RESEARCH ARTICLE

Phytophthora species causing root rot in avocado seedlings at Colombian nurseries: Morphological, molecular, and pathogenic analysis

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Abstract

Expansion of avocado production areas in Colombia has led to an increased demand for plant propagation material. However, this expansion has exacerbated phytosanitary challenges, particularly root rot disease mainly associated with *Phytophthora* spp. Therefore, this study aimed to identify *Phytophthora* species associated with root rot in avocado seedlings within nurseries. Avocado plants exhibiting wilting symptoms were collected from nurseries in the departments of Quindío, Risaralda, and Valle del Cauca (Colombia). Segments of diseased roots were selected, cut, and surface-disinfected, before being planted on Potato Dextrose Agar (PDA) supplemented with antibiotics and fungicides. Microorganism identification was conducted using taxonomic keys and confirmed by molecular techniques employing the identification based on phylogenetic hypothesis, using ITS1-5.8s-ITS2 region encoding for rRNA. Isolates obtained from necrotic avocado roots were identified as *P. cinnamomi* and *P. heveae*. The pathogenicity of the isolates was confirmed in avocado seedlings through inoculation, resulting in symptom reproduction. Consequently, this study identified *P. cinnamomi* and *P. heveae* as causal agents of root rot in avocado during the nursery stage.

Keywords: PCR; *Phytophthora cinnamomi*; *P. heveae*; *Persea americana*; wilting.

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1. Introduction

The primary limitation for avocado (*Persea americana* Mill.) production worldwide are root diseases. Among the pathogens associated with this issue is *Phytophthora cinnamomi* Rands (Oomycota: Peronosporales: Peronosporaceae) (Álvarez et al., 2023). This pathogen has been globally described as causal agent of root rot, a symptom that triggers chlorosis in leaves and plant wilting. The disease progresses with defoliation and dieback of the tree, leading to a reduction in production (Kavroulakis et al., 2024).

Phytophthora cinnamomi has severely affected avocado orchards in Florida and California (USA), causing economic losses of nearly USD $40 million (Belisle et al., 2019). In Greece, the first report of this disease was made in 2024, where it showed a high mortality rate (Kavroulakis et al., 2024). While in Mexico, it caused the death of 100,000 'Hass' avocado trees in Uruapan, Michoacán, with

producers facing economic losses exceeding 32 million pesos (Agapito et al., 2022). *Phytophthora cinnamomi* has also been identified as the causative agent of avocado root rot in various regions and countries, including North America, Latin American, Australia, New Zealand, South Africa, Spain, Morocco, and Israel (Agapito et al., 2022).

In Colombia, avocado crop has experienced a rapid surge in planting areas, escalating from 36,461 ha to 110,183 ha between 2015 and 2022 (FAOSTAT, 2024). The expansion of cultivation areas in this country has been propelled by the goal of positioning itself as a leading exporter of Hass avocados in international markets (Álvarez et al., 2023).

The Root rot caused by *P. cinnamomi* has been reported in several avocado-producing departments at Colombia. This pathogen causes losses in commercial crops ranging from 45% to 90% (Ramírez-Gil, 2018). During the nursery stage an incidence of 28 % was recorded, while mortality, measured as

the number of plants that died due to the disease, reached 60% (Ramírez-Gil et al., 2017). Conditions in nurseries, such as reduced spacing, constant irrigation, high humidity, and excessive use of fertilizers, create an environment conductive to the onset of root rot, leading to the propagation of plant material with low physiological and phytosanitary quality (Ramírez-Gil, 2018).

The expansion of avocado cultivation areas conducts a series of technological challenges related to phytosanitary management, where the health of planting material is crucial to ensure the sustainability of the crop and responding to the demands of producers (Ramírez-Gil, 2018). Therefore, the aim of this study was to identify, through morphological, molecular, and pathogenic analyses, *Phytophthora* species causing root rot in avocado plants in nurseries. This represents a crucial initial step in establishing integrated management alternatives that are economically viable, socially responsible, and environmentally sustainable.

2. Methodology

2.1 Study Area and Sample Collection

Sampling was carried out in 30 avocado-producing nurseries, with 16 located in the Valle del Cauca department, nine in Quindío, and six in Risaralda (Colombia). A total of 138 plants were collected under a collection permit issued by the National Environmental Permits Authority of Colombia (Resolution No. 1466 - December 3, 2014, https://doi.org/10.15472/gcavk3). The avocado plants collected in nurseries showed symptoms such as aerial wilting, chlorosis, and partial root rot, accompanied by a scant volume of roots (Figure 1). The collected samples were processed at Agricultural Microbiology Laboratory of the *Corporación Colombiana de Investigación Agropecuaria - AGROSAVIA, Centro de Investigación Palmira*.

