



Article

Cinnamom verum Plantations in the Lowland Tropical Forest of Mexico Are Affected by *Phytophthora cinnamomi*, Phylogenetically Classified into *Phytophthora* Subclade 7c

Petra Andrade-Hoyos ¹, Omar Romero-Arenas ^{2,*} , Hilda Victoria Silva-Rojas ^{3,*}, Alfonso Luna-Cruz ⁴ , José Espinoza-Pérez ⁵, Aarón Mendieta-Moctezuma ⁶ and José Alberto Urrieta-Velázquez ⁷

- ¹ Instituto Nacional de Investigaciones Forestales Agrícolas y Pecuarias (INIFAP), Campo Experimental Zacatepec, Carretera Zacatepec-Galeana s/n, km 0.5, Colonia IMMS, Zacatepec 62780, Morelos, Mexico
 - ² Centro de Agroecología, Instituto de Ciencias, Benemérita Universidad Autónoma de Puebla, Edificio VAL 1, km 1.7 Carretera a San Baltazar Tetela, San Pedro Zacachimalpa 72960, Puebla, Mexico
 - ³ Producción de Semillas, Colegio de Postgraduados, Campus Montecillo, Montecillo 56230, Mexico, Mexico
 - ⁴ Instituto de Investigaciones Químico-Biológicas, Universidad Michoacana de San Nicolás de Hidalgo, Morelia 27852, Michoacan, Mexico
 - ⁵ El Colegio De La Frontera Sur, Unidad San Cristóbal: El Colegio de la Frontera Sur, San Cristóbal de las Casas 29290, Chiapas, Mexico
 - ⁶ Centro de Investigación en Biotecnología Aplicada, Instituto Politécnico Nacional, Carretera Estatal Santa Inés Tecuexcomac-Tepetitla, km 1.5, Santa Inés Tecuexcomac 90700, Tlaxcala, Mexico
 - ⁷ Agricultura Protegida, CENID-RASPA, INIFAP, Margen Derecho Canal de Sacramento km 6.5 Margen Derecho Canal de Sacramento S/N, Ejido Las Huertas, Gómez Palacio 35079, Durango, Mexico
- * Correspondence: biol.ora@hotmail.com or omar.romero@correo.buap.mx (O.R.-A.); hsilva@colpos.mx (H.V.S.-R.)



Citation: Andrade-Hoyos, P.; Romero-Arenas, O.; Silva-Rojas, H.V.; Luna-Cruz, A.; Espinoza-Pérez, J.; Mendieta-Moctezuma, A.; Urrieta-Velázquez, J.A. *Cinnamom verum* Plantations in the Lowland Tropical Forest of Mexico Are Affected by *Phytophthora cinnamomi*, Phylogenetically Classified into *Phytophthora* Subclade 7c. *Horticulturae* **2023**, *9*, 187. <https://doi.org/10.3390/horticulturae9020187>

Academic Editors: Jian Ling and Jiao Yang

Received: 31 December 2022

Revised: 23 January 2023

Accepted: 23 January 2023

Published: 2 February 2023



Copyright: © 2023 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<https://creativecommons.org/licenses/by/4.0/>).

Abstract: Cinnamon is a tree introduced to the lowlands of Mexico in the mid-16th century, but it spread to other places at the beginning of the 20th century due to its important commercial value as an aromatic spice. In the state of Veracruz, symptoms of dieback have been observed in 12-year-old cinnamon plantations cultivated in an agroforestry system, causing concern among producers. For this reason, the present investigation was carried out to determine the causal agent of these symptoms observed in cinnamon trees. Fifty symptomatic plants were recovered from established plantations. One hundred cinnamon root fragments showing dieback were selected and separated; isolates were made from tissue showing crown and root rot on clarified juice V-8 agar medium. After eight days, the growth of whitish coraloid mycelium with characteristics similar to the *Phytophthora* oomycete was consistently observed. Subsequently, the identity corresponding to *P. cinnamomi* was confirmed by morphological, taxonomic studies and Bayesian inference of the rDNA internal transcribed spacer. The pathogenicity test was performed on 20 6-month-old cinnamon plants grown in pots by inoculating 2.5×10^4 /mL of zoospores around the roots. Control plants were inoculated with sterile distilled water and kept in a greenhouse under conditions controlled. After five weeks, symptoms of root rot were observed in the inoculated plants; however, the control group plants remained healthy. The results showed that *P. cinnamomi* subclade 7c was responsible for the symptoms observed in lowland cinnamon plantations in Mexico. Our findings suggest that this phytopathogen is a new threat for cinnamon growers; likewise, it is recommended that growers implement management strategies to avoid its introduction into nurseries or new plantations that could be susceptible to this pathogen.

Keywords: canker; cinnamon; crown; decay; root rot

1. Introduction

Cinnamon (*Cinnamomum verum*) is a dominant evergreen tree of humid tropical forests globally, native to Sri Lanka and southern India, but also distributed in Southeast Asia,

China, Burma, Indonesia, Madagascar, the Caribbean, Australia, and Africa [1]. Sri Lanka stands out for the most significant production of *C. verum* worldwide, corresponding to 70% [2]. Cinnamon is considered among the main spices for its pleasant flavor and aroma and is used in international cuisine [3]. In addition, medicinal and therapeutic properties have been attributed to this spice, among the most relevant biological activities are anticancer, antidiabetic, and cytotoxic [1,4,5].

Cinnamon is the commercial name of various species and products used in the food industry, such as flavorings and colorings. The systematics of the different *Cinnamomum* species depends mainly on the analysis of morphological characteristics, which is often difficult due to their great diversity, genetic variation, morphological similarity between species, and strict seasonality in flowering and fruiting [6], in addition to including a volatile chemical profile [7]. To date, around 250 species of cinnamon have been identified, distributed in four important groups; Ceylon cinnamon (*Cinnamomum zeylanicum* Blume), native to Sri Lanka; Cassia cinnamon or Chinese cinnamon (*Cinnamomum aromaticum* Nees) from China; Indonesian cassia (*Cinnamomum burmannii* Nees) from Sumatra and Java; and Vietnamese cinnamon (*Cinnamomum loureiroi* Nees) from Vietnam [8].

The health of the plants and the quality of the soil are the determining components to produce cinnamon. A healthy soil in the presence of microorganisms can help improve crop yields. However, there are several limitations on the production of cinnamon around the world. Cinnamon, although a hardy plant, is susceptible to a wide variety of diseases during its various stages of development [9]. The oomycete *Phytophthora cinnamomi* is one of the most important pathogens in the cultivation of cinnamon [10]. *P. cinnamomi* is an important pathogen of horticulture, affecting 319 genera in 90 families—principally forest trees—and found in 56 countries of tropical and subtropical regions [11].

