as wilting, loss of foliage, and dieback of shoots and branches. Trunk cankers were not observed in any of these orchards. These selection criteria were applied in all islands except El Hierro, where no declining orchards were found, and the surveyed orchards on that island were randomly selected at different locations where avocado is grown. Soil samples, each of approx. 1 kg of soil, were taken from three points beneath individual trees in decline (or randomly selected when symptomatic trees could not be clearly noted after visual inspection) at a depth of 10–20 cm below the surface organic layer. Care was taken to collect lateral roots with the soil. The three subsamples from each tree were mixed into one sample, which was passed through a 5 mm mesh sieve. These processed samples were kept at room tempera-

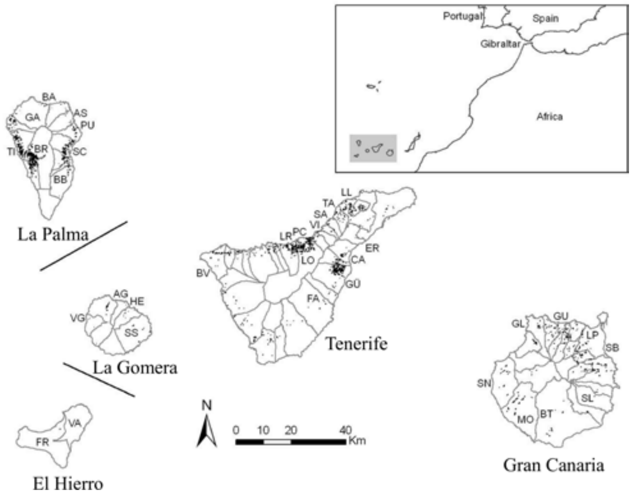

Figure 1. Map showing locations of the Canary Islands (upper square) and enlarged views of La Palma, Tenerife, Gran Canaria, La Gomera and El Hierro (from the shaded square). Avocado plantations in the Canary Islands are highlighted in black. The map indicates the townships and the abbreviated names of places where samples were collected (see Table 2 for abbreviations).

ture in plastic bags to minimize desiccation until they were further processed.

Soil samples (1–5 samples per orchard) were baited with avocado leaves based on standard methods previously described for *Phytophthora* isolation (Erwin and Ribeiro, 1996; Scott *et al.*, 2009). First, 250 g of each soil was pre-moistened for 24 h before being flooded with 500 mL of distilled water. The organic material that floated on the surface of the baiting water was removed with gauze and young leaves of *P. americana* cv. Topa Topa were then floated on the water as baits. Leaves in which brownish lesions appeared after 2–4 d were cut into 10–20 mm^2 sections and plated onto *Phytophthora*-selective P_{10}ARPH medium (Jeffers and Martin, 1986) and potato dextrose agar (PDA; Oxoid). In addition, roots in the soil samples were removed before baiting and used for direct isolation. Sections of roots were each cut with a sterilized scalpel into approx. 1–2 cm long segments, washed under running tap water, surface disinfected by immersion in 70% ethanol for 30 sec, rinsed in sterile distilled water three times, blotted dry on filter paper and then plated onto P_{10}ARPH medium and PDA. Isolation plates were incubated in the dark at 25°C and examined for growth over a 7 d period. Colonies that were suspected to be *Phytophthora* were transferred to PDA, corn meal agar (CMA; Oxoid) and clarified V8 juice agar (Edwin and Ribeiro, 1996) for identification.

For long-term storage, stock cultures were preserved in the culture collection maintained at the Instituto Canario de Investigaciones Agrarias (ICIA, Canary Islands, Spain). Approximately 30 to 40 agar disks (5 mm in diameter) from the margins of colonies on PDA were transferred to 10 mL capacity vials that contained 8 mL of sterile soil extract (10 g of soil were placed in 1 L of distilled water, mixed for 1 h on a magnetic stirrer, filtered through filter paper and then sterilized by autoclaving), and stored at 25°C in the dark. The isolates were also stored on PDA and CMA slants covered with paraffin oil at 25°C in the dark.

Morphological identification

Initial identification of isolates was based on colony and hyphal morphology, maximum growth temperature, and production and morphological features of sporangia, oogonia, antheridia, oospores and chlamydospores, according to Waterhouse *et al.* (1983), Erwin and Ribeiro (1996), Gallegly and Hong (2008), Martin *et al.* (2012) and Abad *et al.* (2014). The maximum temperature for growth was determined by transferring 5 mm diam. mycelial plugs onto PDA plates and checking them for growth after incubation at 25, 30 or 35°C for 5–7 d. To induce the formation of sporangia, 1 cm diam. disks were cut from the growing edges of 5-d-old cultures grown on V8 agar, and were placed in Petri dishes that had been previously flooded with 10 mL of sterile and non-sterile soil extract (Erwin and Ribeiro, 1996). Plates were placed at room temperature under natural light and examined for sporangial development over a 7 d period. Sporangial production on sterile and/or non-sterile soil extracts, and sporangial characteristics such as shape, presence or absence of papillae, persistence, proliferation, and branching of sporangiophores, were assessed by direct observation using a light microscope (Nikon Eclipse 80i). The formation of sexual structures (oogonia, antheridia, and oospores) was examined after 7–15 d of growth on V8 agar at 25°C in the dark. For heterothallic species, production of sexual structures was stimulated by pairing the isolates with tester *P. cinnamomi* strains (A1: BBA 69094; A2: BBA 62660) and *P. cryptogea* (A1: BBA 65909; A2: BBA 63651). Disks (5 mm diam.) of each isolate and tester strain were placed at a 3 cm distance from each other on V8 agar plates, which were then incubated at 25°C in the dark for 2–3 weeks and examined using light microscopy for oogonia, antheridia and oospores. Characteristics such as paragynous or amphigynous antheridial attachment and formation of plerotic or aplerotic oospores were recorded. *Phytophthora* isolates were identified at the species level based on their morphological features.

Molecular identification

The identification of *P. cinnamomi* isolates was confirmed using the *P. cinnamomi*-specific primers LPV2 and LPV3, as described by Kong *et al.* (2003). Identification of other species was based on the amplification of the ITS region using primers ITS-4 (5′-TCCTCCGCTTATTGATATGC-3′)(White *et al.*, 1990) and ITS-6 (5′-GAAGGTGAAGTCGTAACAAGG-3′) (Cooke *et al.*, 2000). In addition, the ITS regions of 13 representative isolates of *P. cinnamomi* that were obtained in this study were also sequenced. The components of PCR reaction mixtures and PCR conditions were as described previously (Pérez-Sierra *et al.*, 2010). PCR products were purified with the High Pure PCR Product Purification Kit (Roche Diagnostic) or the QIAquick PCR Purification Kit (Qiagen), and sequenced in both directions by Macrogen (Amsterdam, The Netherlands) or by the DNA Sequencing Service of the Universidad de La Laguna (La Laguna, Spain). MEGA6 software was used to assemble the forward and reverse sequences into consensus fragments. The chromatograms of the sequences were checked, and trimmed to provide consistency and quality of the sequences. The sequences were subjected to an NCBI BLAST search (http://www.ncbi.nlm.nih.gov/BLAST/) prior to phylogenetic analyses to identify the closest related sequences. Only published sequences were considered.

Phylogenetic analyses

Phylogenetic analyses were carried out separately for *Phytophthora* and *Phytopythium* species using the neighbour-joining (Saitou and Nei, 1987) and maximum-likelihood methods as implemented in MEGA6 (Tamura *et al.*, 2013). ITS sequences representing species from *Phytophthora* clades 1, 2, 4 and 7, and species from the genus *Phytopythium* were downloaded from GenBank and combined with sequences that were derived in this study. The closest published related sequences that were obtained in the initial BLAST search were also included in the phylogenetic analyses. For *Phytophthora*, the reference sequences were (i) the type isolates of *P. cinnamomi*, *P. citricola*, *P. melonis*, *P. mengei*, *P. multivora*, *P. niederhauserii* and *P. plurivora;* (ii) sequences published by Cooke *et al.* (2000) for *P. cactorum*, *P. cambivora*, *P. capsici*, *P. infestans*, *P. megakarya*, *P. multivesiculata*, *P. nicotianae* and *P. palmivora*; (iii) sequences published by Robideau *et al.* (2011) for *P. alni* and *P. tropicalis*; and (iv) the sequence published by Scanu *et al.* (2014) for *P. parvispora*. For *Phytopythium*, the sequences used as references were taken from Cooke *et al.* (2000), Lévesque and De Cock (2004), McLeod *et al.* (2009), Robideau *et al.* (2011) and Spies *et al.* (2011a). They comprise a set of sequences of the *Pp. vexans* complex and other *Phytopythium* species (*Pp. boreale*, *Pp. chamaehyphon*, *Pp. helicoides*, *Pp. litorale* and *Pp. ostracodes*). The ITS sequence of *Pp. sindhum*, the type species of *Phytopythium* (Bala *et al.*, 2010), was also included. Sequence data were initially aligned using the ClustalW program (Thompson *et al.*, 1994), and manual adjustments were made after visual examination by inserting gaps where necessary. In the neighbour-joining method, evolutionary distances were calculated using the Kimura 2-parameter method (Kimura, 1980). Maximum-likelihood analysis of *Phytophthora* sequences was based on the Hasegawa-Kishino-Yano model (Hasegawa *et al.*, 1985) with a discrete gamma distribution (HKY+G) which, among the available combinations, best described the observed substitution patterns. For *Phytopythium* sequences, the Tamura-Nei model (Tamura and Nei, 1993) with a discrete gamma distribution (TN93+G) was used for maximum-likelihood analysis, as it provided the best-fit model using MEGA6. Node support was evaluated through 1,000 bootstrap pseudoreplicates.