2.2 Isolation of microorganisms

Avocado roots were first washed with tap water to remove excess soil. Subsequently, 0.5 cm pieces were cut, incorporating both healthy and necrotic tissue. The tissue underwent superficial disinfection with 0.1% sodium hypochlorite for 45 seconds, followed by 70% alcohol for one minute, and then rinsed three times with sterile distilled water. The tissue was placed on sterile paper, air-dried, and then planted on Potato Dextrose Agar (PDA) supplemented with antibiotics and fungicides, following the method described by Tsao & Guy (1983).

Isolates were purified by hyphal tip method (Abad et al., 2023).

2.3 Morphological characterization

Morphological identification of the isolates was carried out using the taxonomic keys in the IDphy tool (https://idtools.org/tools/1056/index.cfm) (Abad et al., 2023). The isolates were morphologically characterized on PDA medium, where macroscopic features such as the type of mycelial growth, colony color, and shape were assessed.

The microscopic characterization of the isolates was performed using a Nikon ECLIPSE Ci-L microscope, image capture was carried out with the Nikon DS-Ri2 camera, and the NIS-Elements D software was used. Microscopic characteristics including hyphae, chlamydospore shape, and sporangial features such as shape, dimensions (length and width), number of papillae, and type of papillation were also evaluated. For homothallic isolates, the formation of oogonia, along with their size, shape, and surface characteristics were assessed.

2.4 DNA Extraction and PCR

For molecular identification, total genomic DNA was extracted from mycelium grown on PDA medium using the protocol of Raeder & Broda (1985), whit some modifications as described by Ángel-García et al. (2023). The region ITS1-5.8s-ITS2, encoding for rRNA, was amplified by Polymerase Chain Reaction (PCR) using primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (White et al., 1990). Each reaction was performed with 10 ng of genomic DNA, 1X Taq Buffer with KCl, 1.5 mM MgCl2, 0.2 mM dNTPs, 0.2 µM of each primer, 1U Taq DNA polymerase (recombinant) (Thermo Scientific), for a final volume of 25 µl. The PCR was carried out in an Agilent SureCycler 8800 thermocycler with the following thermal profile: an initial cycle at 94 °C for 5 min, followed by 35 cycles at 94 °C for 45 seconds, 55 °C for 30 seconds, 72 °C for 1 min, and a final step at 72 °C for 10 min.

2.5 Sequencing and phylogenetic analysis

PCR products were sequenced in both forward and reverse directions using the Sanger method. Sequence cleaning and assembly were carried out using Geneious 2020.2.1 software. Alignment was conducted with Muscle v3.8.1551 software. Representative ex-type sequences of *Phytophthora* species reported worldwide, belonging to different clades, were included. Sequences were retrieved from GenBank database as reported by Abad et al. (2023).

Figure 1. Symptoms observed in sampled West Indian avocado plants in nurseries; a) Generalized wilting of the plant; b) Aerial wilting, chlorosis, and dry leaves; c) and d) Partial necrosis of roots. Photographs: Research Group of AGROSAVIA, Centro de Investigación Palmira.

The optimal base substitution model for the data was determined using the ModelTest-NG software version 0.1.6 employing the Bayesian information criterion (BIC). Construction of the Phylogenetic tree was executed by Maximum Likelihood method using PhyML software v3.3.20190909. The internal topology support of the dendrograms was assessed through bootstrap analysis with 1,000 replications. Visualization of the Phylogenetic tree was performed in R software using ggtree library.

2.6 Pathogenicity tests

Pathogenicity tests were conducted on three-month-old Hass avocado plants derived from seeds and maintained under plastic cover and shade with a 50% reduction in light. The isolates were cultured on PDA medium and incubated for 15 days at 28 °C. Inoculation was carried out using the stem-wounding method, following the procedure outlined by Dixon et al. (1984): cuts were made 8 cm from the base of the stem, where a 5 mm Ø PDA disk with mycelial growth of the isolate was inserted and covered with Parafilm (Figure 2). For the control treatment, PDA disks without mycelial growth were used.