Phytophthora cinnamomi has been included in the list of the 10 most destructive oomycetes and as one of the 100 worst invasive species and natural enemies worldwide [12]. It may affect 5000 plant species worldwide [13]. The geographic origin of *P. cinnamomi* is not clearly established. However, it was first isolated from striped cankers of cinnamon trees in Sumatra, Indonesia; this suggests that it is indigenous to regions of tropical and subtropical countries and has also spread to other regions of the world [14]. The oomycete *P. cinnamomi* has been reported to affect *Cinnamomum burmannii* in the East Indies [15] and Indonesia [16–18], *C. camphora* in Indonesia [15], *C. culilawan* in China [19] and Indonesia [15], *C. micranthum* in Taiwan [20], and *C. sintok* and *C. verum* in Indonesia [15].

C. verum is the only dominant species of cinnamon cultivated in Mexico, and it has been reported as a host of *P. cinnamomi*. However, since 2019, the planted area has been reduced, in addition to increasing the percentage of dieback, chlorosis, and crown and root rot, with a loss of up to 40% of the plants in production in the Hidalguense region of Totonacapan, Veracruz, Mexico [21]. For this reason, the objective of this work was to identify the causal agent of the dieback of cinnamon plantations established in agroforestry systems in the lowlands of Totonacapan, using morphological and phylogenetic approaches, as well as reproducible symptoms in laboratory.

2. Materials and Methods

2.1. Sample Collection

In spring 2019, roots with rhizosphere soil were recovered from fifty cinnamon plants exhibiting symptoms of chlorosis, foliage wilt, crown rot, and root rot (Figure 1) in lowland tropical forests of the state of Veracruz ($n = 50$) located at 300 m altitude in eastern Mexico, with a subhumid warm climate (A-W1) and an average rainfall of 2000 mm [22]. All samples were kept in plastic bags in a cooler until they were transferred to the laboratory to be processed.



Figure 1. Traditional *Cinnamomum verum* plantations of 12 years old located in the lowland of Veracruz State, Mexico. (A) Cinnamon shrub with evident dieback, that began with chlorotic foliage and decline, and (B) healthy plants showing normal leaves.

2.2. Isolation

One hundred 25-cm cinnamon root fragments showing root rot from fifty symptomatic plants were selected and separated. Subsequently, the outer part was washed with running water and disinfected by immersion for 1 min in a 1.5% *w/v* aqueous sodium hypochlorite solution and rinsed three times with sterile distilled water. Finally, samples were wrapped with sterile paper towels and placed in a laminar flow chamber at 20 °C for 15 min [23]. Small samples (3 cm) showing a rusty-looking reddish-brown discoloration within the roots were excised with the aid of a sterile scalpel and placed upright in Petri dishes with a selective medium of agar from clarified PARPH-V-8 juice (pimaricin 10 $\mu\text{g L}^{-1}$, ampicillin 292 $\mu\text{g L}^{-1}$, rifampicin 10 $\mu\text{g L}^{-1}$, pentachloro-nitrobenzene 0.10 g L^{-1} , and himexazol 0.25 $\mu\text{g L}^{-1}$). Samples were then incubated at 28 ± 2 °C for 72 h in the dark [24,25].

After eight days, the isolates were transferred to V-8 juice agar plates by the hyphal tip method to identify them at the genus level with the morphological keys [26,27]. All *Phytophthora* isolates obtained in pure culture were preliminarily grouped into morphotypes, as well as gametangia characteristics. To enhance sporangia production, 10 plugs of mycelial agar (50 mm in diameter) taken from the margin of actively growing colonies of 4 days old were placed and transferred to 9 cm Petri dishes containing 10 mL of liquid medium V-8 juice. The plates were kept in the dark at 25 °C and examined every 24 h for 96 h. Once the sporangia were formed, the Petri dishes were placed at 4 °C for 30 min, and then incubated at 25 °C for 1 h, to promote the release of zoospores, where this was verified under an optical microscope (Zeiss Axioskop plus) at 400 \times magnification [28].

Detailed studies of colony morphology, sporangia, and chlamydospores were carried out in clarified V-8 juice plates, incubated at 28 °C in the dark for 7 days. Samples were then mounted onto a microscope slide with clear 1% lactic acid, and 30 were selected for measurement using ImageJ software (<https://imagej.nih.gov/ij/>, accessed on 17 August 2022) through photographs taken by Infinity 1–2 C implemented on an Olympus BX41 microscope (Tokyo, Japan).

2.3. DNA Extraction and PCR Amplification

Once the isolates were characterized, the most representative strain of the study area was selected. Genomic DNA (gDNA) was extracted with the CTAB 2% (Tris-HCL 10 Mm, pH 8; H₂O 20 Mm, pH 8; CTAB 2%; NaCl 1.4 M at 60 °C) protocol of [29] with some modifications (Rivera-Jiménez et al., 2018). The DNA was suspended in 100 μL of sterile HPLC water and quantified by spectrophotometry in a Nanodrop 2000 C (Thermo Scientific, Waltham, MA, USA), DNA was considered acceptable when the ratio of absorbance at $A_{260/280}$ and $A_{260/230}$ nm ranged between 1.8 and 2.2. Finally, the DNA was diluted to 20 $\text{ng } \mu\text{L}^{-1}$ and stored at -20 °C for PCR amplification.

PCR was performed in a C1000 Touch Thermal Cycler (BioRad, Hercules, CA, USA) with a 15 μL reaction mixture containing 0.18 μL of each primer ITS6 (5'-GAAGGTGAAGTC GTAACAAGG-3') and ITS4 (5'-TCCTCCGCTTATTGAGC-3') [30,31], 0.18 μL of dNTPs, 0.9 U of GoTaq DNA polymerase (Promega, Madison, WI, USA), and 3 μL of DNA template (20 ng mL^{-1}). The thermocycler program consisted of a first step at 95 °C for 4 min, followed by 35 cycles at 95 °C for 1 min, 58 °C for 1 min, and 72 °C for 2 min, and a final step at 72 °C for 10 min. Amplicons were verified by horizontal electrophoresis in 1.5% agarose Sea Kem LE (Lonza, Morristown, NJ, USA), stained with GelRed (Biotium, Fremont, CA, USA). The gels were visualized using the Infinity imaging systems in the Infinity-3026 WL/LC/26MX transilluminator (Vilber Lourmat, Eberhardzell, Germany). Before sequencing, amplicons were cleaned with the enzyme ExoSAP-IT (Affymetrix, Santa Clara, CA, USA) according to the manufacturer's instructions. Both strands were directly sequenced with amplifying primers using the BigDye Terminator v3.1 Cycle Sequencing Kit in a 3130 Genetic Analyzer Sequencer (Applied Biosystems, Waltham, MA, USA) at Postgraduate College facilities, Mexico.