Pathogenicity tests

Avocado root and stem pathogenicity tests were performed to assess root rot and canker formation. Representative isolates of the different species that were obtained during this study were used, including four isolates of *P. multivora* (C002, C003, C005, C023), three of *P. niederhauserii* (C051, C052, C063), one of *P. nicotianae* (C013), one of *P. palmivora* (C011) and six belonging to the *Pp. vexans* complex (C001, C010, C015, C020, C022, C024). In addition, a *P. cinnamomi* isolate

(PcH15, ITS GenBank accession LM650983) that was obtained from an experimental avocado orchard that showed severe symptoms of root rot was included as a positive control. All pathogenicity tests were carried out using avocado seedlings (cv. Topa Topa). Koch's postulates were confirmed by re-isolation from necrotic tissues of the infected plants.

Root inoculation

Root pathogenicity was first tested by avocado root inoculation in hydroponic conditions and, in a second experiment, by inoculating potted plants as described below.

To inoculated roots in hydroponic conditions, 6-month-old avocado seedlings growth in 1.5 L capacity plastic pots were suspended by purpose-made brackets in containers (three plants per container) that contained 12 L of nutrient solution (Bingham and Zentmyer, 1954) covering the plant roots. Each container was then inoculated with one of the test isolates. Inoculum was prepared by growing each isolate on PDA at 25°C until the mycelium completely covered the surface of the plate (9 cm diam.). Mycelium and agar was then removed from the Petri dish (one Petri dish per container), cut into pieces of approx. 0.5 cm^2 and divided between two bags made with gauze sponges that were suspended with thread at opposite sides of the container, keeping them in the nutrient solution. Pieces of uninoculated culture medium were used for non-inoculated control plants. The solution was kept ventilated using air pumps that provided constant bubbling during the first month of the trial, and then ventilation was supplied 1 d per week to facilitate development of symptoms. The water that was lost by evaporation was periodically replaced to maintain the solution level in the containers. The assay was carried out in an inner courtyard from August to December in 2013. The monthly mean temperature ranged from 22.9°C in August (mean daily minimum 20.0°C, maximum 26.6°C) to 18.2°C in December (mean daily minimum 15.2°C, maximum 22.1°C). Plants were inspected weekly for symptoms. At the end of the trial, the height and collar diameter of the plants were measured, and root rot was severity assessed using a necrosis index from 0 to 3 (0 = no obvious symptoms; 1 = less than 30% of the root system necrotic, 2 = 30–70% of the root system necrotic, and 3 = more than 70% of the root system necrotic). In addition, and separately, the primary and lateral roots of each plant were weighed after they were dried at 65°C, and the weight percentage of lateral root with respect to the total root was calculated.

To inoculate the roots of potted plants, 2-year-old plants (three plants for each isolate) were transplanted into plastic pots (18 cm diam.) that contained potting mix consisting of soil, peat moss and lapilli (1:1:1 v/v/v). Inocula were prepared by growing each isolate on PDA as described above for root inoculation in hydroponic solution. The agar with mycelium was cut and buried in the pots around the roots (one Petri dish per pot). Uninoculated PDA plates were used for control plants. To provide a conducive environment for *Phytophthora* root rot, plants were flooded for 24 h every week (Simamora *et al*., 2017) by submerging each pot into a 10 L capacity plastic bucket that was filled with irrigation water to approx. 2 cm above the substrate surface. The plants were kept in a shade house from August 2015 to April 2016. The monthly mean temperature ranged from 23.6°C in August 2015 (mean daily minimum 20.7°C, maximum 27.7°C) to 16.7°C in January 2016 (mean daily minimum 13.7°C, maximum 20.3°C). At the end of the experiment, plants were carefully removed from the pots, and the roots were gently washed free of substrate with tap water. Root damage was assessed for each plant as described above for the hydroponic experiment, both for severity of necrosis (necrosis index) and by calculating the percentage (by weight) of lateral roots relative to the entire root system.

Stem inoculation

Stem pathogenicity tests were performed on 1-year-old seedlings (three plants for each isolate) that were inoculated with the tested isolates that had been grown on PDA. A lengthwise wound of 1 cm was made on the stem of each test plant, 5 cm from the collar region. A fragment of agar with mycelia (1 cm diam.) was inserted into the wound, and it was then sealed with Parafilm. Sterile PDA plugs without mycelium were used for control plants. The plants were kept in a controlled temperature greenhouse (25 ± 5°C) for 3 months, and were watered and fertilized regularly. The canker size on each plant was determined by tracing the outline of the canker edge with a permanent felt marker pen on a sheet of clear plastic that was put over the canker. The image obtained for each canker was scanned with a desktop scanner, and its perimeter was measured using a computer image analysis system (WinFOLIA, 2007b, Regent Instruments Inc.).

Statistical analyses

Analysis of variance (ANOVA) using Statistix 9 (Analytical Software) was applied to data that were obtained in the pathogenicity tests, and means were separated by Fisher's Least Significant Difference test (LSD) at $P<0.05$. In addition, relationships between the necrosis index and the percentage of lateral root weight were analysed using Pearson correlation coefficients.

Results

Isolate identification and phylogenetic analysis

During this study, a set of *Phytophthora* isolates was obtained from samples taken in 99 avocado orchards in the Canary Islands. Five *Phytophthora* species were identified, based on their phenotypic features. The species identified were *P. cinnamomi, P. citricola sensu lato*, *P. nicotianae, P. niederhauserii* and *P. palmivora*. In addition, young colonies of a group of isolates that were able to grow on P_{10}ARPH medium showed hyphal growth patterns that were similar to those of *Phytophthora* spp. Sporangia and oogonia of these isolates resembled those of *Phytophthora*, but zoospore discharge was *Pythium*-like, and the characteristics conformed to *Pythium* clade K (Lévesque and De Cock, 2004), whose members have been formally transferred to the new genus *Phytopythium* (Bala *et al.*, 2010; De Cock *et al.*, 2015).

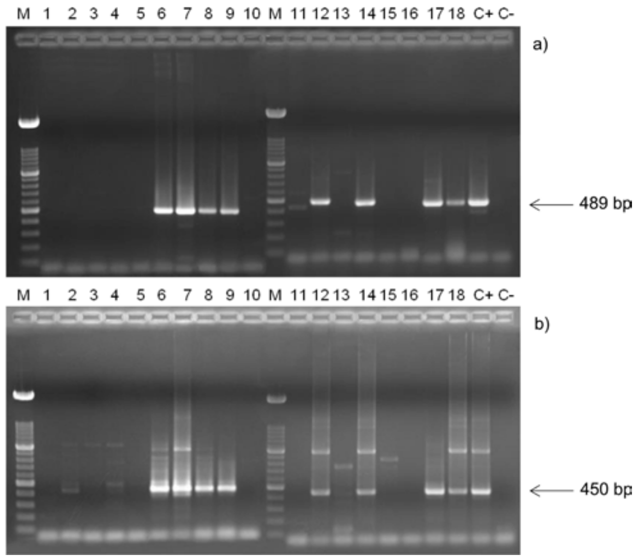

Figure 2. Agarose gel electrophoresis of PCR products that were amplified with *Phytophthora cinnamomi*-specific primer pairs LPV2 (a) and LPV3 (b), showing amplified fragments of sizes 489 and 450 bp, respectively. M, 100-bp size marker. C+, positive control (*P. cinnamomi* PcH15); C–, negative control. *P. cinnamomi*, lines 6-9, 12, 14, 17-18 (isolates C006, C007, C012, C014, C017, C018, C025, C045); *P. multivora*, lines 2-5 (isolates C002, C003, C005, C023); *P. palmivora,* line 11 (isolate C011); *P. nicotianae,* line 13 (isolate C013); *Phytopythium vexans*, lines 1, 10, 15-16 (isolates C001, C010, C015, C020).