The experiment was arranged in a completely randomized design (CRD) with one plant as experimental units and six replications assigned to each treatment (isolate).

Figure 2. Diagram of the pathogenicity test.

Evaluations were conducted at 3, 6, 9, 12, 15, 18, 21, 24 and 27 days a post-inoculation (dpi), determining the degree of severity based on the visual symptom scale designed for *P. cinnamomi* in Hass avocado by Ramírez & Morales (2020), the size of the necrotic lesions was also recorded and the Area Under the Disease Progress Curve (AUDPC) was determined. To confirm Koch's postulates, necrotic stem tissue was cultured on PDA medium, and microscopic observations were conducted for isolate identification. An analysis of variance (ANOVA) and mean comparison using the Duncan test (p < 0.05) were performed with the average ABCPE using R software, Version: 4.3.2.

The trial was conducted under aphid-proof mesh house conditions, with periodic watering every three days. The relative humidity ranged between 46.9% and 59.7%, and temperatures fluctuated between 24.6 °C and 26.3 °C. Environmental conditions were monitored using a WatchDog meteorological station, programmed to record variables every 30 minutes for 27 days.

3. Results and discussion

Isolation of microorganisms

Out of the 138 processed avocado plants, 11 isolates of *Phytophthora* sp. were obtained, with 10 originating from nurseries in Valle del Cauca and one from a nursery in Quindío (Table 1). This study found a low frequency of *Phytophthora* sp. isolates, a finding consistent with reports by Saltarén et al. (1998). However, these results differ from those obtained by Ramírez-Gil et al. (2017), who identified *P. cinnamomi* as the main causal agent of the wilting complex in avocado plants in nurseries in the department of Antioquia, with a frequency of 45 %, followed by abiotic problems such as hypoxia and anoxia. The scarce presence of *Phytophthora* sp. in the analyzed nurseries could be related to the employed management practices, including the use of sterile substrates and the application of active ingredients such as Dazomet for substrate disinfection, aimed at controlling fungi and oomycetes

(Villarino et al., 2021). Additionally, the use of biological products based on *Trichoderma* spp. may impact colony-forming units of *Phytophthora* sp. (Leal et al., 2014). Likewise, it's conceivable that the sampled avocado plants in the nurseries may be affected by other causal agents associated to root rot (Parkinson et al., 2017).

Morphological characterization

Out of the 11 isolates of *Phytophthora* sp. obtained from necrotic roots of avocado plants produced in the nurseries, six isolates were selected for morphological and molecular identification as well as pathogenic characterization. Morphological characterization revealed that three isolates were identified as *P. cinnamomi* (Per2PH, Profru1PH, Ses4PH) and three isolates as *P. heveae* A.W. Thomps. (Profru2PH, Profru4PH, Rena1PH).

Distinctive patterns were observed between the two *Phytophthora* species through morphological analysis. *Phytophthora cinnamomi* showed colonies with trailing mycelium, petaloid pattern, the presence of hyphal swellings and chlamydospores, unbranched sporangiophores, and non-papillate sporangia, with ellipsoidal or lemon-shaped forms (Figure 3), with lengths between 48–60 µm (Table 1). *Phytophthora heveae* displayed colonies with aerial mycelium, absence of hyphal swellings or chlamydospores, its sporangiophores have a simple sympodial branching pattern, with papillate sporangia (1 to 2 papillae) of globular or ovoid shape (Figure 4) and with lengths between 34–39 µm (Table 1), making them smaller compared to the sporangia of *P. cinnamomi*. These observed characteristics align with previous reports by Abad et al. (2023).

Analysis phylogenetic

Phytophthora spp. molecular identification was carried out by amplifying the ITS region of ribosomal DNA using the primers ITS1 and ITS4. The six consensus sequences generated in this study have been deposited in GenBank for reference and public access.