2.4. Phylogenetic

The DNA sequences from both strands were assembled with BioEdit v7.0.5 [32] to create a consensus sequence for each isolate. To compare sequences obtained in this study with those deposited in GenBank, 51 sequences belonging to different *Phytophthora* species were retrieved from this database (Table 1). Multiple sequences were aligned using the option Muscle [33] implemented in Mega v7.0.26 software (Estado de México, H.V.S.-R.) [34] under default parameters. To obtain sequences with the same length, alignment was trimmed at both ends. Sequences derived from this study have been deposited in GenBank (<https://www.ncbi.nlm.nih.gov/WebSub/>, accessed on 23 September 2019).

Bayesian inference (BI) was performed using four Monte Carlo Markov Chains (MCMC) in Mr. Bayes software v.3.2.6 [35]. Two analyses of four MCMC strings from random trees were run for 1,500,000 iterations, and samples were taken every 1000 iterations. In total, 25% of the generated trees were discarded in the burn phase, and posterior probabilities were determined for the remaining trees (75%). Figtree v1.4.4 software was used as a graphic viewer of the resulting tree (<http://tree.bio.ed.ac.uk/software/figtree/>). The phylogenetic consensus tree was based on 51 sequences belonging to different *Phytophthora* species. *Phytophthora vexans* (GenBank accession number AY598713) is the outgroup in the analysis.

2.5. Pathogenicity Test

Actively growing strain PC-C4 mycelial discs were grown on PARPH antibiotic-modified clarified V-8 juice agar plates and incubated at room temperature (24 to 26 °C) for 10 days. Subsequently, from these plates, 10 discs with mycelial growth were removed and placed into transparent 500 mL glass flasks containing V-8 juice liquid medium and incubated at 28 ± 2 °C for 14 days. Sporangia were obtained from 2-week-old cultures as described above. Zoospore concentration was adjusted to 2.5×10^4 zoospores per mL with a hemocytometer.

Twenty 6-month-old cinnamon plants were inoculated by adding 25,000 mL^{-1} zoospores around the roots [24]. Each plant was sown individually in 25 cm diameter \times 30 cm deep plastic pots with a sterilized mixture of peatmoss and agrellite (1:1 v/v). In the case of control plants, sterile distilled water was added. After that, inoculated cinnamon plants were kept in a greenhouse under controlled conditions; during the experiments, the soil moisture remained at 90%. A pathogenicity test was performed twice.

The plants were kept under observation for 60 days [36]. During the first five weeks, the first symptoms of wilting and root rot appeared; after the seventh week, the plants showed tissue death.

Table 1. Database of sequences belonging to different species of *Phytophthora* spp.

Phytophthora Species	Subclade	Strain	Host	Country	GenBank Accession
<i>P. agathidicida</i>	5	ICMP 16471	<i>Agathis australis</i>	New Zealand	KP295318
<i>P. asiatica</i>	7b	Ex-type CPHST BL 124	<i>Pueraria lobata</i>	Japan	MG783378
<i>P. asparagi</i>	6	VHS17644	<i>Dryandra squarrosa</i>	Australia	EU301168
<i>P. cambivora</i>	7a	CBS 111329	<i>Malus pumila</i>	South Korea	KU899158
<i>P. capensis</i>	2c	CBS 128321	<i>Olea capensis</i>	South Africa	NR_147872
<i>P. castaneae</i>	5	Ex-type CPHST BL 47G	<i>Castanea crenata</i>	Japan	MG865470
<i>P. chrysanthemi</i>	9	Ex-type CPHST BL 94	<i>Chrysanthemum</i> sp.	Japan	MG865472
<i>P. cinnamomi</i>	7c	Pc	<i>Quercus</i> sp.	Mexico	FJ361037
<i>P. cinnamomi</i>	7c	CRM-R6A	<i>Vaccinium corymbosum</i>	Mexico	MF536298
<i>P. cinnamomi</i>	7c	Munoz-Perez-001	<i>Persea americana</i>	Mexico	DQ173250
<i>P. cinnamomi</i>	7c	Cerritos	<i>Quercus</i> sp.	Mexico	KP773294
<i>P. cinnamomi</i>	7c	Valle de Bravo	<i>Quercus</i> sp.	Mexico	KP773293
<i>P. cinnamomi</i>	7c	Tecoanapa	<i>Quercus</i> sp.	Mexico	KP773292
<i>P. cinnamomi</i>	7c	Manantlan	<i>Quercus peduncularis</i>	Mexico	KP773291
<i>P. cinnamomi</i>	7c	Arrayanal	<i>Quercus salicifolia</i>	Mexico	KP773290
<i>P. cinnamomi</i>	7c	CPO-PCU	<i>Persea Americana</i>	Mexico	JQ266267
<i>P. cinnamomi</i>	7c	242	<i>River water</i>	USA	KF750569
<i>P. cinnamomi</i>	7c	CBS 144.22	<i>Cinnamomum</i> sp.	Indonesia	KC478663
<i>P. cinnamomi</i>	7c	Ex-type CPHST BL 12	<i>Cinnamomum</i> sp.	Sumatra	MG865473
<i>P. citricola</i>	2c	CBS 221.88	<i>Citrus sinensis</i>	Taiwan	JX545153
<i>P. citricola</i>	2c	CBS 295.29	<i>Citrus</i> sp.	Japan	KC855336
<i>P. citrophthora</i>	2a	CBS 581.69	<i>Hevea brasiliensis</i>	Malaysia	MH401211
<i>P. cocois</i>	5	ICMP 19685	<i>Cocos nucifera</i>	Cote d'Ivoire	KP295306
<i>P. colcasiae</i>	2a	CBS 358.30	<i>Hevea brasiliensis</i>	Sri Lanka	MH401210
<i>P. cryptogea</i>	8a	CBS 418.71	<i>Gerbera</i> sp.	The Netherlands	KX017611
<i>P. drechsleri</i>	8a	CBS 292.35	<i>Beta vulgaris</i>	USA	KJ744314
<i>P. europea</i>	7a	CBS 109051	<i>Quercus</i> sp.	France	KU899157
<i>P. europea</i>	7a	CBS 109049	<i>Quercus</i> sp.	France	NR_147861
<i>P. europea</i>	7a	CBS 109049	<i>Quercus</i> sp.	France	DQ275190
<i>P. heveae</i>	5	CBS 296.29	<i>Hevea brasiliensis</i>	Malaysia	HQ643238
<i>P. ilicis</i>	3	PH046	<i>Ilex aquifolium</i>	Italy	KJ458956
<i>P. morindae</i>	10	Ex-type CPHST BL 49G	<i>Morinda citrifolia</i>	USA	MG865543
<i>P. multivora</i>	2c	CBS 124094	<i>Eucalyptus marginata</i>	Australia	FJ237521
<i>P. nicotianae</i>	1	CBS 535.92	<i>Soil under citrus tree</i>	Unknow	AY946253
<i>P. nicotianae</i>	1	CBS 114343	<i>Piper betel</i>	Unknow	DQ403794
<i>P. niederhauserii</i>	7b	465/10	<i>Acacia dealbata</i>	Italy	JF900371
<i>P. palmivora</i>	4	CBS 148.88	<i>Chamaedorea seifrizii</i>	USA	MH401200
<i>P. palmivora</i>	4	CBS 236.30	<i>Cocos nucifera</i>	India	KY475624
<i>P. palmivora</i>	4	CBS 1113.46	<i>Cymbidium</i> sp.	South Korea	KY475633
<i>P. parvispora</i>	7c	CBS 411.96	<i>Beaucarnea</i> sp.	Germany	KC478672
<i>P. parvispora</i>	7c	CBS 413.96	<i>Beaucarnea</i> sp.	Germany	KC478668
<i>P. parvispora</i>	7c	CBS 132771	<i>Arbutus unedo</i>	Italy	KC478670
<i>P. parvispora</i>	7c	CBS 132772	<i>Arbutus unedo</i>	Italy	KC478667
<i>P. pluvialis</i>	3	3661-NDL-041514	<i>Pseudotsuga menziesii</i>	USA	KM491217
<i>P. pseudosyringae</i>	3	RR4-L4-021712	<i>Notholithocarpus densiflorus</i>	USA	KT719238
<i>P. quercetorum</i>	4	CBS 121119	<i>Quercus rubra</i>	USA	KX759518
<i>P. quercina</i>	4	CBS 789.95	<i>Quercus cerris</i>	Germany	KX062206
<i>P. ramorum</i>	8c	Pr-400	<i>Smilicina racemosa</i>	USA	AY526570
<i>P. stricta</i>	8	Ex-type CPHST BL 127	<i>Surface water</i>	USA	MG865589
<i>P. tropicalis</i>	2b	CBS 434.91	<i>Macadamia integrifolia</i>	USA	DQ464057
<i>P. tropicalis</i>	2b	Ex-type CPHST BL 58	<i>Macadamia integrifolia</i>	USA	MG865596

