

Cross-Infection Analysis of *Phytophthora* spp. Isolated from Cacao (*Theobroma cacao* L.) Pods at Nagcarlan, Laguna, Philippines

Ricci Julia De Mesa

Polytechnic University of the Philippines

Myles David Florano

mdavidflorano@gmail.com

Polytechnic University of the Philippines

Jarvic Louis Suguitan

Polytechnic University of the Philippines

Lourdes Alvarez

Polytechnic University of the Philippines

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Abstract

Despite its high demand, the production of cacao (*Theobroma cacao* L.) remains low, which can be attributed to phytopathogens responsible for the destruction of cacao crops. Among these phytopathogens include *Phytophthora* spp. - pathogenic oomycetes that target plant species through their zoospores and cause black pod rotting. This research studied the ability of *Phytophthora* spp. to infect fruits commonly intercropped with cacao, such as papaya (*Carica papaya* L.) and banana (*Musa* spp.). Cacao pods exhibiting black pod rot symptoms were collected to isolate *Phytophthora* spp. from two cacao farms in Nagcarlan, Laguna, Philippines. Twenty-two isolates were identified as *Phytophthora palmivora* through their culture morphology, zoospore morphology, and molecular identity through ITS gene sequencing and confirmed through phylogenetic analysis. Five selected isolates were inoculated with their original host, cacao, and cross-infected with banana and papaya, which exhibited varying degrees of black pod rot symptoms. The lesions in cacao exhibited a higher degree of disease progression compared to banana and papaya. However, statistical analysis showed that bananas were more susceptible to the infection than papayas. Quantifying the degree of infection of *P. palmivora* from its host to commonly intercropped plants provides information on which plants are most vulnerable to infection within intercrop systems.

Introduction

Cacao is the raw material for the world-renowned delicacy known as chocolate. Despite a high demand for cacao, its production remains low [1]. Among many factors, phytopathogens contribute significantly to the low production of cacao.

Phytophthora spp. are pathogenic oomycetes that target numerous plant species. They infect plant species through their zoospores, which are motile, asexual spores. Using their flagella, these can move according to physical, electrical, and chemical cues, allowing them to navigate to sites within the plant that they can infect, including the stomata, wound sites, and roots [2]. This mechanism of infection proved to be incredibly effective, with *Phytophthora* species having already caused numerous historical social crises during the 1800s, including the European Famine, the Great Famine of Ireland, and the Highland Famines. The species that caused these famines was identified as *P. infestans*, which originated in Central Mexico and was then unknowingly introduced to Europe, where it wrought devastation [3].

Despite causing such ravaging events in history nearly 200 years ago, the full extent of the genus *Phytophthora* is yet to be thoroughly studied, with research suggesting an estimated number of about 500 species of *Phytophthora* exist all around the world but are yet to be identified [4]. Having unknown species of *Phytophthora* out in the wild can have new implications, such as a far more extensive host range and varying degrees of pathogenicity compared to what is currently known [5]. This uncertainty, along with the dangerous precedent set two centuries ago for the ruin it can bring, is indeed enough to

raise concerns, not only for cacao but also for entire farms that implement intercropping cacao with other potential hosts.

Numerous studies have been conducted and published to identify unknown species of *Phytophthora* in the wild and understand their capacity to cause damage to different plant hosts. According to the United States Department of Agriculture's research headed by Brasier in 2009, novel *Phytophthora* species are constantly being discovered worldwide, each adapting to different plant species as their hosts [4].

Phytophthora spp. are known for their diverse host range and highly virulent characteristics. Aside from cacao, other fruits are also devastated by various *Phytophthora* spp. Since cacao is usually intercropped with other plants, the chances of passing on *Phytophthora* spp. from one plant species to another is high. Upon an interview with the farmers from Nagcarlan, Laguna, Philippines, they revealed their usual intercrops with cacao, which include rambutan (*Nephelium lappaceum* L. var. *lappaceum*), banana (*Musa* spp.), coconut (*Cocos nucifera* L.), lanzones (*Lansium domesticum*), jackfruit (*Artocarpus heterophyllus*), avocado (*Persea americana*), and durian (*Durio zibethinus* L.). This study focuses on bananas and papayas for convenience since they are of the optimum size and not seasonal.