Figure 3. Macroscopic and microscopic appearance of *P. cinnamomi* on PDA medium. a) Trailing white mycelium with a petaloid pattern; b) Hyphal swellings (40x); c) Globose chlamydospores with thin walls (40x); d) Non-papillate ellipsoidal sporangium (40x). Photographs: Research Group of AGROSAVIA, Centro de Investigación Palmira.
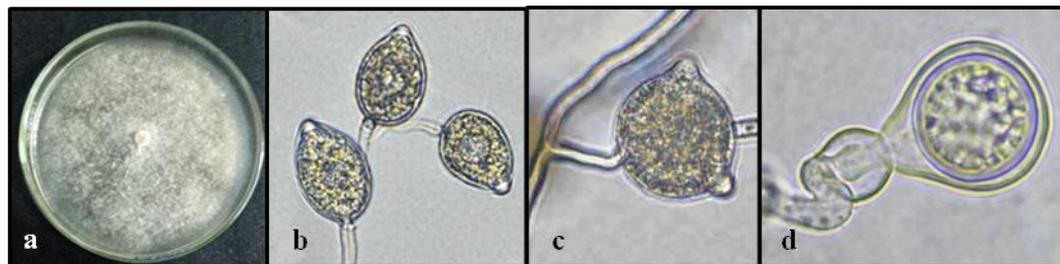
Figure 4. Macroscopic and microscopic appearance of *P. heveae* on PDA medium. a) Trailing white mycelium without a characteristic pattern; b) Sporangiophore with simple sympodial branching and ovoid papillate sporangia (40x); c) Globose sporangium with two papillae (40x); d) Globose oogonium with a conical base and round oospore, amphigynous antheridium (100x). Photographs: Research Group of AGROSAVIA, Centro de Investigación Palmira.

Table 1

Macro and microscopic characterization of isolates of *P. cinnamomi* and *P. heveae*

Isolated	Location	Phytophthora specie	Colony			Asexual phase									Sexual phase		
			CP	TC	CC	HS	FC	SF	SP	NP	SL	SW	SH	ER	OW	AA	TO
Per2PH	Valle del Cauca	*P. cinnamomi*	P	C	W	HS	CH	UN	NP	0	49,9	33	EL	HT	-	-	-
Profru1PH	Valle del Cauca	*P. cinnamomi*	NP	C	W	HS	CH	UN	NP	0	57,3	33,4	EL	HT	-	-	-
Ses4PH	Valle del Cauca	*P. cinnamomi*	P	C	W	HS	CH	UN	NP	0	48,2	32,7	EL	HT	-	-	-
Profru2PH	Valle del Cauca	*P. heveae*	NP	A	W	-	-	SS	P	2	37,4	24,8	GO	HO	S/tb	A	21,9
Profru4PH	Valle del Cauca	*P. heveae*	NP	A	W	-	-	SS	P	2	34,2	24,6	GO	HO	S/tb	A	20,3
Rena1PH	Quindío	*P. heveae*	NP	A	W	-	-	SS	P	1	38,3	25,1	OI	HO	S/tb	A	20

CP Colony Pattern NP = Not Present, P = Petaloid, TC Colony growth type C = Creeping, A = Aerial, CC Colony color W= White, HS Formation of hyphal swellings HS = formed,"-" = not formed., FC Formation of chlamydospores CH = formed, CH/- = sometimes formed, and "-" = not formed., SF Sporangiophore form SS = Simple sympodial, UN = unbranched, SP Sporangia papillae P = papillate, SP = semi-papillate, NP = non-papillate., NP Number of papillae 0 = No papillae, 1 = one papillae, 2 = one and two papillae, SL Sporangia length (µm), SW Sporangia width (µm), SH Sporangia shape E= ellipsoid, O = ovoid, L = limoniform, G = globose, I = irregular, RE Reproductive strategy HO = homothallic and HT = heterothallic., OW Oogonial wall S/tb = smooth wall and tapered base, "-" = was not determined, AA Antheridial attachment P = paragynous, A = amphigynous, and "-" = was not determined, OS Oospore size (diameter µm), and "-" = was not determined.

For *P. cinnamomi*, the accession numbers OR378296, OR378297, and OR378303 were assigned for Per2PH, Profru1PH and Ses4PH, respectively. For *P. heveae*, the accession numbers OR378298, OR378299 and OR378301 were assigned for Profru2PH, Profru4PH and Rena1PH, respectively.

Phylogenetic analyses of the complete ITS region dataset for three isolates of *P. cinnamomi* showed a clear clustering with the ex-type strain CPHST BL 12 (MG865473) and different isolates obtained from avocado plants (**Figure 5**). Three isolates clustered with the ex-type strain of *P. heveae* (MH136899), alongside isolates from Ecuador, Guatemala, and Hawaii. The constructed tree exhibited a similar topology and high cladistic consistency for Phytophthora species, where *P. cinnamomi* is included in clade 7a and a *P. heveae* belongs to clade 5 (**Abad et al., 2023**; **Lanete et al., 2023**).