The causal agent was re-isolated from the inner of the stem, crown, and root in compliance with Koch's postulates, and their identities were confirmed by molecular and morphological tests.

3. Results

3.1. Isolation, Characterization, and Identification

Twenty-three representative isolates from 50 different plants (symptomatic stem, crown, and root) developed white colonies displaying coraloid mycelium on V-8 juice agar medium, with coenocytic hyphal swellings in clusters up to 8 µm wide. Short, ovoid, non-papillary sporangia were observed proliferating through the empty sporangium or occasionally branching.

The length and width of the sporangia varied widely for each isolate, similar to the findings reported by Al-Hedaithy [37]—broadly ellipsoid to ovoid, as shown in Figure 2b (26.8 to 37.9 μm wide, and 35.3 to 66.7 μm long). Terminal spherical or globose chlamydospores of variable size (Figure 2), spherical intercalated and terminal (Figure 2b) with thick walls of 24.3 to 44.6 μm in diameter were identified, which agrees with taxonomic keys proposed by Erwin and Waterhouse [26,38] for the *Cinnamomi* species.

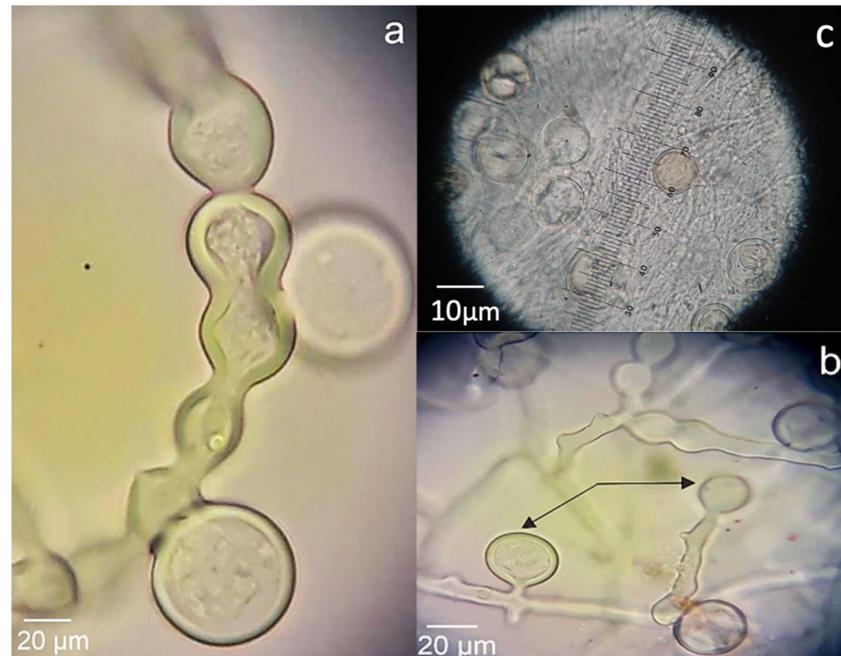


Figure 2. *Phytophthora cinnamomi* oomycete isolated from root rot of *Cinnamomum verum*. (a) Mycelial coenocytic hyphae showing swellings on clarified V-8 juice medium after 7 days, (b) arrow shows intercalated and terminal spherical chlamydospores, and (c) ripe oogonia.

3.2. Phylogenetic Reconstruction

For these analyses, a stem and root rot isolate called PC-C4 (MN497236) originating from the study area of Veracruz and the strain PC-C5 (MN497237) recovered from Koch's postulates were selected. The alignment comprises 53 taxa, including the outgroup, and 978 characters, including a gap. The best evolutionary model for ITS sequences implemented in BI was the General Time Reversible with an Invgamma distribution (GTR + I).