Tan (2021) wrote that cacao and bananas are promising intercropping duos because they use the same fertilizer and do not attract the same types of pests [6]. Previously, *Phytophthora* spp. was not viewed as a threat in bananas (Stover 1972) until Thompson (1981) recorded the presence of *P. nicotinae* B. de Haan var. *parasitica* (Dastur) Waterh. in a devastating *Phytophthora* finger rot in South Africa [7, 8]. He described the symptoms as brown-margined with a firm black lesion on the peel and pulp, sometimes covered by a superficial, felty, white mycelium. Moreover, the possibility that bananas could be an alternate host for *P. palmivora* in cacao plantations was also reported [9]. In recent reports, *P. cryptogea* and *P. inundata* causing root and basal stem rot were detected on dwarf bananas in Italy [10].

Another good intercrop for cacao and banana is papaya [11, 12]. However, papaya is a known susceptible host to *Phytophthora*. The known species that cause *Phytophthora* blight in papaya include *P. palmivora* and *P. nicotinae*. The symptoms of *Phytophthora* blight in papaya include water-soaked lesions with milky latex, fruit rot with white mycelium growth, stem cankers, and dark, rotten roots.

Research conducted by Tocafuldo et al. (2021) studied the direct and cross-infection of *P. palmivora* on cacao intercropped with papaya and peach palm [13]. It was found that while the derived *P. palmivora* isolates can infect other plant species, the specificity of the *Phytophthora* species meant that they were far more aggressive on their original host. While this means there would be less damage inflicted than anticipated in intercrop systems, it still exists. Moreover, given enough time to evolve according to their new host, the *Phytophthora* species may become just as pathogenic or even more.

Local research was also conducted in South-Central Mindanao, where *Phytophthora* spp. isolated from infected cacao and durian trees were cross-infected with potential hosts, rubber and coconut. These four were used as these were often grown in intercrop systems in that part of Mindanao. The research found that *P. palmivora* was pathogenic to all the tested hosts [14].

Studies have made promising progress in combating *Phytophthora* infections in genetics. As zoospores infect hosts via taxis, researchers have avoided host recognition by silencing target genes, such as the *Avr* gene [15]. While this breakthrough was successful in the laboratory, it may take some time and more research before this method can be deployed in real-world applications. Furthermore, as the fear of cross-infection caused by *Phytophthora* within intercrop systems sets in, it may be misconstrued that the solution to the problem is monocropping, but it is not. As mentioned earlier, the Great Famine of Ireland was partially caused by the country's dependence on potato and tomato monocropping [16].

With this situation in hand, this research investigated the possible cross-infection of *Phytophthora* species isolated from cacao collected within the local geographic region, Nagcarlan, Laguna, Philippines, to papaya and banana. This serves as a stepping stone in understanding these pathogenic species even more and learning which plants are in danger of infection within intercrop systems. Specifically, this study aims to isolate *Phytophthora* spp. from diseased cacao at Nagcarlan, Laguna, Philippines, and identify these through polyphasic methods. After successful isolation, the extent of cross-infection of these *Phytophthora* spp. was analyzed in banana and papaya.

Results

The collection conditions of the cacao pods were considered factors in successfully isolating the samples. During the cacao pod collection on October 22, 2023, a heavy downpour of rain ensued throughout the collection period at the collection sites in Nagcarlan, Laguna. From previous studies, it has been observed in other hot-humid countries in Southeast Asia that *P. palmivora* infects faster and spreads more during the wet seasons as the water brought on by the rain allows the flagellated zoospores of the *P. palmivora* to swim and infect nearby hosts [17].

In addition, the geographical structure of the two farms, while different from each other, was still a considerable factor in the success of isolating the samples. The steep slopes of the first farm help propagate the *P. palmivora* spores as water-carrying zoospores from infected plants at the top of the hill flow downward to the other plants at the bottom, promoting infection. The second farm has a flat and open field where water could accumulate on its surface, spreading disease around it [17].