Pathogenicity tests

In the pathogenicity tests conducted with isolates of *P. cinnamomi* and *P. heveae* on Hass avocado seedlings, symptoms similar to those observed in nurseries were reproduced. Symptoms of infection and pathogen colonization during the first six days post-infection (dpi) manifested as wilting and chlorosis of the seedlings (**Figure 6a and c**). Between nine and 12 dpi, the seedlings developed symptoms of wilting and downward death (**Figure 6b and d**). Plants in the control treatment showed no symptoms and continued with normal growth throughout the evaluation period (**Figure 6e**). These results are consistent with those reported by **Marulanda (2018)**, who evaluated a collection of *P. cinnamomi* isolates in Hass avocado plants using the stem wound method, where symptoms described as wilting, and chlorosis were observed between the third and sixth dpi. *P. cinnamomi* and *P. heveae* were re-isolated from necrotic lesions, and in all cases, the identity of the oomycetes was confirmed, supporting the pathogenic nature of both Phytophthora species in avocado plants.

The analysis of variance conducted on the AUDPC of the six evaluated isolates showed highly significant differences ($p < 0.001$), confirming variability in the pathogenicity of the *Phytophthora* spp. isolates. Three groups of means with highly significant differences ($p < 0.001$) were formed, as depicted in **Figure 7**. Isolate Per2PH (*P. cinnamomi*) exhibited higher AUDPC and necrotic lesions area than other isolates, thereby emerging as the most virulent in this study. Profru2PH (*P. heveae*), exhibited an average AUDPC of 2,117 and an average necrotic lesion area of 10.1 cm², did not differ from isolates Rena1PH (*P. heveae*), Ses4PH (*P. cinnamomi*), and Profru1PH (*P. cinnamomi*) means.
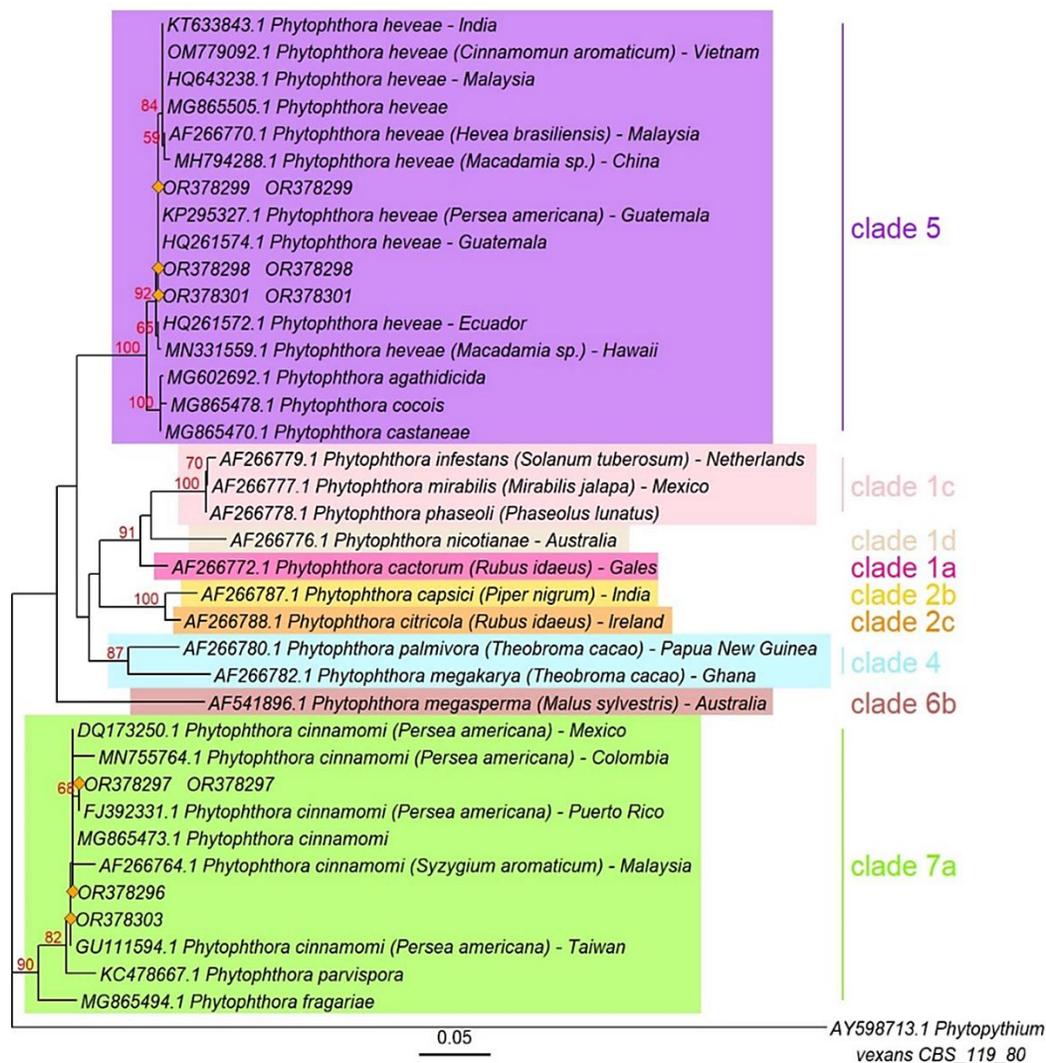
Figure 5. Phylogenetic tree of *Phytophthora* spp. isolates, generated with sequences of the ITS1-5.8s-ITS2 region, obtained by maximum likelihood method. The tree was constructed using the PhyML software (bootstrap=1,000), employing the Tim3 substitution model including a gamma distribution rate (+G4). Bootstrap values are located above the branches.