After 1,500,000 iterations, the standard deviation of the split frequencies was 0.008053 with 2252 trees sampled. The consensus tree obtained for the Bayesian analysis showed that the two isolates obtained in this study were grouped with *Phytophthora cinnamomi* subclade 7c with a posterior probability of 100%, and they differed from the reference isolates. *P. cinnamomi* diverged from *P. parvispora*, the most closely related species with 100% support (Figure 3).

3.3. Pathogenicity Tests

Initial symptoms of wilting and decaying were observed in cinnamon plants 30 days after inoculation (DAI). At this time, stem, crown, and root were analyzed, making a cross section, and the infection advanced progressively from the stem to the crown, and the base of the stem became brown with exudate (Figure 4b). Root rot and canker in cinnamon includes cracking of the bark, with bluish depressed necrosis. One discoloration of the roots was observed frequently, with roots turning brown in color. Black necrosis was evident in the roots. In the large roots connected to the collar, generally only the periderm was necrotic. In the smaller roots, the wood was also affected (Figure 4a,b). Bark cankers occurred at the base of the main stem up to 30–50 cm (Figure 4).

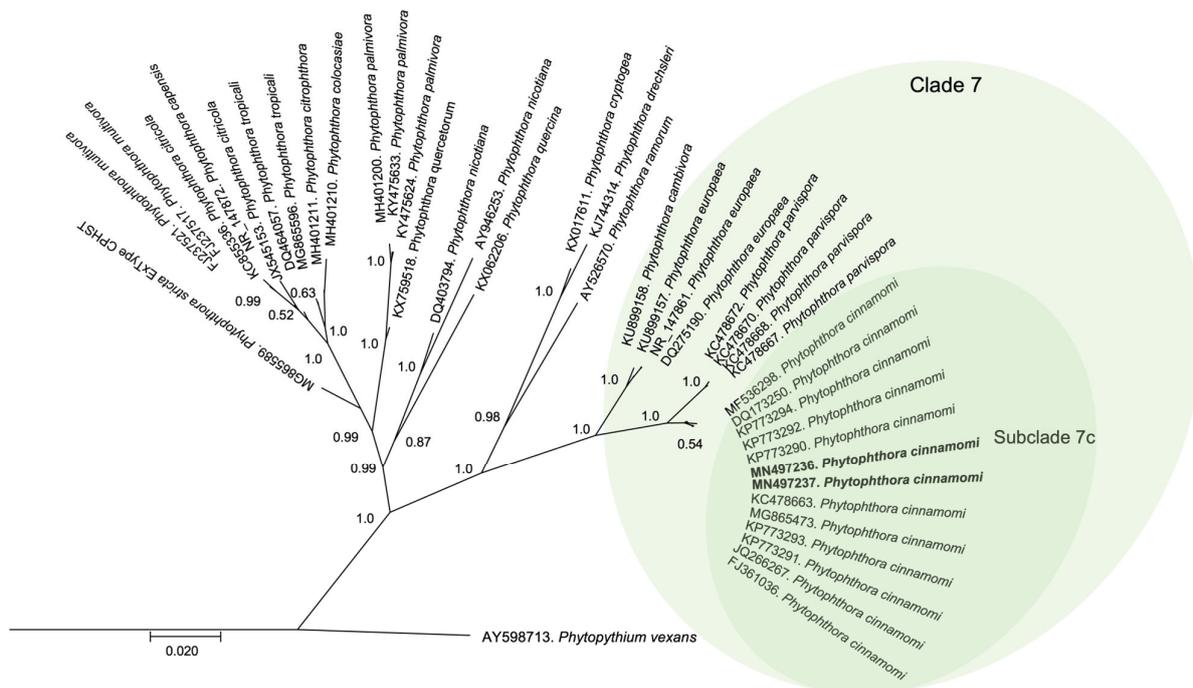


Figure 3. Bayesian phylogenetic tree reconstructed with 16S rDNA sequences from *Phytophthora* species. *Phytophthora vexans* type (AY598713) was used as an outgroup. Bayesian posterior probabilities are shown at nodes, and the scale bar indicates expected changes by site.

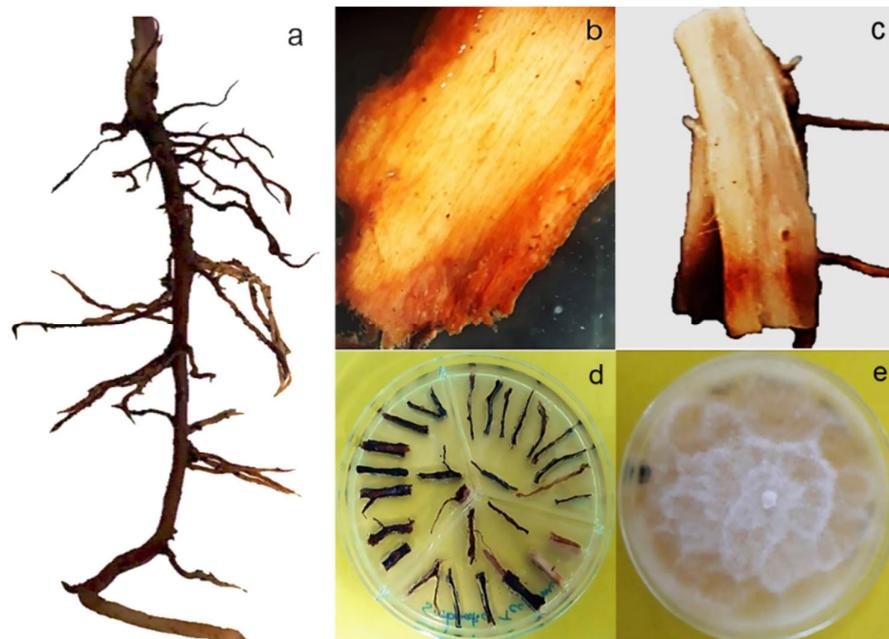


Figure 4. Pathogenicity test on 6-month-old cinnamon plants (*Cinnamomum verum*) artificially inoculated with *Phytophthora cinnamomi* under nursery conditions. (a) Symptoms of root rot and canker showing a dark brown color, (b) crown canker symptoms of the cinnamon plant, (c) internal root rot necrosis, (d) roots with rot symptoms in selective medium PARPH clarified V-8 juice agar, and (e) mycelium of *P. cinnamomi* grown at 25 °C for 8 days.