Phytophthora palmivora Morphology

Only five (5) isolates were described morphologically due to their high percentage identity. Table 1 shows the macroscopic and microscopic morphology of *P. palmivora* isolates in V8 agar. Overall, there were three major colony patterns observed: (1) stellate striated pattern (n = 3), (2) cottony with central rosette/chrysanthemum pattern and stellate radiate edge (n = 1), and a consistent (3) rosette/chrysanthemum pattern (n = 1). These patterns are congruent with the morphology of the isolated *P. palmivora* of Solpot and Cumagun (2022), whose isolation is done in the same country but in a different region [14]. The stellate pattern is typical in all *Phytophthora* species and not just *P. palmivora* as seen in the *P. megakarya* isolates of Akrofi et al. (2015) [18]. Moreover, the rosette or chrysanthemum growth pattern in *P. palmivora* was also reported by Solpot and Cumagun (2022) [14]. Still, this unique

growth pattern can also be seen in the novel species of *P. theobromicola* sp. nov. reported by Decloquement et al. (2021) in Brazil [14, 19]. Based on its colony growth, the CP1 colony can reach 35 ± 1 mm in 2 days, the CP2 colony can reach 36 ± 1 mm in 2 days, the CP3 and CP5 colonies can reach 40 ± 0.6 mm in 2 days, and the CP4 colony can reach 38 ± 0.6 mm in 2 days. Combined, *P. palmivora* isolates exhibited a growth rate of 38 ± 2 mm in 2 days.

Table 1
Description of colony pattern, sporangia morphology, and chlamydospores morphology of the isolated *P. palmivora*.

Isolate Code	Identity	Colony Pattern	Sporangia Morphology	Chlamydospores Morphology
CP1	<i>P. palmivora</i>	stellate striated pattern	Shape: ovoid, globose, limoniform Length: 37 ± 5 mm	Shape: globose Location: terminal Length: 38 ± 4 mm
CP2	<i>P. palmivora</i>	stellate striated pattern	Shape: ovoid, globose Length: 24 ± 3 mm	Shape: globose Location: terminal, intercalary, lateral Length: 29 ± 3 mm
CP3	<i>P. palmivora</i>	stellate striated pattern	Shape: ovoid, globose Length: 28 ± 9 mm	Shape: globose Location: terminal, intercalary Length: 30 ± 6 mm
CP4	<i>P. palmivora</i>	cottony with central rosette / chrysanthemum pattern and stellate radiate edge	Shape: ovoid, globose Length: 30 ± 7 mm	Shape: globose, semi globose Location: terminal Length: 28 ± 5 mm
CP5	<i>P. palmivora</i>	rosette / chrysanthemum pattern	Shape: ovoid, globose, ellipsoid, limoniform Length: 28 ± 7 mm	Shape: globose Location: terminal, intercalary Length: 30 ± 4 mm

There was also variation in the sporangia shape, but all isolates uniformly presented common shapes, which are ovoid and globose with evident papillae. Kuswinati et al. (2023) reported a varying papilla of *Phytophthora* sporangia, which can be papillated or non-papillated [20]. However, the ovoid sporangia shape was the most abundant of all isolates. This is also true for the *P. palmivora* isolated by Alsultan et al. (2022) in Malaysia [21]. Moreover, CP1 and CP5 showed a limoniform sporangia shape, and CP5

showed a unique ellipsoid sporangia shape. The lengths of all sporangia vary even within the same isolate.

In terms of chlamydospores, all isolates showed uniform terminal globose chlamydospores, while some isolates showed intercalary and lateral chlamydospores. Some chlamydospores showed a semi-globose shape. All these morphological characteristics of chlamydospores are common in *P. palmivora* and other *Phytophthora* spp., as reported by Kurbeti et al. (2020), Scanu et al. (2021), Alsultan et al. (2022), and Kuswinati et al. (2023) [22, 23, 21, 20].

Different *Phytophthora* spp. have similar morphological characteristics, while the same species of *Phytophthora* may show varying morphological characteristics. Thus, morphological characterization is only helpful in identifying *Phytophthora* at the genus level. Several studies also report variations in colony morphology and microscopic morphology between the same species of *P. palmivora* [24, 22, 21, 20].