Figure 6. Symptoms observed in three-month-old Hass avocado plants inoculated with *Phytophthora* spp. a) Chlorosis and wilting caused by *P. cinnamomi* six dpi, and b) Wilting and death caused by *P. cinnamomi*, 15 dpi. c) Wilting caused by *P. heveae*, six dpi. d) Wilting and death caused by *P. heveae* nine dpi. e) Control treatment. Photographs: Research Group of AGROSAVIA, Centro de Investigación Palmira.
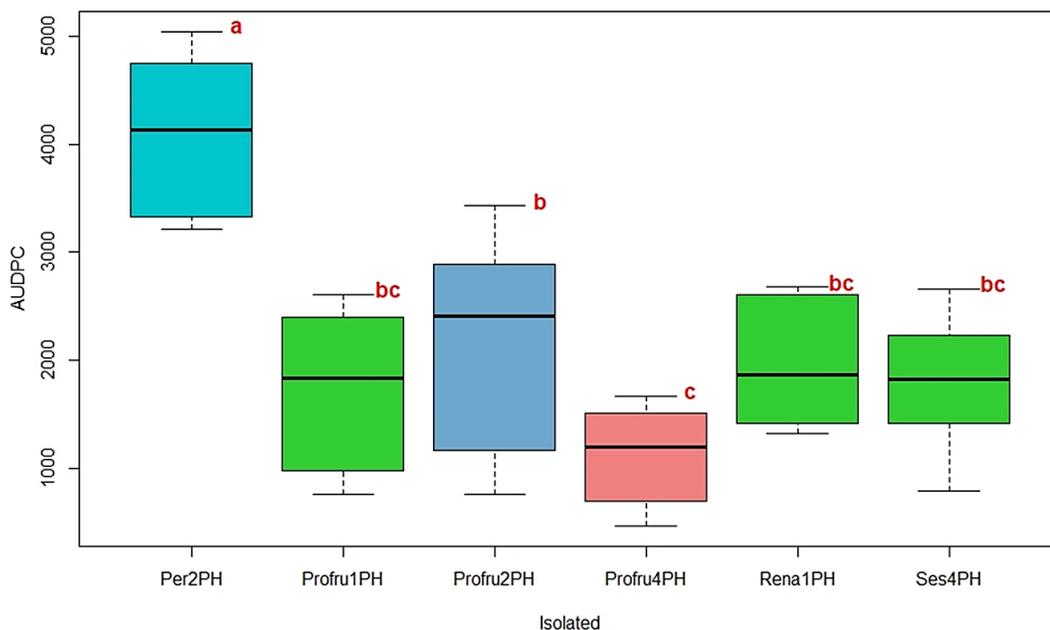
Figure 7. Boxplot of the Area Under the Disease Progress Curve (AUDPC) in Hass avocado seedlings inoculated with different isolates of *Phytophthora* spp.