The humidity conditions of 90% and the temperature of 25 to 28 °C favored the infection in the crown neck or base of the stem, inducing symptoms of canker, the control plants did not present symptoms. The PC-C4 strain was re-isolated from tissues taken from

the stem, crown, and root of diseased plants. The strain was registered in GenBank as strain PC-C5 with accession number MN497237 and included in the phylogenetic reconstruction (Figure 3). Having successfully overcome Koch's postulate, we establish that *P. cinnamomi* is the etiological agent of the disease under study.

4. Discussion

Cinnamon is an introduced valuable aromatic spice originating from Indonesia, where the greatest amount of cinnamon is produced today [39]. Although the production of cinnamon in Mexico is not comparable with China, Sri-Lanka, and Vietnam, the quality of cinnamon obtained—mainly from the inner part of the bark and its quills—has an important commercial value considered in the national market, as well as the diversification of crops to improve the family economy in rural regions [40,41].

It has been mentioned that cinnamon prospers well as a forest tree at 300–350 masl, with a cultivation temperature between 20 and 30 °C, and rainfall between 1250 and 2500 mm. In Mexico, cinnamon plantations are well established at sea level, and it is very well adapted to 300 mm; these areas are characterized by frequent rains that favor the successful growth of the bush and the production of cinnamon bark, as well as agroforestry. However, these prevailing climatic conditions in this area, which favor crop development, also allow soil pathogens to start their infection cycle in agroforestry systems, as determined in the present investigation.

The first evidence of this fact was given by Rands [42], who described the oomycete *Phytophthora cinnamomi* as the causal agent of the stripe cankers on cinnamon plantations from Sumatra, Indonesia [38]. It has since been reported in an ample host range over 5000 plant species in 319 genera [11]. The spread of *P. cinnamomi* primarily found in the southern hemisphere has been considered as indigenous in tropical and subtropical areas, and it is currently found in 56 countries around the world (Figure 5).

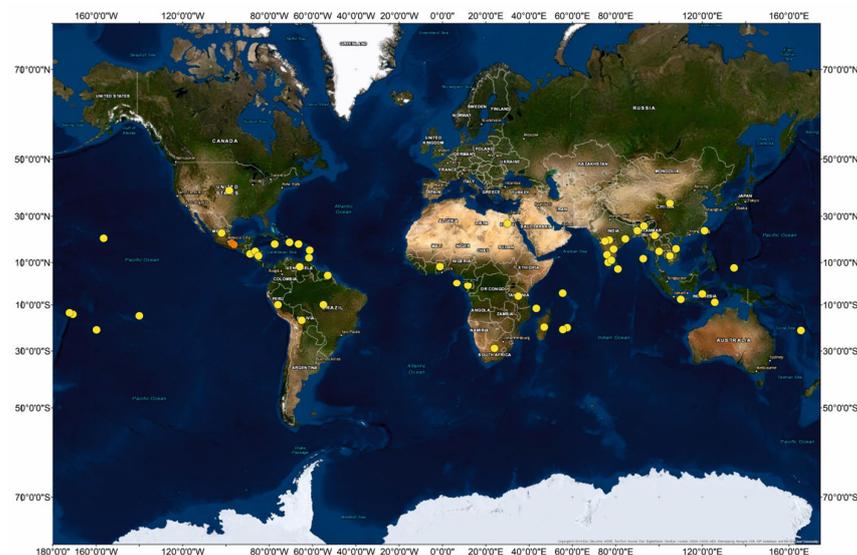


Figure 5. Distribution reports of *P. cinnamomi* in tropical and subtropical areas of the southern hemisphere in 56 countries. For Mexico they are observed in orange color.

In cinnamon plantations located in lowland Mexico, the dieback symptom began with top-down leaf chlorosis, then rot, depressed areas, inner bark necrosis, and root rot. From these symptoms, the oomycete *P. cinnamomi* was consistently isolated in V-8 clarified juice medium and identified by its morphological structures.

A sophisticated phylogeny based on the sequences of seven nuclear genetic markers including ITS divided 82 *Phytophthora* species into 10 phylogenetically well-supported clades [18,43]. The results of the phylogenetic reconstruction performed in this study using sequences belonging to the ITS region identified the isolates analyzed here as *P. cinnamomi*,

a member of *Phytophthora* subclade 7c; they were very well differentiated from *P. parvispora*, which was previously considered a subspecies of *P. cinnamomi*.

Thus, we decided to use Bayesian analysis to carry out the phylogenetic approach, using ITS sequences combined with morphological traits, where *P. cinnamomi* was clearly identified as the causal agent of dieback in cinnamon plantations in the Hidalguense region of Totonacapan, Veracruz, Mexico—this oomycete is included as one of the most destructive pathogens [12]. In addition, it has been reported to affect members of two different genera in Mexico, such as *Persea americana* [15,44], *P. schiedeana* [15], *Quercus glaucooides*, *Q. peduncularis*, and *Q. salicifolia* [45].

With the advent of molecular-based techniques, the systematic and evolutionary understanding of the *Phytophthora* genus has advanced, and the detection and description of new *Phytophthora* species have become a priority [46]. This work represents the first report of *P. cinnamomi* as responsible for the symptoms observed in lowland cinnamon plantations in Mexico. Therefore, we suggest that management strategies should be implemented to avoid its introduction into nurseries or new plantations that could be susceptible to this pathogen.

5. Conclusions

This study provides evidence for and confirms the widespread presence of the wide distribution of *Phytophthora cinnamomi* subclade 7c in plantations in the Hidalguense region of Totonacapan, Veracruz, Mexico. Our findings suggest that this phytopathogen is a new threat to cinnamon growers—presenting 40% of regressive death, chlorosis, and crown and root rot. We recommend that growers implement management strategies to avoid its introduction into nurseries or new plantations that could be susceptible to this pathogen.

Author Contributions: Conceptualization, P.A.-H. and O.R.-A.; methodology, P.A.-H. and H.V.S.-R.; software, H.V.S.-R.; validation, O.R.-A., J.A.U.-V. and A.L.-C.; formal analysis, P.A.-H., A.M.-M. and O.R.-A.; investigation, P.A.-H. and O.R.-A.; resources, P.A.-H., H.V.S.-R. and O.R.-A.; data curation, J.E.-P.; writing—original draft preparation, P.A.-H. and O.R.-A.; writing—review and editing, H.V.S.-R.; visualization, A.M.-M.; supervision, O.R.-A.; project administration, P.A.-H.; funding acquisition, P.A.-H. and O.R.-A. All authors have read and agreed to the published version of the manuscript.

Funding: This work was financed through the National Institute of Agricultural and Livestock Forestry Research (INIFAP), Campo Experimental Zacatepec, Morelos, Mexico, identification number ID: 4119-AAh.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Informed consent was obtained from all subjects involved in the study.