The identity of *P. cinnamomi* isolates was confirmed using the species-specific primer pairs LPV2 and LPV3, which successfully discriminated the *P. cinnamomi* isolates from other species that were obtained in this study (Figure 2). The identification of the other species was first confirmed by their homology with sequences that have been deposited in GenBank (≥99% similarity) (Table 1), and secondly by alignment with sequences of reference isolates. The sequences that were derived in this study and used for phylogenetic analysis were deposited in GenBank with the accession numbers shown in Table 1. The ITS phylogeny placed the *P. cinnamomi* and *P. niederhauserii* isolates into *Phytophthora* clade 7 as defined by Cooke *et al.* (2000) (Figure 3). The ITS sequences of the *P. cinnamomi* isolates obtained formed a monophyletic clade (99% bootstrap support) which included the ex-type isolate of *P. cinnamomi* (HQ643189; CBS 144.22). The ITS sequences of *P. niederhauserii* grouped with high bootstrap values (97%) with the ex-type isolate of *P. niederhauserii* (AY550915; WPC P10616), other *P. niederhauserii* isolates from Italy (JX494411) and Spain (GQ385965), and the ex-type isolate of *P. melonis* (HQ643283; CBS 582.69), a representative species from subclade 7b. All the isolates of the *P. citricola* species-complex that were obtained in this study grouped with the ex-type isolate of *P. multivora* (FJ237521; CBS 124094) in a well-supported terminal clade (98% bootstrap support), that was clearly distinct from the ex-type strain of *P. citricola* (FJ237526; IMI021173) and other taxa from the *P. citricola* complex within ITS clade 2 (Cooke *et al*., 2000), and the ex-type strain of *P. mengei* (EU748545, ATCC MYA-4554) which is responsible of trunk canker in avocado in California (Hong *et al*., 2009) (Figure 3). The four isolates of *P. nicotianae* resided in ITS clade 1 and formed a monophyletic clade (100% bootstrap support) with the *P. nicotianae* isolate (AF266776) used in the study of Cooke *et al.* (2000). The only isolate of *P. palmivora* obtained from Canary Island avocado orchards was

Table 1. Identity of *Phytophthora* and *Phytopythium* isolates based on morphology and ITS sequence data (one representative isolate of each species isolated per orchard). GenBank accessions numbers of isolates that were used for phylogenetic analysis are provided.

Species	Isolate code	Location[a]	GenBank accession ITS
Phytophthora cinnamomi[b]	C006	Tenerife (CA)	LM650978
	C007	La Palma (GA)	LM650979
	C012	Tenerife GÜ)	LM650980
	C014	La Palma (SC)	LM650981
	C017	La Palma (BR)	LM650982
	C025	La Palma (SC)	LM650984
	C045	La Palma (BR)	LM650985
	C046	Tenerife (PC)	LM650986
	C053	Gran Canaria (SB)	LM650987
	C103	La Palma (AS)	LM650988
	C104	La Palma (BA)	LM650989
	C106	La Palma (PU)	LM650990
	C107	La Palma (PU)	LM650991
P. multivora	C002	Tenerife (ER)	LM650997
	C003	Tenerife (VI)	LM650998
	C005	Tenerife (LO)	LM650999
	C023	La Palma (BA)	LM651000
	C028	La Palma (SC)	LM651001
	C030	La Palma (BA)	LM651002
	C034	Tenerife (ER)	LM651003
	C057	Tenerife (LL)	LM651004
	C095	Tenerife (GÜ)	LM651005
	C105	La Palma (BA)	LM651006
P. niederhauserii	C051	Gran Canaria (MO)	LM651007
	C052	Gran Canaria (BT)	LM651008
	C063	La Gomera (SS)	LM651009
	C093	Tenerife (GÜ)	LM651010
P. nicotianae	C013	Tenerife (GÜ)	LM650993
	C041	Tenerife (LL)	LM650994
	C054	Tenerife (LL)	LM650995
	C064	La Gomera (HE)	LM650996
P. palmivora	C011	Tenerife (LL)	LM650992
Phytopythium vexans	C001	Tenerife (ER)	-
	C010	Tenerife (SA)	LM651011
	C015	La Palma (BR)	LM651012
	C020	Tenerife (CA)	LM651013
	C022	La Palma (SC)	LM651014
	C024	La Palma (SC)	LM651015
	C029	La Palma (SC)	LM651016
	C031	La Palma (BA)	-
	C032	La Palma (BA)	-
	C033	La Palma (PU)	-
	C040	Tenerife (LO)	LM651017
	C055	Tenerife (LL)	LM651018
	C056	Gran Canaria (LP)	-
	C058	La Gomera (SS)	-
	C059	La Gomera (VG)	-
	C060	Tenerife (LR)	LM651019
	C061	La Gomera (SS)	LM651020
	C062	La Gomera (HE)	LM651021
	C094	Tenerife (GÜ)	LM651022
	C096	Tenerife (GÜ)	LM651023

[a] See Table 2 for abbreviations.
[b] A total of 26 *P. cinnamomi* isolates were identified morphologically and by PCR using the *P. cinnamomi*-specific primer pairs LPV2 and LPV3; a subset of 13 of these isolates had their ITS regions subsequently sequenced.

placed in ITS clade 4, grouping with high bootstrap support (100%) with AF266780, the *P. palmivora* isolate used by Cooke *et al.* (2000).

ITS sequences of the isolates identified as *Phytopythium* sp. showed high homology with the published ITS sequences of the *Pp. vexans* complex

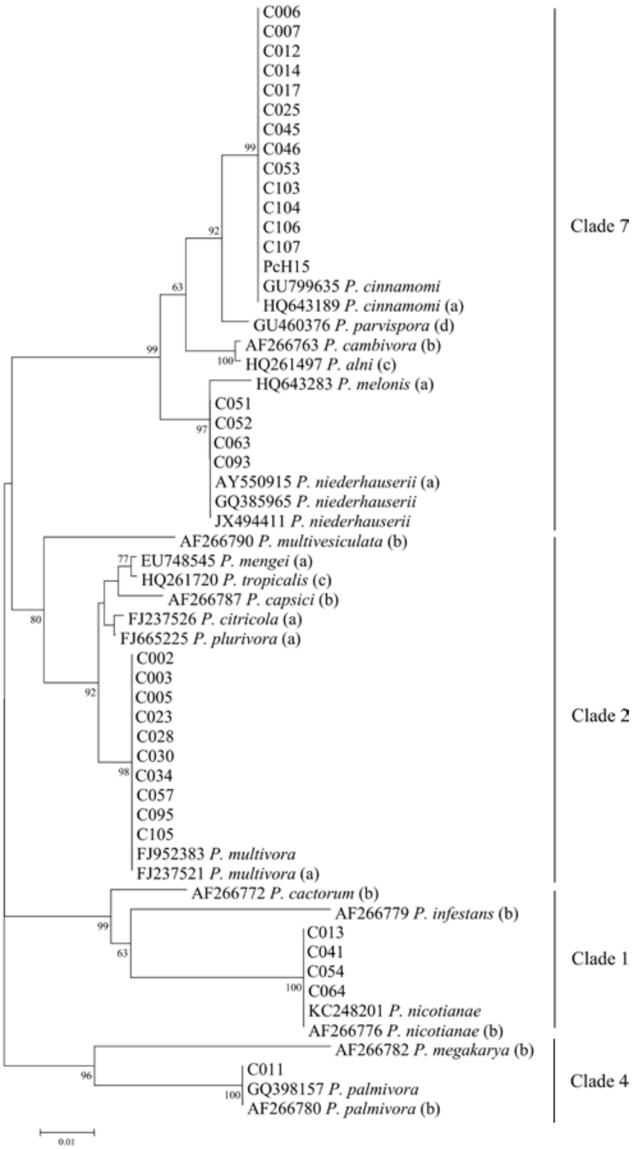

Figure 3. Phylogenetic tree constructed using the neighbour-joining method based on rDNA-ITS sequences that show the positions of *Phytophthora* isolates that were obtained in this study and were found to belong to clades 1, 2, 4 and 7, as defined by Cooke *et al.* (2000). Type isolate of the species (a); sequences used by Cooke *et al.* (2000) (b), Robideau *et al.* (2011) (c), and Scanu *et al.* (2014) (d). Numbers at the nodes of clusters represent bootstrap values that were generated from 1,000 pseudoreplicates (only values greater than 60% are shown).