Molecular and Phylogenetic Analysis

Following the isolation of the samples from the cacao pods, molecular identification of the samples was started simultaneously with the morphological analysis. The samples were sent to the Plant Pathology Department of the University of Florida for DNA sequencing. Out of the twenty-two isolates sent, twenty-one were revived successfully following the delivery of the samples overseas, which were then sequenced. The resulting sequences sent back were cross-checked with sequences from the NCBI GenBank for identification. It was found that all twenty-one isolates were identified as *Phytophthora palmivora*. From those, five isolates that presented with high percent identities were chosen as representatives for this study, which are shown in Table 2. The selected five isolates, CP1, CP2, CP3, CP4, and CP5, all matched with *P. palmivora* isolate UHO-1 (MT644188.1) on NCBI's GenBank [25] with 99.39%, 99.39%, 100%, 99.85%, and 99.23% percent identities, respectively.

Table 2
Molecular identification of the five isolates.

Isolate Code	Identification	Percent Identity	Accession Number
CP1	<i>Phytophthora palmivora</i>	99.39%	MT644188.1
CP2		99.39%	
CP3		100%	
CP4		99.85%	
CP5		99.23%	

These sequences then underwent phylogenetic analysis with the use of MEGA X [26], along with additional reference sequences for comparison retrieved from NCBI's GenBank, including ten reference sequences of *P. palmivora* isolated from *Theobroma cacao* (isolates 227, TARS1, I320, CNC-P8, UHO-1,

PPM1, H12, Co-17, CPR22, and CBS179.26), two reference sequences of *P. palmivora* isolated from *Carica papaya* (isolates CHA134 and BRIP 40506a), five reference sequences of *Phytophthora sp.* from Type Material: *P. litoralis*, *P. multivora*, *P. plurivora*, *P. capsici*, and *P. tropicalis*, two reference sequences of *Phytophthora sp.* isolated from *Musa sp.* (*P. cryptogea* PHBan1 and *P. inundata* PHBan3), and one reference sequence of *Pythium zingiberum* (isolate UOP389) as the outgroup. The resulting Phylogenetic Tree is shown in Fig. 4.

All the formed clades in the Phylogenetic Tree are strongly supported, with bootstrap values ranging between 98–100%. Starting from the bottom of the tree, the *Phytophthora sp.* clade can be seen diverging from the *Pythium zingiberum* by a large margin, which is to be expected from the outgroup. Among the referenced Type Material *Phytophthora sp.* isolates, *P. tropicalis* and *P. capsici* were the first to diverge away from the group, which was then followed by the clade containing *P. plurivora* and *P. multivora*. The remaining isolates were then divided into two groups; the first comprised of *P. cryptogea* and a minor clade containing the *P. inundata* and *P. litoralis*. The second clade comprised the *P. palmivora* isolates, including the PUP Cacao Isolates.

Pathogenicity Test and Cross-Infection Test

The results of the pathogenicity and cross-infection tests of the five isolates of *Phytophthora palmivora* from infected cacao are shown in Table 3. Two isolates infected unripe cacao pods with varying degrees of severity. Brown-reddish lesions were first observed on the third day of C1T2 with an average diameter of 50mm. On the fifth day, all replicates of C1 varied in the severity of infection, showing brown-reddish to dark brown lesions with the appearance of mycelia and spores over them. Concurrently, dark brown lesions were observed around the wound of C3T2. The average lesion diameter for infected cacao on the fifth day ranged from 29 mm to 61.78 mm. On the seventh day, all replicates of C1 showed widened and darkened brown lesions with increased manifestation of mycelia and spores. At the same time, two replicates of C3 showed varying degrees of infection; C3T1 exhibited an irregular brown lesion near the wound, while C3T2 presented a spread-out dark brown lesion with an increased amount of mycelia and spores. The average lesion diameter for infected cacao on the seventh day ranged from 55 mm to 99 mm. The lesion description of the cacao pods for the third, fifth, and seventh-day are elucidated in Supplementary Table 1. None of the negative control setups developed lesions.

Table 3

Mean lesion diameter of *Phytophthora palmivora* isolates on cacao, banana, and papaya after 7 days of inoculation. *Means within the same column followed by a common letter are not significantly different at $p < 0.05$ using Tukey's Studentized Range (HSD) test. The mean of lesions was calculated with three independent replicates per sample unit.