Acknowledgments: The authors are grateful to cinnamon producers from Veracruz and Puebla states, Mexico, for the information provided for this study. In addition, we would like to thank the Laboratory of Seed Biotechnology and Plant Pathology of the Postgraduate College at Montecillo Campus for allowing us the use of the sequencing facility unit.

Conflicts of Interest: All authors declare no conflict of interest.

References

1. Xavier, J.K.A.M.; Baia, T.G.C.; Alegria, O.V.C.; Figueiredo, P.L.B.; Carneiro, A.R.; Moreira, E.C.d.O.; Maia, J.G.S.; Setzer, W.N.; da Silva, J.K.R. Essential Oil Chemotypes and Genetic Variability of *Cinnamomum verum* Leaf Samples Commercialized and Cultivated in the Amazon. *Molecules* **2022**, *27*, 7337. [CrossRef]
2. Ministry of Development Strategies and International Trade (MDSIT) and Sri Lanka Export Development Board (SLEDB). *Government of Sri Lanka National Export Strategy of Sri Lanka—Spices and Concentrates Strategy. 13–14*; Government of Sri Lanka: Sri Jayawardenepura Kotte, Sri Lanka, 2018.
3. Singh, N.; Rao, A.S.; Nandal, A.; Kumar, S.; Yadav, S.S.; Ganaie, S.A.; Narasimhan, B. Phytochemical and pharmacological review of *Cinnamomum verum* J. Presl—a versatile spice used in food and nutrition. *Food Chem.* **2021**, *338*, 127773. [CrossRef] [PubMed]

4. Chen, P.; Jianghao, S.; Ford, P. Differentiation of the four major species of *Cinnamons* (*C. burmannii*, *C. verum*, *C. cassia*, and *C. loureiroi*) using a flow injection mass spectrometric (FIMS) fingerprinting method. *J. Agric. Food Chem.* **2014**, *62*, 2516–2521. [CrossRef]
5. Sadeghi, S.; Davoodvandi, A.; Pourhanifeh, M.H.; Sharifi, N.; ArefNezhad, R.; Sahebnaasagh, R.; Moghadam, S.A.; Sahebkar, A.; Mirzaei, H. Anti-cancer effects of cinnamon: Insights into its apoptosis effects. *Eur. J. Med. Chem.* **2019**, *178*, 131–140. [CrossRef] [PubMed]
6. Geethakumary, M.P.; Pandurangan, A.G.; Santhoshkumar, E.S. *Cinnamomum litseaefolium* (Lauraceae)—A new distributional record for India. *Rheedea* **2012**, *22*, 127–130.
7. Ananthkrishnan, R.; Santhoshkumar, E.S.; Rameshkumar, K.B. Comparative chemical profiles of essential oil constituents of eight wild *Cinnamomum* species from the Western Ghats of India. *Nat. Prod. Commun.* **2018**, *13*, 1934578X1801300525. [CrossRef]
8. Menggala, S.R.; Damme, P.V. Improving *Cinnamomum burmannii* Blume value chains for farmer livelihood in Kerinci, Indonesia. *Eur. J. Med. Nat. Sci.* **2022**, *5*, 74–103.
9. Rajapakse, R.H.S.; Kumara, K.L.W. A Review of identification and management of pests and diseases of cinnamon (*Cinnamomum zeylanicum* Blume). *Trop. Agric. Res. Ext.* **2007**, *10*, 1–10. [CrossRef]
10. Khan, A.U.; Khan, A.U.; Khanal, S.; Gyawali, S. Insect pests and diseases of cinnamon (*Cinnamomum verum* Presl.) and their management in agroforestry system: A review. *Acta Entomol. Zool.* **2020**, *1*, 51–59. [CrossRef]
11. Farr, D.F.; Rossman, A.Y. Fungal Databases, U.S. National Fungus Collections, ARS, USDA. 2019. Available online: <https://nt.ars-grin.gov/fungalDATABASES/> (accessed on 8 October 2022).
12. ISSG. Global Invasive Species Database (GISD). Invasive Species Specialist Group of the IUCN Species Survival Commission. 2012. Available online: <http://www.issg.org/database> (accessed on 23 January 2023).
13. Engelbrecht, J.; Duong, T.A.; Berg, N.v.d. New microsatellite markers for population studies of *Phytophthora cinnamomi*, an important global pathogen. *Sci. Rep.* **2017**, *7*, 17631. [CrossRef]
14. Zentmyer, G.A. Origin and distribution of four species of *Phytophthora*. *Trans. Br. Mycol. Soc.* **1988**, *91*, 367–378. [CrossRef]
15. Spaulding, P. Foreign Diseases of Forest Trees of the World. In *Agriculture Handbook*; U.S. Department of Agriculture: Washington, DC, USA, 1961; Volume 197, pp. 1–361.
16. Oudemans, P.; Coffey, M.D. Isozyme comparison within and among worldwide sources of three morphologically distinct species of *Phytophthora*. *Mycol. Res.* **1991**, *95*, 19–30. [CrossRef]
17. Martin, F.N.; Blair, J.E.; Coffey, M.D. A combined mitochondrial and nuclear multilocus phylogeny of the genus *Phytophthora*. *Fungal Genet. Biol.* **2014**, *66*, 19–32. [CrossRef] [PubMed]
18. Yang, X.; Tyler, B.M.; Hong, C. An expanded phylogeny for the genus *Phytophthora*. *IMA Fungus* **2017**, *8*, 355–384. [CrossRef]
19. Zhuang, W.Y. *Higher Fungi of Tropical China*; Mycotaxon, Ltd.: Ithaca, NY, USA, 2001; 485p.
20. Jung, T.; Jung, M.H.; Cacciola, S.O.; Cech, T.; Bakonyi, J.; Seress, D.; Scanu, B. Multiple new cryptic pathogenic *Phytophthora* species from Fagaceae forests in Austria, Italy and Portugal. *Ima Fungus* **2017**, *8*, 219–244. [CrossRef] [PubMed]
21. FAOSTAT. Food and Agriculture Organization of the United Nations. Statistical, Agricultural Production. 2019. Available online: <https://www.fao.org/faostat/en/#data/QC> (accessed on 12 September 2019).
22. García, E. *Modificaciones al Sistema de Clasificación Climática de Köppen*, 5th ed.; Instituto de Geografía, Universidad Autónoma de México: México City, México, 2004; pp. 11–90.
23. Morales-Mora, L.A.; Andrade-Hoyos, P.; Valencia-de Ita, M.A.; Romero-Arenas, O.; Silva-Rojas, H.V.; Contreras-Paredes, C.A. Characterization of strawberry associated fungi and in vitro antagonistic effect of *Trichoderma harzianum*. *Rev. Mex. Fitopatol.* **2020**, *38*, 434–449. [CrossRef]
24. Andrade-Hoyos, P.; De León, C.; Molina-Gayosso, E.; Espíndola, B.M.C.; Alvarado, R.D.; López, J.A. Totipotency in avocado seedling resistance to *Phytophthora cinnamomi*. *Rev. Mex. Cienc. Agrícolas* **2015**, *6*, 361–373. [CrossRef]
25. Belisle, R.J.; McKee, B.; Hao, W.; Crowley, M.; Arpaia, M.L.; Miles, T.D.; Manosalva, P. Phenotypic characterization of genetically distinct *Phytophthora cinnamomi* isolates from avocado. *Phytopathology* **2019**, *109*, 384–394. [CrossRef]
26. Erwin, D.; Ribeiro, O. *Phytophthora Diseases Worldwide*; American Phytopathological Society Press: St. Paul, MN, USA, 1996.
27. Gallegly, M.E.; Hong, M. *Phytophthora: Identifying Species by Morphology and DNA Fingerprints*. American Phytopathological Society Press: St. Paul, MN, USA, 2008.
28. Wang, Y.; Sun, Y.; Zhang, Y.; Zhang, X.; Feng, J. Antifungal activity and biochemical response of cuminic acid against *Phytophthora capsici* Leonian. *Molecules* **2016**, *21*, 756. [CrossRef]
29. Doyle, J.J.; Doyle, J.L. Isolation of plant DNA from fresh tissue. *Focus* **1990**, *12*, 13–15.
30. White, T.J.; Bruns, T.; Lee, S.; Taylor, J.W. 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.; Academic Press: New York, NY, USA, 1990.
31. Cooke, D.E.L.; Drenth, A.; Duncan, J.M.; Wagels, G.; Brasier, C.M. A molecular phylogeny of *Phytophthora* and related oomycetes. *Fungal Genet. Biol.* **2000**, *30*, 17–32. [CrossRef]
32. Hall, T.A. BioEdit: A user friendly biological sequence alignment editor and analysis program for windows 95/98/NT. *Nucleic Acids Symp. Ser.* **1999**, *41*, 95–98.
33. Edgar, R.C. MUSCLE: Multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res.* **2004**, *32*, 1792–1797. [CrossRef] [PubMed]