Figure 4. Phylogenetic tree of *Phytopythium* spp. that was constructed by the neighbour-joining method based on their rDNA-ITS sequences, showing the position of *Phytopythium* isolates that were obtained in this study within the *Pp. vexans* complex. Sequences from the studies by Robideau *et al.* (2011) (a); Lévesque and De Cock (2004) (b); Spies *et al.* (2011a) (c); McLeod *et al.* (2009) (d); Cooke *et al.* (2000) (e); and Bala *et al.* (2010) (f). Numbers at the nodes of clusters represent bootstrap values that were generated from 1,000 pseudoreplicates (only values greater than 60% are shown).

(Table 1). Phylogenetic analysis with a subset of 13 out 20 of these isolates grouped them into a single highly supported clade (100% bootstrap support) that contained sequences of the *Pp. vexans* complex used in the studies of Cooke *et al.* (2000), Lévesque and De Cock (2004), McLeod *et al.* (2009), Robideau *et al.* (2011) and Spies *et al.* (2011a). These sequences were clearly distinct from those of other *Phytopythium* species (Figure 4). The major *Pp. vexans* clade can be subdivided into two subclades; one which shows a high bootstrap value (99%), includes four Canary Island isolates (C010, C015, C020, C096), the *Pp. vexans* (previously *Pythium vexans*) reference strain (AY598713; CBS 119.80) used by Lévesque and De Cock (2004), and two *Pp. vexans* strains isolated from *Persea americana*, one in South

Table 2. Occurrence of *Phytophthora* spp. and *Phytopythium vexans* in avocado orchards of the Canary Islands.

Island	Location	No. orchards[a]	Positive isolations[b]						Observations[c]
			P.c	P.m	P.nd	P.nt	P.p	Pp.v	
Tenerife	Buenavista (BV)	1	-	-	-	-	-	-	
	Candelaria (CA)	2	1	-	-	-	-	1	
	El Rosario (ER)	2	-	2	-	-	-	1	†
	El Sauzal (SA)	1	-	-	-	-	-	1	
	Fasnia (FA)	1	-	-	-	-	-	-	
	Güímar (GÜ)	4	2	1	1	1	-	2	†
	La Laguna (LL)	6	-	1		2	1	1	†
	La Orotava (LO)	8	1	1	-	-	-	1	
	La Victoria de Acentejo (VI)	1	-	1	-	-	-	-	
	Los Realejos (LR)	2	-	-	-	-	-	1	
	Puerto de la Cruz (PC)	2	1	-	-	-	-	-	
	Tacoronte (TA)	2	-	-	-	-	-	-	
	Total	32	5	6	1	3	1	8	
La Palma	Barlovento (BA)	9	5	3	-	-	-	2	†
	Breña Alta (BR)	5	3	-	-	-	-	1	†
	Breña Baja (BB)	1	-	-	-	-	-	-	
	Garafía (GA)	1	1	-	-	-	-	-	
	Puntallana (PU)	5	4	-	-	-	-	1	
	San Andrés y Sauces (AS)	4	1	-	-	-	-	-	
	S/C de La Palma (SC)	5	4	1	-	-	-	3	†
	Tijarafe (TI)	6	2	-	-	-	-	-	
	Total	36	20	4	0	0	0	7	
Gran Canaria	Gáldar (GL)	1	-	-	-	-	-	-	
	Guía (GU)	1	-	-	-	-	-	-	
	Las Palmas de Gran Canaria (LP)	1	-	-	-	-	-	1	
	Mogán (MO)	3	-	-	1	-	-	-	
	San Bartolomé de Tirajana (BT)	2	-	-	1	-	-	-	
	San Nicolás de Tolentino (SN)	2	-	-	-	-	-	-	
	Santa Brígida (SB)	1	1	-	-	-	-	-	
	Santa Lucía (SL)	1	-	-	-	-	-	-	
	Total	12	1	0	2	0	0	1	
La Gomera	Agulo (AG)	1	-	-	-	-	-	-	
	Hermigua (HE)	3	-	-	-	1	-	1	

(Continued)

Table 2. (Continued).

Island	Location	No. orchards[a]	Positive isolations[b]						Observations[c]
			P.c	P.m	P.nd	P.nt	P.p	Pp.v	
	San Sebastián de La Gomera (SS)	5	-	-	1	-	-	2	
	Valle Gran Rey (VG)	2	-	-	-	-	-	1	
	Total	11	0	0	1	1	0	4	
El Hierro	Frontera (FR)	2	-	-	-	-	-	-	
	Valverde (VA)	6	-	-	-	-	-	-	
	Total	8	0	0	0	0	0	0	
Total		99	26	10	4	4	1	20	

[a] Numbers of surveyed orchards.
[b] Numbers of orchards from which isolates were obtained. *P. cinnamomi* (P.c), *P. multivora* (P.m), *P. niederhauserii* (P.nd), *P. nicotianae* (P.nt), *P. palmivora* (P.p), and *Pp. vexans* (Pp.v)
[c] † Locations with orchards from which more than one species were isolated: ER, P.m+Pp.v (one orchard); GÜ, P.c+P.nt (one orchard) / P.m+P.nd+Pp.v (one orchard); LL, P.m+P.nt+Pp.v (one orchard); BA, P.c+Pp.v (two orchards); BR, P.c+Pp.v (one orchard); SC, P.c+Pp.v (two orchards) / P.c+P.m+Pp.v (one orchard).

Africa (FJ415977, McLeod *et al.*, 2009) and the other in mainland Spain (AM701798, unpublished). The second subclade of *Pp. vexans* (82% bootstrap support) includes the remaining Canary Island isolates along with many other *Pp. vexans* sequences, the ex-type *Pp. cucurbitacearum* (AY598667; CBS 748.96) used by Lévesque and De Cock (2004), and a sequence submitted as *Pp. amazonianum* (HQ261725, Robideau *et al.*, 2011). Further subdivision of this subclade only had low (<60%) or moderate (62-77%) bootstrap support.

Distribution of *Phytophthora* spp. and *Phytopythium vexans* in the surveyed avocado orchards

Phytophthora spp. were isolated from 41 of the 99 avocado orchards that were surveyed in the Canary Islands (Table 2). They were present in 64% of the orchards that were sampled on La Palma (23 of 36 orchards), 41% of those on Tenerife (13 of 32), 25% on Gran Canaria (three of 12) and 18% on La Gomera (two of 11). No *Phytophthora* spp. were recovered from samples taken on El Hierro. The most frequently isolated species was *P. cinnamomi*, which was present in 26 orchards; followed by *P. multivora* (ten orchards), *P. niederhauserii* (four orchards), *P. nicotianae* (four orchards) and *P. palmivora* (one orchard) (Table 2). In orchards from which *P. cinnamomi* was isolated, trees showed clear root rot symptoms. Severely diseased trees were also present in the four orchards where *P. niederhauserii* was detected. In three of these orchards, this species was exclusively isolated. The affected trees showed symptoms indistinguishable from those caused by *P. cinnamomi* (obvious wilting, heavy defoliation beginning in the upper canopy and dieback of branches; cankers were not observed). Some trees were completely dry (Figure 5).

The composition of species differed among islands. Tenerife had the greatest diversity of *Phytophthora* spp., as five species were isolated from there in this study. In contrast, only two *Phytophthora* spp. were isolated in each of La Palma (*P. cinnamomi* and *P. multivora*), Gran Canaria (*P. cinnamomi* and *P. niederhauserii*) and La Gomera (*P. nicotianae* and *P. niederhauserii*) (Table 2). On the other hand, *Pp. vexans* was recovered from 20 avocado orchards on four islands: eight were from Tenerife, seven from La Palma, one from Gran Canaria and four from La Gomera. As noted in Table 2, more than one species were found in ten orchards; four of these orchards were located on Tenerife and six on La Palma.

Pathogenicity

The results of the pathogenicity tests are shown in Table 3. Several isolates were pathogenic to avocado

Figure 5. Avocado tree in advanced decline infected with *P. niederhauserii*; isolate C051 was obtained from roots that were collected from this tree. No other *Phytophthora* was detected in the orchard that was located in Mogán (Gran Canaria, Canary Islands).

causing significant root necrosis, loss of lateral roots, and/or cankers. Regarding the root pathogenicity tests, a significant negative correlation (P<0.001) was found between the necrosis index and the percentage of lateral roots when plants were either inoculated in hydroponics or in pots (Pearson's correlation coefficients of -0.7559 for root inoculation in hydroponic conditions, and -0.7605 in potted plants), as a consequence of fine root losses that were caused by necrosis of the root systems.