Isolate Code	Inoculum source	Cacao (7th day)	Banana (7th day)	Papaya (7th day)
CP1	<i>Phytophthora palmivora</i> (cacao)	99 _a	13 _a	9.11 _{ab}
CP2		9 _b	9.89 _b	8 _{ab}
CP3		55 _{ab}	10.78 _{ab}	8.67 _{ab}
CP4		10.33 _b	10.67 _{ab}	8.5 _{ab}
CP5		9.22 _b	11.78 _{ab}	11.5 _a
CONTROL	-	5 _b	5 _c	5 _b

Following the measurement of the lesions on Day 7, the diseased cacao pods were sliced open horizontally to visualize the cross-section of the cacao pods after being infected by *P. palmivora*. C1T2 was opened to reveal some mild shrinkage of the cacao's pulp and browning of the placenta. With this cross-section, the spread of the *P. palmivora*-induced lesion on the 7th day is also visualized, and it was observed to have encompassed the entire cacao pod's pericarp. C1T3 was also opened to reveal an entirely devastated interior, with its shriveled placenta and dried-out pulp, leaving the pinkish-brown seeds without support. The cross-section of C1T3 also shows the lesion progression of the *P. palmivora* infection, penetrating through all the layers of the pericarp and spreading through more than half of the entire pod. Meanwhile, CTT1 was also opened for comparison as a control. The cacao pod was healthy – from its pericarp to its entire milky white pulp covering the seeds- with no signs of disease.

All isolates caused lesions on unripe banana fruits. However, there is a difference in the severity of the lesions compared to its native host, cacao; the lesions were smaller in bananas than in cacao. On the third day, small reddish-brown to irregularly shaped reddish-brown lesions were observed around the wound of the infected banana. The average lesion diameter for an infected banana on the third day ranged from 8.78 mm to 10.89 mm. Moreover, the banana fruits infected with *P. palmivora* ripened on the 3rd day; earlier than the control. On the fifth day, most lesions for the isolates darkened in color, while the rest did not have any significant changes in appearance compared to the third day. The average lesion diameter for infected bananas on the fifth day widened, ranging from 11.89 mm to 13.88 mm. The lesions did not darken further on the seventh day, but the wound had shrunk. The average lesion diameter for infected bananas ranged from 9.89 mm to 13 mm. The lesion description of the banana fruits for the 3rd, 5th, and 7th day are elucidated in Supplementary Table 2. No lesions were observed for the negative control setups.

After the measurement of lesions during the cross-infection analysis of *P. palmivora* on bananas on Day 7, select infected banana replicates and control banana replicates were sliced open horizontally to visualize the pathogen's infection inside the fruit. C1BT2 and C5BT1 were opened to reveal the cross-section of the infected banana fruit. Both replicates show the progression of the disease from inside the fruit through the spreading darkening areas with hints of green underneath the inoculation site. The green color is more pronounced on C5BT1, right below the dark lesion on the peel. CTBT1 was also opened as the control pod to compare with the infected pods. Several dark spots were seen on the skin of the banana – these were not considered as infection-induced lesions as the cross-section of CTBT1 revealed a healthy banana fruit with no lesion formation on the peel and no other signs of disease as seen in Fig. 8.

Like bananas, all isolates caused lesions in papaya but exhibited a lower degree of pathogenicity than cacao. On the third day, all isolates caused darkening on the inner circumference of the wound, with a few cases of brown lesions surrounding them. The average lesion diameter for an infected papaya on the third day ranged from 7.22 mm to 8.11 mm. On the fifth day, C1PT1 was observed with slight browning around its wound, C1PT2 showed browning around the wound while exhibiting reddish brown lesions on the upper and right side of the wound together with increased mycelia outside the wound, three of the isolates had an irregularly-shaped translucent brown lesion around their wound, some of the isolates exhibited slight darkening of the translucent brown lesion around their wound, C2PT3 was observed with minor translucent browning on the upper right side of the wound, C4PT3 slightly darkened with the manifestation of a translucent brown lesion and mycelia growing out the wound, C5PT1 exhibited irregularly-shaped translucent brown lesion around its wound with mycelia growing out of it, C5PT2 had increased areas with irregularly-shaped translucent to reddish brown lesion around the wound, C5PT3 showed darkening of its irregularly shaped brown lesions below the wound, and C2PT1 did not have any significant changes in terms of appearance compared to its third day. The average lesion diameter for an infected papaya on the fifth day ranged from 7.83 mm to 9.61 mm. On the seventh day, the majority of the isolates manifested a slight darkening of the brown lesions around their wound; both C1PT2 and C3PT1 had increased mycelia growing out of their wound, C2PT1 exhibited a slight browning on the right side of its wound, C2PT3 had translucent yellowish lesions around the wound, C4PT3 showed darkening of its brown lesion around its wound, and both C5PT2 and C5PT3 manifested a slight darkening of the irregularly shaped brown lesions below their wound. The average lesion diameter for an infected papaya on the seventh day ranged from 8 mm to 11.50 mm. The lesion description of the papaya fruits for the 3rd, 5th, and 7th day are elucidated in Supplementary Table 3. There were no significant changes in the appearance of the negative control nor the manifestation of wound shrinkage.