However, it did differ from Per2PH and Profru4PH. The isolates Rena1PH (*P. heveae*), Ses4PH (*P. cinnamomi*), Profru1PH (*P. heveae*), and Profru4PH (*P. heveae*) did not show significant differences among them. They exhibited intermediate infective capacity and lower infection speed, with an average AUDPC ranging between 1,961 and 1,121 and an average necrotic lesion area ranging between 7.4 cm^2 and 8.5 cm^2. The control treatment (mock inoculation) did not produce necrotic lesions or any other symptoms.

Isolates Per2PH and Profru2PH of *P. cinnamomi* and *P. heveae* exhibited a faster symptom expression rate and seedling mortality in this study. At 15 dpi, 83% of the material inoculated with these two isolates died. Profru2PH showed more virulence, affecting between 30% and 50% of the inoculated material within the first six to nine dpi. Isolate Rena1PH caused the death of 33% of the material at six dpi and 50% at 15 dpi. Isolates Profru1PH and Profru4PH demonstrated lower virulence, causing only mild wilting in the plants without resulting in mortality during the evaluation period. These findings indicate variability in the virulence of the isolates, which aligns with studies by **Ochoa-Fuentes et al. (2015)** and **Belisle et al. (2019)**, who also observed variability in the virulence of *P. cinnamomi* isolates infecting avocado plants.

Phytophthora cinnamomi can infect plants at various developmental stages. This microorganism causes damage to the roots and triggers the appearance of various symptoms such as yellowing, leaf wilting, defoliation, regressive branch death, and ultimately, tree decline (**Castaño & Leal, 2018**). The presence of *P. cinnamomi* affecting avocado plants has been extensively documented worldwide (**EPPO, 2004**; **Miyambo et al., 2022**). In Colombia, its presence has also been confirmed in various departments, including Antioquia, Cauca, Risaralda, Tolima, and Valle del Cauca (**Ramírez-Gil, 2018**; **Marulanda, 2018**).

Phytophthora cinnamomi is considered as the most important species within the avocado wilt complex due to its frequency, aggressiveness, and the considerable losses it causes (**Miyambo et al., 2022**). This study confirmed the high degree of virulence exhibited by *P. cinnamomi* in avocado seedlings. Several research works have highlighted its ability to infect and cause the death of avocado plants rapidly, characteristics associated with factors such as its speed in penetrating and colonizing plant tissue (**Castaño & Leal, 2018**).

Phytophthora heveae has been documented in Guatemala, where it was identified as the causal agent for avocado stem canker (**Zentmyer et al., 1978**). Later, its presence was reported in Mexico, showing similar symptoms of stem canker (**Ceja-Torres et al., 2000**). **Ochoa-Ascencio et al. (2011)** identified *P. heveae* as the cause of avocado fruit rot in Mexico. In Colombia, there have been no reports of this pathogen causing root rot in avocado seedlings in nurseries. However, in 2014, its presence was reported in the Antioquia department, where it induced cankers at the base

of the stems of mature avocado trees in the field (**Ramírez-Gil et al., 2014**).

This research makes a significant contribution to the advancing scientific knowledge by identifying pathogenic agents associated with avocado root rot disease in the nursery phase.

4. Conclusions

Phytophthora cinnamomi and *P. heveae* were identified through morphological, molecular, and pathogenic analyses, confirming their role as causative agents of wilting and root rot in avocado plants in nursery material. Both species demonstrated a high pathogenic capacity, leading to symptoms such as leaf wilting, chlorosis, progressive wilting, and plant death. The presence of *P. cinnamomi* and *P. heveae* poses a significant risk during the crop establishment phase.

This work serves as the foundation for the development and implementation of comprehensive disease management strategies, as well as the selection of pathogen-resistant rootstocks.

Author contributions

L. P. Palacios-Joya: Methodology, Investigation, Formal analysis, Writing – original draft, Writing – review & editing. **K. A. Rodríguez-Arévalo:** Methodology, Investigation, Formal analysis. Writing – review & editing. **M. F. Martínez:** Investigation, Resources, Funding acquisition, Project administration, Writing – review & editing. **N. Murcia-Riaño:** Methodology, Resources, Supervision, Writing – review & editing. **D. M. Rodríguez-Mora:** Methodology, Investigation, Formal analysis, Supervision, Writing – original draft, Writing – review & editing.

Declaration of interest statement

The authors declare no affiliation or involvement with any entity that could have financial or equity interest that jeopardizes the validity of the presented results. The funders were not involved in the design of the study, methodology, data analysis, results interpretation or writing of the manuscript.

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