Results from the pathogenicity tests indicated that *P. niederhauserii* is the most virulent of the species evaluated. All the tested isolates of this species (C051, C052, C063) gave necrosis indices and caused reductions in lateral roots that did not differ significantly from those of *P. cinnamomi* after root inoculation in hydroponics (Table 3 and Figure 6), although no statistically significant differences were found between isolates and controls for mean plant height or collar diameter (data not shown). The same results were obtained in potted plants, except for isolate C051, which caused less lateral root losses than *P. cinnamomi* (Table 3). In the stem inoculation experiment, all the *P. niederhauserii* isolates also caused stem cankers (Figure 7) that were significantly larger than those from the uninoculated control (Table 3). The only other species that caused stem cankers was *P. palmivora*, although the isolate was unable to cause significant root necrosis or reductions in mean lateral root mass. In contrast, *P. nicotianae* caused root damage as extensive as *P. cinnamomi*, both in hydroponics and potted plants, while stem inoculation did not result in canker formation (Table 3).

Not all isolates of *P. multivora* caused significant root necrosis. Isolates C003 and C005 gave significantly greater mean necrosis indices than uninoculated control plants in hydroponic conditions, while isolate C023 did the same, but in potted plants, and isolate C002 did not cause significant root necrosis in either pathogenicity test. Moreover, necrosis caused by isolates C003 and C005 in hydroponic conditions and by C023 in potted plants was significantly less than that produced by *P. cinnamomi*, and these isolates did not cause significant losses of lateral roots (Table 3). For root inoculation with *Pp. vexans*, large variation in pathogenicity was observed among the six isolates tested: three (C010, C015, C024) were non-pathogenic, one (C001) was moderately pathogenic, and the other two (C020 and C022) gave results that were inconsistent between the two root pathogenicity tests. Isolate C022 was as damaging as *P. cinnamomi* in hydroponic conditions (Figure 6) but did not cause significant root necrosis in potted plants, and the opposite results were obtained with isolate C020 (Table 3). In addition, no isolates of *P. multivora* or *Pp. vexans* caused stem cankers (Table 3), probably due to the formation of host callus barriers that halted canker expansion (Figure 7).

Discussion

This study was conducted to investigate the presence and diversity of *Phytophthora* spp. in commercial avocado orchards in the Canary Islands. The predominant species that was isolated was *P. cinnamomi*, the main causal agent of avocado root rot on a global

Table 3. Results of pathogenicity tests in avocado seedlings, showing the mean root necrosis indices, percentages of lateral roots with respect to the total root weights (root inoculation), and canker perimeters (stem inoculation). Root inoculation tests were carried out under hydroponic conditions and in potted plants.

Species	Isolate	Root inoculation[a]				Stem inoculation[a]
		Hydroponics		Potted plants		Lesion perimeter (cm)
		Necrosis index[b]	Lateral roots (%)	Necrosis index[b]	Lateral roots (%)	
P. cinnamomi	PcH15	3.0 ± 0.0 a	38.8 ± 3.9 bcde	3.0 ± 0.0 a	29.1 ± 0.7 a	6.1 ± 0.5 abc
P. palmivora	C011	0.7 ± 0.3 cd	45.2 ± 0.6 cdef	0.3 ± 0.3 ef	47.9 ± 1.9 cde	9.8 ± 2.6 a
P. niederhauserii	C051	3.0 ± 0.0 a	28.2 ± 9.7 bcd	3.0 ± 0.0 a	48.0 ± 3.4 cde	7.6 ± 2.4 ab
	C052	3.0 ± 0.0 a	26.2 ± 10.0 abc	3.0 ± 0.0 a	28.3 ± 1.4 a	9.8 ± 4.2 a
	C063	2.7 ± 0.3 a	42.8 ± 7.7 cdef	3.0 ± 0.0 a	33.4 ± 5.1 ab	10.1 ± 2.3 a
P. multivora	C002	0.0 ± 0.0 d	67.2 ± 4.7 g	0.3 ± 0.3 ef	60.2 ± 5.8 ef	3.8 ± 0.2 bc
	C003	1.7 ± 0.3 b	43.2 ± 6.8 cdef	0.7 ± 0.3 def	54.2 ± 7.3 def	4.7 ± 0.7 bc
	C005	1.3 ± 0.3 bc	46.9 ± 7.1 cdef	0.3 ± 0.3 ef	58.9 ± 1.7 def	5.1 ± 0.8 bc
	C023	0.7 ± 0.3 cd	47.8 ± 6.4 defg	1.3 ± 0.9 cde	47.1 ± 5.3 bcde	4.2 ± 0.4 bc
P. nicotianae	C013	2.7 ± 0.3 a	21.9 ± 4.7 ab	2.7 ± 0.3 ab	35.5 ± 3.0 abc	4.8 ± 1.0 bc
Pp. vexans	C001	1.3 ± 0.9 bc	38.8 ± 10.8 bcde	1.7 ± 0.9 bcd	47.7 ± 9.9 cde	3.3 ± 0.3 bc
	C010	0.3 ± 0.3 d	50.9 ± 10.4 efg	1.0 ± 0.0 def	38.9 ± 0.9 abc	3.9 ± 0.1 bc
	C015	0.0 ± 0.0 d	56.9 ± 5.3 efg	0.3 ± 0.3 ef	65.5 ± 2.3 f	3.8 ± 0.1 bc
	C020	0.0 ± 0.0 d	50.6 ± 6.1 efg	2.3 ± 0.7 abc	45.9 ± 8.7 bcd	3.8 ± 0.2 bc
	C022	3.0 ± 0.0 a	6.4 ± 6.3 a	0.3 ± 0.3 ef	63.7 ± 2.1 f	3.9 ± 0.2 bc
	C024	0.0 ± 0.0 d	51.2 ± 4.7 efg	1.0 ± 0.6 def	57.8 ± 7.0 def	4.2 ± 0.3 bc
Uninoculated control		0.0 ± 0.0 d	61.5 ± 8.2 fg	0.0 ± 0.0 f	55.2 ± 1.5 def	2.8 ± 0.1 c

[a] Values are means ± standard errors of tests on three plants per isolate. Within columns, values followed by the same letter are not significantly different (*P*<0.05) according to Fisher's least significant difference (LSD) test.
[b] Root necrosis was rated on a 0–3 scale (0 = no obvious symptoms, 1 = less than 30% of the root system was necrotic, 2 = 30–70% of the root system was necrotic, and 3 = more than 70% of the root system was necrotic).

scale, which causes the most important avocado disease worldwide. The most likely hypothesis is that *P. cinnamomi* was introduced and disseminated with infected plant material once avocado production became commercial in the Canary Islands. The presence of this pathogen in the archipelago was reported almost 40 years ago (Gallo-Llobet *et al.*, 1978), and it has since been consistently isolated from declining avocado orchards in Tenerife, La Palma and Gran Canaria. The present study shows that the current distribution of *P. cinnamomi* among islands is unchanged, with avocado orchards from La Gomera and El Hierro remaining free of *P. cinnamomi*. The limited development of avocado production in La Gomera and El Hierro, together with sanitary measures against *P. cinnamomi* that were adopted in nursery plant production and introduction of avocado plants to the region, could explain why *P. cinnamomi* has not spread to these two islands.

The results of this study demonstrate that, in addition to *P. cinnamomi*, other species of *Phytophthora* are also present in avocado orchards in the Canary

Figure 6. Root rot symptoms in avocado seedlings at the end of the root pathogenicity test under hydroponic conditions: a, Control (uninoculated plants); b, Positive control (*P. cinnamomi* isolate PcH15); c, *P. palmivora* C011; d, *P. niederhauserii* C052; e, *P. multivora* C005; f, *P. nicotianae* C013; g, *Pp. vexans* C022.

Islands. The most common among these was *P. multivora* (isolated from 10% of surveyed orchards), followed by *P. nicotianae* and *P. niederhauserii* (each from 4% of orchards). *Phytophthora palmivora* was isolated from only one orchard. To the best of our knowledge, this study constitutes the first report of *P. multivora* and *P. niederhauserii* infecting avocado plants. The other *Phytophthora* spp. found in Canary Island avocado orchards, including *P. palmivora* (Machado *et al.*, 2012) and *P. nicotianae* (Machado *et al.*, 2013), have been previously reported as pathogens of avocado.