After measuring lesions during the cross-infection analysis of *P. palmivora* on papaya fruits at Day 7, some infected papayas and control papaya replicates were sliced open horizontally to visualize the pathogen's infection inside the fruits. C1PT3 and C5PT2 were opened to show the fruits' cross-section, revealing mycelial formations and dark brown lesions formed at the inoculation sites with a light yellow-brownish area radiating from the circumference, as seen in Fig. 10. Meanwhile, CTPT2 was also opened

for comparison as the control fruit. Its cross-section revealed a healthy fruit without any darkening around the wounded site and no other observed signs of infection.

Although only two isolates infected their host cacao, a higher degree of pathogenicity of *P. palmivora* is observed in cacao compared to banana and papaya. Similar findings were also observed in other cross-infection analyses in which the aggressiveness of the lesions was more advanced in their native hosts than their potential hosts [14, 27]. *P. palmivora* presented a higher degree of pathogenicity in cacao, producing a lesion diameter ranging from 55 mm to 99 mm. In contrast, the isolates manifested a smaller lesion diameter of 9.89 mm to 13 mm in banana and 8 mm to 11.50 mm in papaya.

Plants possess innate immunity, which pathogens such as *P. palmivora* must overcome. Phytophthora species adapted to impede the immunity of their specific plant hosts by utilizing effector proteins to manipulate and interfere with the plant's immune response [28]. Cross-infection studies indicate that the isolates of *P. palmivora* are less virulent in bananas than in cacao. The lesions observed in bananas were smaller and less aggressive than those found in cacao. This suggests a lower degree of pathogenicity in bananas compared to their native host, cacao. This is expected as the phylogenetic tree infers that the *P. palmivora* infecting cacao and the *Phytophthora* species infecting banana were separated into different lineages.

However, the observed shrinking of the wound on the banana on the 7th day can be attributed to its faster ripening process compared to the control group. As the banana ripens, the peel "shrinks" as its water content decreases [29].

The lesions in papaya had the most minor and slowest disease progression compared to both cacao and banana. Similarly to bananas, the degree of pathogenicity varied in terms of severity. Papaya is a known host of *P. palmivora* [30, 31]. Manifestation of the Phytophthora infection in papaya fruit is shown through off-white mycelium covering the wound and water-soaked lesions that turn brown as the disease progresses [32, 33]. The slow progression of these symptoms may be attributed to the minute genetic differences of different *P. palmivora* strains that infect cacao and papaya. *P. palmivora* can inhibit plants' intrinsic immune response against pathogens through protease inhibitors [34]. However, the expression of these inhibitors varies from one plant host to another [35, 36, 37, 38]. The varying degree of pathogenicity of the cacao isolates to the papaya fruits revealed differences in the isolated pathogen's ability to propagate from its native host to other potential plant hosts. The phylogenetic tree also supports this, wherein the cacao isolates were quite distant from the *P. palmivora* strain, whose native host is the papaya.

Discussion

The main objective of this study was to conduct a cross-infection analysis of *Phytophthora* spp. on intercropped fruits isolated from cacao collected at Nagcarlan, Laguna, Philippines. While examining cacao pods that manifested black pod rot symptoms using *Phytophthora* spp.-selective agar, only one species was isolated, *P. palmivora*. Despite being the same species, the five selected *P. palmivora*

isolates exhibited variations in their macroscopic and microscopic morphologies. However, molecular identification and phylogenetic analysis confirmed that all isolates belonged to the *P. palmivora* species regardless of morphological differences. The results showed that the isolates had a higher degree of pathogenicity in cacao, with mean lesion diameters ranging from 55 mm to 99 mm. In contrast, the isolates caused smaller mean lesion diameters of 9.89 mm to 13 mm in banana and 8 mm to 11.50 mm in papaya. The cross-infection studies revealed that the mean lesion diameter of bananas exhibited a marginally higher degree of pathogenicity when cross-infected with cacao isolates compared to the mean lesion diameter of papayas.