34. Kumar, S.; Stecher, G.; Tamura, K. MEGA7: Molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Mol. Biol. Evol.* **2016**, *33*, 1870–1874. [[CrossRef](#)]
35. Ronquist, F.; Teslenko, M.; van der Mark, P.; Ayres, D.L.; Darling, A.; Höhna, S.; Larget, B.; Liu, L.; Suchard, M.A.; Huelsenbeck, J.P. MrBayes 3.2: Efficient Bayesian phylogenetic inference and model choice across a large model space. *Syst. Biol.* **2012**, *61*, 539–542. [[CrossRef](#)]
36. Andrade-Hoyos, P.; Silva-Rojas, H.; Romero-Arenas, O. Endophytic *Trichoderma* Species Isolated from *Persea americana* and *Cinnamomum verum* Roots Reduce Symptoms Caused by *Phytophthora cinnamomi* in Avocado. *Plants* **2020**, *9*, 17. [[CrossRef](#)] [[PubMed](#)]
37. Al-Hedaithy, S.S.; Tsao, P.H. Sporangium pedicel length in *Phytophthora* species and the consideration of its uniformity in determining sporangium caducity. *Trans. Br. Mycol. Soc.* **1979**, *72*, 1–13. [[CrossRef](#)]
38. Waterhouse, G.M.; Waterston, J.M. *Phytophthora cinnamomi*. In *CMI Descriptions of Pathogenic Fungi and Bacteria*; CABI Digital Library: Boston, MA, USA, 1966; Volume 113, pp. 1–2.
39. FAOSTAT. Food and Agriculture Organization of the United Nations. Statistical, Agricultural Production. 2022. Available online: <https://www.fao.org/faostat/en/#data/QC> (accessed on 23 January 2023).
40. Hagggar, J.; Ayala, A.; Díaz, B.; Reyes, C.U. Participatory design of agroforestry systems: Developing farmer participatory research methods in Mexico. *Dev. Pract.* **2001**, *11*, 417–424. [[CrossRef](#)]
41. Hoffmann, H.; Uckert, G.; Sieber, S.; Fasse, A. Development and adjustment of sustainability indicators to evaluate outgrower schemes in bioenergy production: The case of Tanzania. In Proceedings of the 9th European IFSA Symposium, Vienna, Austria, 4–7 July 2010; pp. 4–7.
42. Rands, R.D. Streepkanker van Kaneel, Veroorzaakt door *Phytophthora cinnamomi* n. sp. [Stripe canker of cinnamon caused by *Phytophthora cinnamomi* n. sp.]. *Mededeelingen Inst. Plantenziekten* **1922**, *54*, 53.
43. Blair, J.E.; Coffey, M.D.; Park, S.Y.; Geiser, D.M.; Kang, S. A Multilocus phylogeny for *Phytophthora* utilizing markers derived from complete genome sequences. *Fungal Genet. Biol.* **2008**, *45*, 266–277. [[CrossRef](#)] [[PubMed](#)]
44. McGuire, J.U., Jr.; Crandall, B.S. *Survey of Insect Pests and Plant Diseases of Selected Food Crops of Mexico, Central America and Panama*; International Agricultural Development Service, U.S. Dept. of Agriculture: Washington, DC, USA, 1967; 157p.
45. Tainter, F.H.; O'Brien, J.G.; Hernandez, A.; Orozco, F.; Rebolledo, O. *Phytophthora cinnamomi* as a cause of oak mortality in the state of Colima, Mexico. *Plant Dis.* **2000**, *84*, 394–398. [[CrossRef](#)] [[PubMed](#)]
46. Jung, T.; Jung, M.H.; Scanu, B.; Seress, D.; Kovacs, G.M.; Maia, C.; Perez-Sierra, A.; Chang, T.-T.; Chandelier, A.; Heungens, K.; et al. Six new *Phytophthora* species from ITS clade 7a including two sexually functional heterothallic hybrid species detected in natural ecosystems in Taiwan. *Persoonia* **2017**, *38*, 100–135. [[CrossRef](#)] [[PubMed](#)]

Disclaimer/Publisher's Note: The statements, opinions and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions or products referred to in the content.