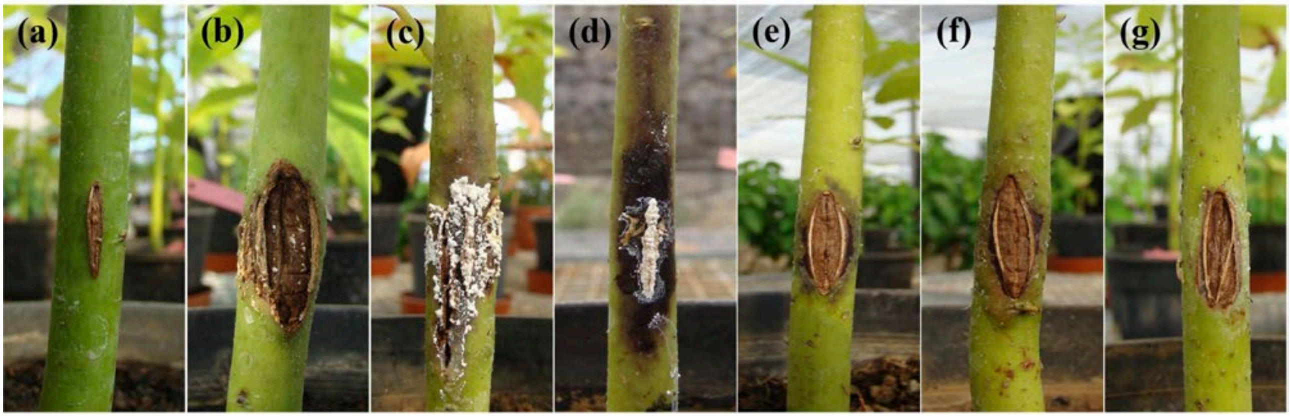

Figure 7. Lesions on stems of avocado seedlings at the end of the stem pathogenicity test: a, Control (uninoculated plants); b, Positive control (*P. cinnamomi* isolate PcH15); c, *P. palmivora* C011; d, *P. niederhauserii* C051; e, *P. multivora* C005; f, *P. nicotianae* C013; g, *Pp. vexans* C015. Note the absence of callus tissue at the edges of the wounds that were inoculated with *P. palmivora* and *P. niederhauserii*, as well as the exudation of white, sugary material that is commonly associated with avocado cankers.

Phytophthora niederhauserii is a newly described species from *Phytophthora* clade 7b (Abad *et al.*, 2014). It was first isolated in 2001 from necrotic collars, stems and roots of arborvitae (*Thuja occidentalis*) and English ivy (*Hedera helix*) in North Carolina, USA, and subsequently from 33 plant species in 13 countries including Australia, South Africa and several European countries (Abad *et al.*, 2014). Phylogenetic analysis revealed previous mis-identifications of *P. niederhauserii* as *P. drechsleri* in Japan and Israel and *P. melonis* in Taiwan. According to Abad *et al.* (2014), *P. niederhauserii* is a polyphagous pathogen that is emerging from nurseries of ornamental plants in several countries.

On the Spanish mainland, *P. niederhauserii* was first isolated from diseased potted plants in ornamental nurseries (Moralejo *et al.*, 2009) and later, from young almond trees in two field-grown nurseries, that had shown symptoms associated with root rot (leaf chlorosis, defoliation, wilting) as well as stem cankers and gummosis (Pérez-Sierra *et al.*, 2010). *Phytophthora niederhauserii* has also been reported in Turkey (Kurbetli and Degirmenci, 2011) and California (Browne *et al.*, 2015), causing death of almond trees in orchards. Therefore, it seems likely that this species could represent a general risk for fruit crops. Our results support this concern since, *P. niederhauserii* was isolated from trees that showed severe decline and dieback in four avocado orchards, and in three of these orchards, this pathogen was exclusively isolated. The results of the pathogenicity tests that were carried out with *P. niederhauserii* isolates demonstrated they were highly virulent, causing root rot as severe as *P. cinnamomi*, and cankers in experimentally inoculated avocado seedlings. *Phytophthora niederhauserii* was not isolated as frequently as *P. cinnamomi* or *P. multivora*, suggesting a more recent introduction in the Canary Islands, but there is an obvious risk considering that this pathogen has been detected in three out of the five surveyed islands.

Phytophthora multivora is the other *Phytophthora* species which was reported for the first time in avocado. This is a species in the *P. citricola* complex described by Scott *et al.* (2009). It appears to be widespread in natural ecosystems in Western Australia (Burgess *et al.*, 2009; Scott *et al.*, 2009) and South Africa (Bezuidenhout *et al.*, 2010; Oh *et al.*, 2013; Nagel *et al.*, 2015). *Phytophthora multivora* has also been described in Central Europe, where it has been recovered from declining oak forests in Hungary (Szabó *et al.*, 2013) and Czech Republic (Mrázková *et al.*, 2013). Because this species was identified previously as *P. citricola* (Burgess *et al.*, 2009; Scott *et al.*, 2009), its global distribution is uncertain and could be underestimated. For example, *P. multivora* isolates from mango and ornamental nurseries on the Spanish mainland were reported at the time as *P. citricola* (Zea-Bonilla *et al.*, 2007; Moralejo *et al.*, 2009).

In the Canary Islands, *P. multivora* was detected in a native laurel forest (laurisilva) in Tenerife (Catalá *et al.*, 2014). The origin of *P. multivora* in the laurisilva and its role as a pathogen in this forest are unknown, but it could be a threat to this unique and valuable ecosystem that is the subject of considerable conservation efforts. In Western Australia, *P. multivora* shows a distribution that is similar to that of *P. cinnamomi*, and it is thought to be involved in the decline of tuart woodland, although the collapse of the whole ecosystem is most likely driven by *P. cinnamomi* (Scott *et al.*, 2009). *Phytophthora multivora* has been shown to cause root rot and/or stem lesions under controlled conditions in multiple hosts such as *Agathosma* spp. (Bezuidenhout *et al.*, 2010), *Eucalyptus* spp. (Scott *et al.*, 2012), *Rhododendron* spp. (Henricot *et al.*, 2014) and *Rubus* spp. (Aghighi *et al.*, 2016), but to cause mild damage than other *Phytophthora* spp. and show variable aggressiveness among isolates. Similar results were obtained in the present study by inoculating avocado seedlings; no canker development was observed upon stem inoculation, and variable root necrosis, from moderate to non-significant, was observed in two independent root pathogenicity tests, depending on isolate and experiment.

In addition to *Phytophthora* species, isolates of the *Pp. vexans* group were obtained from all the surveyed islands except El Hierro. Although the main aim of this study was to investigate *Phytophthora* spp., the *Pp. vexans* isolates were examined because they were repeatedly isolated throughout the survey. The taxonomic arrangement of the *Pp. vexans* group is under revision, but it is assumed to comprise more than one species based on the heterogeneity of the ITS sequences in different isolates of this complex (Lévesque and De Cock, 2004; McLeod *et al.*, 2009; Robideau *et al.*, 2011; Spies *et al.*, 2011a; De Cock *et al.*, 2015). *Phytopythium vexans* is widespread worldwide, and recent studies carried out in South Africa have noted that *Pp. vexans* is pathogenic towards woody hosts such as grapevine (Spies *et al.*, 2011b) and apple (Tewoldemedhin *et al.*, 2011). *Phytopythium vexans* was previously isolated on the Spanish mainland from avocado trees that showed root rot symptoms (Martín-Sánchez *et al.*, 2008), but to our knowledge, no further studies were made in this respect. As in *P. multivora*, the pathogenicity to avocado seedlings of different isolates of *Pp. vexans* was variable, and some were non-pathogenic. Moreover, pathogenicity of several isolates was inconsistent between root inoculations in hydroponic and potted plants, indicating that isolates varied in their aggressiveness, depending on test conditions.

This study increases the knowledge of oomycetes beyond *P. cinnamomi* associated with avocado crops, with the first report of *P. niederhauserii* and *P. multivora* from avocado, and the isolation of the previously described avocado pathogens *P. nicotianae* and *P. palmivora* in the Canary Islands. In addition, isolates belonging to the *Pp. vexans* complex were also consistently obtained from soils and roots of avocado orchards. Among these species, *P. niederhauserii* has proven to be the most aggressive pathogen of avocado plants. Therefore, this pathogen may be a new threat to this crop in the Canary Islands, and also to other avocado-growing regions considering its occurrence in the nurseries (Abad *et al.*, 2014; Jung *et al.*, 2016). The presence of *P. niederhauserii* has been recently reported in almond orchards in California (Browne *et al.*, 2015), the largest avocado growing area in the USA. The pathogen is also present in mainland Spain (Moralejo *et al.*, 2009; Pérez-Sierra *et al.*, 2010), the only country that produces avocados in the European Union in significant quantities. However, further investigation is required to clarify the involvement of *P. multivora* and *Pp. vexans* in avocado decline. The results of this study suggest that both species could be weak pathogens of avocado, but their contribution to crop damage in the Canary Islands should be considered, since both species were found quite often and they were isolated together with other very aggressive species in nearly 10% of prospected orchards.