Based on morphological and molecular analysis, *Phytophthora palmivora* is the only species isolated from the diseased cacao pods collected at the two intercropping farms at Nagcarlan, Laguna, Philippines. Cross-infection analysis suggested the potential for cross-infection of *P. palmivora* from its native host, cacao, to other plants, such as banana and papaya, when grown under the same environmental conditions. The lesions in papaya exhibited the most minor and slowest disease progression compared to cacao and banana. Meanwhile, bananas had a slightly higher degree of pathogenicity when compared to papaya. This variation in pathogenicity may be due to differences in the innate plant immune responses, indicating that bananas are more susceptible to infection by *P. palmivora* than papayas when intercropped with infected cacao plants.

Isolating the same plant pathogen from various plant hosts is recommended. Future studies should also include more fruits for cross-infection analysis, considering their common intercropping with cacao. While pathogenicity testing is typically observed and quantified in the exocarp lesion, further studies should also explore its effects on the endocarp. Identifying these phytopathogens enables downstream applications such as discovering potential biocontrol agents. Additionally, sequencing effector proteins can enhance our understanding of the evolutionary relationships between *P. palmivora* isolates and their infection mechanisms across different plant hosts.

Methods

Collection of Cacao Pods

Two intercropping farms in Nagcarlan, Laguna, Philippines, were the sites for the research's survey and collection of *Theobroma cacao* pods. The two farms intercropped cacao with various plants, such as *Artocarpus heterophyllus*, *Cocos nucifera*, *Carica papaya*, and *Musa sp.* These farms were chosen as they reported experiencing an outbreak of a disease that causes black pod rot. Twenty-two cacao pods exhibiting black pod rot symptoms were collected through purposive sampling from the two farms. During the sample collection, the weather conditions experienced by the researchers were cloudy with rain showers and thunderstorms, which was confirmed by the Philippine Atmospheric, Geophysical, and Astronomical Services Administration's regional weather forecast in Laguna for October 22, 2023 [39]. The exact coordinates of the cacao trees in which the cacao pod isolates were collected are listed in Table 4.

Table 4

Information on the cacao pod collection sites. This contains the date of collection, coordinates, altitude, and weather conditions during collection.

Cacao Accession Number	Plant ID	Date of Collection	Latitude	Longitude	Altitude	Weather
CP1	<i>Theobroma cacao</i>	10/22/2023	Pre-harvested			Cloudy with intermittent rain showers
CP2		10/22/2023	14.143404	121.378279	253 m	
CP3		10/22/2023	14.13692	121.385413	198 m	
CP4		10/22/2023	14.137016	121.385518	196 m	
CP5		10/22/2023	14.137067	121.385527	196 m	
CP6		10/22/2023	14.143439	121.378233	273 m	
CP7		10/22/2023	Pre-harvested			
CP8		10/22/2023				
CP9		10/22/2023	14.143763	121.377948	253 m	
CP10		10/22/2023	14.143763	121.377911	253 m	
CP11		10/22/2023	14.143779	121.377791	249 m	
CP12		10/22/2023	14.143779	121.377791	249	
CP13		10/22/2023	14.143756	121.377799	248	
CP14		10/22/2023	14.143696	121.377904	249	
CP15		10/22/2023	14.143734	121.377758	249	
CP16		10/22/2023	14.143725	121.377677	249	
CP17		10/22/2023	14.143725	121.377677	249	
CP18		10/22/2023	14.143722	121.377727	249	
CP19		10/22/2023	14.144206	121.377217	255	
CP20		10/22/2023	14.137217	121.385637	257	
CP21		10/22/2023	14.136738	121.385835	195	
CP22		10/22/2023	Pre-harvested			

Isolation of *Phytophthora* spp.