Plant health measures for avocado are focused on the control of *P. cinnamomi* because it is recognized as the major phytosanitary threat to worldwide crop production. However, our results indicate that other species should also be considered in disease control programmes. The Canary Islands suffer from continuous import of exotic plant species, enabling the entry of many potentially harmful associated organisms that overcome phytosanitary barriers despite the region having regulations that are independent from those applied in the rest of Spain (BOE, 1987). Some *Phytophthora* spp. have been mainly introduced into new regions by the international trade of exotic or ornamental plants (Brasier, 2008; 2009; Jung *et al.*, 2016), so it is likely that most of the introductions of these pathogens into the Canary Islands have occurred via this pathway. This could be particularly the case with *P. niederhauserii*, a well-known harmful organism that is emerging in nurseries, whose recent worldwide spread has been attributed to plant trade. A recent large-scale study revealed that this species has been found in forest and ornamental nurseries in different European regions (Jung *et al*., 2016), and it has been suggested that multiple introductions of this pathogen may have occurred in Italian nurseries that produce ornamental and fruit tree plantlets (Prigigallo *et al*., 2015). The present study proves that *P. niederhauserii* is highly damaging to avocado, and that it should be considered in future sanitary control protocols for avocado nurseries.

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Literature cited

Abad Z.G., J.A. Abad, S.O. Cacciola, A. Pane, R. Faedda, E. Moralejo, A. Pérez-Sierra, P. Abad-Campos, L.A. Álvarez-Bernaola, J. Bakonyi, A. Józsa, M.L. Herrero, T.I. Burgess, J.H. Cunnington, I.W. Smith, Y. Balci, C. Blomquist, B. Henricot, G. Denton, C. Spies, A. McLeod, L. Belbahri, D. Cooke, K. Kageyama, S. Uematsu, I. Kurbetli and K. Değirmenci, 2014. *Phytophthora niederhauserii* sp. nov., a polyphagous species associated with ornamentals, fruit trees and native plants in 13 countries. *Mycologia* 106, 431–447.

Aghighi S., T.I. Burgess, J.K. Scott, M. Calver and GEStJ Hardy, 2016. Isolation and pathogenicity of *Phytophthora* species from declining *Rubus anglocandicans*. *Plant Pathology* 65, 451–461.

Bala K., G.P. Robideau, C.A Lévesque, A.W.A.M. de Cock, G. Abad, M.A. Lodhi, S. Shahzad, A. Ghaffar and M.D. Coffey, 2010. *Phytopythium* Abad, de Cock, Bala, Robideau, Lodhi & Lévesque, gen. nov. and *Phytopythium sindhum* Lodhi, Shahzad & Lévesque, sp. nov. Fungal Planet 49. *Persoonia* 24, 136–137.

Bezuidenhout C.M., S. Denman, S.A. Kirk, W.J. Botha, L. Mostert and A. McLeod, 2010. *Phytophthora* taxa associated with cultivated *Agathosma*, with emphasis on the *P. citricola* complex and *P. capensis* sp. nov. *Persoonia* 25, 32–49.

Bingham F.T. and G.A. Zentmyer, 1954. Relation of hydrogen-ion concentration of nutrient solution to Phytophthora root rot of avocado seedlings. *Phytopathology* 44, 611–614.

BOE, 1987. Ministerio de Agricultura, Pesca y Alimentación del Gobierno de España. Orden Nº 72: 8540-56 de 12 de marzo de 1987 por la que se establecen para las Islas Canarias las Normas Fitosanitarias relativas a la importación, exportación y tránsito de vegetales y productos vegetales. 8540–8556.

Brasier C.M., 2008. The biosecurity threat to the UK and global environment from international trade in plants. *Plant Pathology* 57, 792–808.

Brasier C.M., 2009. *Phytophthora* biodiversity: how many *Phytophthora* species are there? In Phytophthoras in Forests and Natural Ecosystems, *Proceedings of the Fourth Meeting of the International Union of Forest Research Organizations* (IUFRO), edited by E. M. Goheen and S. J. Frankel. Albany, CA: USDA Forest Service, pp. 101–115.

Browne G.T., L.S. Schmidt and G. Brar, 2015. First report of *Phytophthora niederhauserii* causing crown rot of almond (*Prunus dulcis*) in California. *Plant Disease* 99, 1863.

Burgess T.I., J.L. Webster, J.A. Ciampini, D. White, GEStJ Hardy and M.J.C. Stukely, 2009. Re-evaluation of *Phytophthora* species isolated during 30 years of vegetation health surveys in Western Australia using molecular techniques. *Plant Disease* 93, 215–223.

Catalá S., A. Pérez-Sierra, C. Rodríguez-Padrón, F. Siverio de la Rosa and P. Abad-Campos, 2014. Discovering *Phytophthora* species in the laurel forest in Tenerife and La Gomera islands (Canary Islands, Spain). In: *7th Meeting of IUFRO WP 7.02.09 Phytophthora in Forests & Natural Ecosystems.* Esquel, Argentina, 78.

Cooke D.E.L., A. Drenth, J.M. Duncan, G. Wagels and C.M. Brasier, 2000. A molecular phylogeny of *Phytophthora* and related oomycetes. *Fungal Genetics and Biology* 30, 17–32.

De Cock A.W.A.M., A.M. Lodhi, T.L. Rintoul, K. Bala, G.P. Robideau, Z. Gloria Abad, M.D. Coffey, S. Shahzad and C.A. Lévesque, 2015. *Phytopythium*: molecular phylogeny and systematics. *Persoonia* 34, 25–39.

Erwin D.C. and O.K. Ribeiro, 1996. *Phytophthora Diseases Worldwide*. The American Phytopathological Society Press, St. Paul, Minnesota, USA.

Gallegly M.E. and C.X. Hong, 2008. *Phytophthora: Identifying species with morphology and DNA fingerprints*. The American Phytopathological Society Press, St. Paul, Minnesota, USA.

Gallo-Llobet L., F. Miralles Ciscar and F.J. Álvarez de la Peña, 1978. *La podredumbre de las raíces del aguacate*. Hoja divulgadora Nº 26-78 HDX. Ministerio de Agricultura.

Gallo L., J. Hernández, M.C. Jaizme and L. Sala, 1988. Especies fúngicas encontradas en los cultivos de Canarias en el periodo 1974-84. In: *III Congreso de la Sociedad Española de Fitopatología*. Puerto de la Cruz, Canary Islands, Spain, 43–50.

Hansen E.M., P.W. Reeser and W. Sutton, 2012. *Phytophthora* beyond agriculture. *Annual Review of Phytopathology* 50, 359–378.

Hasegawa M., H. Kishino and T. Yano, 1985. Dating the human-ape split by a molecular clock of mitochondrial DNA. *Journal of Molecular Evolution* 22, 160–174.

Henricot B., A. Pérez-Sierra and T. Jung, 2014. *Phytophthora pachypleura* sp. nov., a new species causing root rot of *Aucuba japonica* and other ornamentals in the United Kingdom. *Plant Pathology* 63, 1095–1109.

Hong C.X., M.E. Gallegly, G.T. Browne, R.G. Bhat, P.A. Richardson and P. Kong, 2009. The avocado subgroup of *Phytophthora citricola* constitutes a distinct species, *Phytophthora mengei* sp. nov. *Mycologia* 101, 833–840.

Jeffers S.N. and S.B. Martin, 1986. Comparison of two media selective for *Phytophthora* and *Pythium* species. *Plant Disease* 70, 1038–1043.

Jung T., A.M. Vettraino, T.L. Cech and A. Vannini, 2013. The impact of invasive *Phytophthora* species on European forests. In: Lamour K, ed. *Phytophthora*: A Global Perspective. Wallingford, UK: CABI, pp. 146–158.

Jung T., L. Orlikowski, B. Henricot, P. Abad-Campos, A.G. Aday, O. Aguín Casal, J. Bakonyi, S.O. Cacciola, T. Cech, D. Chavarriaga, T. Corcobado, A. Cravador, T. Decourcelle, G. Denton, S. Diamandis, H.T. Doğmuş-Lehtijärvi, A. Franceschini, B. Ginetti, S. Green, M. Glavendekić, J. Hantula, G. Hartmann, M. Herrero, D. Ivic, M. Horta Jung, A. Lilja, N. Keca, V. Kramarets, A. Lyubenova, H. Machado, G. Magnano di San Lio, P.J. Mansilla Vázquez, B. Marçais, I. Matsiakh, I. Milenkovic, S. Moricca, Z.Á. Nagy, J. Nechwatal, C. Olsson, T. Oszako, A. Pane, E.J. Paplomatas, C. Pintos Varela, S. Prospero, C. Rial Martínez, D. Rigling, C. Robin, A. Rytkönen, M.E. Sánchez, A.V. Sanz Ros, B. Scanu, A. Schlenzig, J. Schumacher, S. Slavov, A. Solla, E. Sousa, J. Stenlid, V. Talgø, Z. Tomic, P. Tsopelas, A. Vannini, A.M. Vettraino, M. Wenneker, S. Woodward and A. Peréz-Sierra, 2016. Widespread *Phytophthora* infestations in European nurseries put forest, semi-natural and horticultural ecosystems at high risk of *Phytophthora* diseases. *Forest Pathology* 46, 134–163.

Kimura M., 1980. A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. *Journal Molecular Evolution* 16, 111–120.

Kong P., C.X. Hong and P.A. Richardson, 2003. Rapid detection of *Phytophthora cinnamomi* using PCR with primers derived from the *Lpv* putative storage protein genes. *Plant Pathology* 52, 681–693.

Kurbetli I. and K. Değirmenci, 2011. First report of *Phytophthora* taxon *niederhauserii* causing decline of almond in Turkey. *New Disease Reports* 23, 14.

Lévesque C.A. and A.W.A.M. De Cock, 2004. Molecular phylogeny and taxonomy of the genus *Pythium*. *Mycological Research* 108, 1363–1383.

Machado M., C. Collazo, M. Peña, M.A. Renaud, M.O. López, O. Coto, V. Zamora, R.I. Cabrera, M. Aranguren and G.J. Boland, 2012. First report of *Phytophthora palmivora* Butler causing root rot on avocado (*Persea americana*) in Cuba. *Canadian Journal of Plant Pathology* 34, 338.

Machado M., C. Collazo, M. Peña, O. Coto and M.O. López, 2013. First report of root rot caused by *Phytophthora nicotianae* in avocado trees (*Persea americana*) in Cuba. *New Disease Reports* 28, 9.

Martín-Sánchez P.M., T. Zea-Bonilla and R.M. Pérez-Jiménez, 2008. Nuevas enfermedades del aguacate causadas por hongos y oomicetes descritas en Andalucía. In: *XIV Congreso de la Sociedad Española de Fitopatología*. Lugo, Spain. 84.

Martin F.N., Z.G. Abad, Y. Balci and K. Ivors, 2012. Identification and detection of *Phytophthora*: reviewing our progress, identifying our needs. *Plant Disease* 96, 1080–1103.

McLeod A., W.J. Botha, J.C. Meitz, C.F.J. Spies, Y.T. Tewoldemedhin and L. Mostert, 2009. Morphological and phylogenetic analyses of *Pythium* species in South Africa. *Mycological Research* 113, 933–951.

Moralejo E., A.M. Pérez-Sierra, L.A. Álvarez, L. Belbahri, F. Lefort and E. Descals, 2009. Multiple alien *Phytophthora* taxa discovered on diseased ornamental plants in Spain. *Plant Pathology* 58, 100–110.

Mrázková M., K. Černý, M. Tomšovský, V. Strnadová, B. Gregorová, V. Holub, M. Pánek, L. Havrdová and M. Hejná, 2013. Occurrence of *Phytophthora multivora* and *Phytophthora plurivora* in the Czech Republic. *Plant Protection Science* 49, 155–164.

Nagel J.H., B. Slippers, M.J. Wingfield and M. Gryzenhout, 2015. Multiple Phytophthora species associated with a single riparian ecosystem in South Africa. *Mycologia* 107, 915–925.

Oh E., M. Gryzenhout, B.D. Wingfield, M.J. Wingfield and T.I. Burgess, 2013. Surveys of soil and water reveal a goldmine of *Phytophthora* diversity in South African natural ecosystems. *IMA Fungus* 4, 123–131.

Pérez-Sierra A., M. León, L.A. Álvarez, S. Alaniz, M. Berbegal, J. García-Jiménez and P. Abad-Campos, 2010. Outbreak of a new *Phytophthora* sp. associated with severe decline of almond trees in Eastern Spain. *Plant Disease* 94, 534–541.

Prigigallo M.I., S. Mosca, S.O. Cacciola, D.E.L. Cooke and L. Schena, 2015. Molecular analysis of Phytophthora diversity in nursery-grown ornamental and fruit plants. *Plant Pathology* 64, 1308–1319.

Robideau G.P., A.W.A.M. De Cock, M.D. Coffey, H. Voglmayr, H. Brouwer, K. Bala, D.W. Chitty, N. Désaulniers, Q.A. Eggertson, C.M.M. Gachon, C.H. Hu, F.C. Küpper, T.L. Rintoul, E. Sarhan, E.C.P. Verstappen, Y. Zhang, P.J.M. Bonants, J.B. Ristaino and C.A. Lévesque, 2011. DNA barcoding of oomycetes with cytochrome c oxidase subunit I and internal transcribed spacer. *Molecular Ecology Resources* 11, 1002–1011.

Saitou N. and M. Nei, 1987. The neighbor-joining method: A new method for reconstruction phylogenetic trees. *Molecular Biology and Evolution* 4, 406–425.

Scanu B., G.C. Hunter, B.T. Linaldeddu, A. Franceschini, L. Maddau, T. Jung and S. Denman, 2014. A taxonomic re-evaluation reveals that *Phytophthora cinnamomi* and *P. cinnamomi* var. *parvispora* are separate species. *Forest Pathology* 44, 1–20.

Scott P.M., T.I. Burgess, P.A. Barber, B.L. Shearer, M.J.C. Stukely, GEStJ Hardy and T. Jung, 2009. *Phytophthora multivora* sp. nov., a new species recovered from declining *Eucalyptus*, *Banksia*, *Agonis* and other plant species in Western Australia. *Persoonia* 22, 1–13.

Scott P.M., T. Jung, B.L. Shearer, P.A. Barber, M. Calver and GEStJ Hardy, 2012. Pathogenicity of *Phytophthora multivora* to *Eucalyptus gomphocephala* and *Eucalyptus marginata*. *Forest Pathology* 42, 289–298.

Simamora A.V., M.J.C. Stukely, P.A. Barber, GEStJ Hardy and T.I. Burgess, 2017. Age-related susceptibility of *Eucalyptus* species to *Phytophthora boodjera*. *Plant Pathology* 66, 501–512.

Spies C.F.J., M. Mazzola, W.J. Botha, M. Van Der Rijst, L. Mostert and A. McLeod, 2011a. Oogonial biometry and phylogenetic analyses of the *Pythium vexans* species group from woody agricultural hosts in South Africa reveal distinct groups within this taxon. *Fungal Biology* 115, 157–168.

Spies C.F.J., M. Mazzola and A. McLeod, 2011b. Characterization and detection of the *Pythium* and *Phytophthora* species associated with grapevines in South Africa. *European Journal of Plant Pathology* 131, 103–119.

Szabó I., F. Lakatos and G. Sipos, 2013. Occurrence of soilborne *Phytophthora* species in declining broadleaf forests in Hungary. *European Journal of Plant Pathology* 137, 159–168.

Tamura K. and M. Nei, 1993. Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. *Molecular Biology and Evolution* 10, 512–526.

Tamura K., G. Stecher, D. Peterson, A. Filipski and S. Kumar, 2013. MEGA6: Molecular evolutionary genetics analysis version 6.0. *Molecular Biology and Evolution* 30, 2725–2729.

Tewoldemedhin Y.T., M. Mazzola, W.J. Botha, C.F.J. Spies and A. McLeod, 2011. Characterization of fungi (*Fusarium* and *Rhizoctonia*) and oomycetes (*Phytophthora* and *Pythium*) associated with apple orchards in South Africa. *European Journal of Plant Pathology* 130, 215–229.

Thompson J.D., D.G. Higgins and T.J. Gibson, 1994. CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position specific gap penalties and weight matrix choice. *Nucleic Acids Research* 22, 4673–4680.

Waterhouse G.M., F.J. Newhook and D.J. Stamps, 1983. Present criteria for classification of *Phytophthora*. In: *Phytophthora. Its Biology, Taxonomy, Ecology, and Pathology* (Erwin D.C., S. Bartnicki-Garcia and P. Tsao, eds), The American Phytopathological Society Press. St Paul, Minnesota, USA, pp. 139–147.

White T.J., T. Bruns, S. Lee and J. Taylor, 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: *PCR Protocols: A Guide to Methods and Applications* (Innis M.A., Gelfand D.H., Sninsky J.J., White T.J., eds) San Diego: Academic Press, pp. 315–322.

Zea-Bonilla T., P.M. Martín-Sánchez, J.M. Hermoso, M.P. Carmona, E. Segundo and R.M. Pérez-Jiménez, 2007. First report of *Phytophthora citricola* on *Mangifera indica* in Spain. *Plant Pathology* 56, 356.

Zentmyer G.A., 1980. *Phytophthora cinnamomi* and the diseases it causes. Monograph nº 10. The American Phytopathological Society. St. Paul, Minnesota. USA

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