Pathogen samples were collected from the pods of *Theobroma cacao* L. exhibiting *Phytophthora* blight symptoms. The collected samples were brought to the PUP Mycology Laboratory for isolation. Before

isolation, the samples were surface sterilized using 70% ethanol for one minute, rinsed three times with sterile distilled water, and dried on sterile tissue paper.

Samples of 0.5 x 0.5 cm were excised from the cacao pericarp with partly necrotic and healthy tissues. After excision, these samples are embedded in a selective media of 20% clarified V8 agar buffered with 2 g/L calcium carbonate and amended with 100 ml/L nystatin, 200 mg/L ampicillin, and 100 mg/L rifampicin. Cultures were incubated at 25°C or room temperature in the dark for 2–3 days.

Morphological Characterization of the Isolates

Five *Phytophthora* isolates were used to characterize the culture and spore morphology based on their high percentage identity. Microscopic observation of cultural morphology was done using a stereomicroscope. Aseptic techniques were implemented while observing the mycelia and spores using a compound microscope. The tape method was used for observation, and the specimen was stained using lactophenol cotton blue.

Molecular Identification and PCR Amplification

Samples of *Phytophthora* isolates were sent to the Department of Plant Pathology at the University of Florida for DNA extraction, amplification, and sequencing. V8 agar blocks from the colony edges of 5-day-old *Phytophthora* isolates containing mycelia were prepared in a sterile microcentrifuge tube and then shipped to the Department of Plant Pathology at the University of Florida, following the approval of the Material Transfer Agreement and permit from the Bureau of Plant Industry in the Philippines.

Sequence and Phylogenetic Analysis

Sequence and phylogenetic analysis were performed using two bioinformatics solutions, specifically NCBI Blast and Mega X. The processed sequences sent by the Department of Plant Pathology, University of Florida, were converted into FASTA format. The multiple and pairwise sequence alignment of the DNA nucleotides of the different *Phytophthora* species were rendered using MUSCLE via the MEGA program. The *Pythium zingiberum* strain (UOP389) from the NCBI GenBank is the chosen outgroup used in generating the phylogenetic tree. The best model generated using the MEGA program was chosen based on the lowest BIC scores. The phylogenetic tree was constructed using a bootstrap value of 1000 replicates [14].

Pathogenicity Test and Cross-Infection Test

5 *Phytophthora* isolates were used for the pathogenicity test on cacao and cross-infection tests on papaya and banana based on their high percentage identity. Pathogenicity and cross-infection tests were performed by artificial inoculation using a sterile 5 mm borer. Each isolate was tested in triplicate, with one sample unit per replicate. A 7-day-old *Phytophthora* culture, grown 5 days in the dark and 2 days in

light, in 2 g/L calcium carbonate-buffered 20% V8 agar was used for the inoculum blocks or agar blocks. The healthy pods and fruits were surface sterilized using 70% ethanol for one minute, rinsed with sterile distilled water twice, and blot-dried with sterile tissue paper. Using the modified protocol of Solpot & Cumagun (2022), a 5 mm *Phytophthora* agar block was placed at the center of the artificially wounded cacao pods, papaya fruits, and banana fruits obtained in commercial markets [14]. The samples were incubated in the dark at 25°C. After three to seven days, successful inoculation was measured based on the extent of discoloration or lesions around the inoculation plugs by calculating the average lesion diameter in millimeters three times at different angles. Another triplicate control setup was prepared using the same 5 mm V8 agar blocks without the pathogen.

Statistical Analysis

The pathogenicity and cross-infection tests were conducted using a completely randomized design (CRD). The mean lesion diameter of all isolates underwent statistical analysis using the Statistical Tool for Agricultural Research (STAR), version 2.0.1 (<http://bbi.irri.org/products>), and data were analyzed using Tukey's Studentized Range (HSD) test to group isolates that are not statistically different at $p < 0.05$ [14].

Declarations

Competing Interests

The authors declare that the research was conducted with no competing interest.

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Author Contribution

Ricci Julia A. De Mesa, Myles David A. Florano, Jarvic Louis D. Suguitan, and Dr. Lourdes V. Alvarez equally contributed to this study. All authors reviewed the manuscript.

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Data Availability

The datasets used and/or analysed during the current study is available from the corresponding author on reasonable request.

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Figures

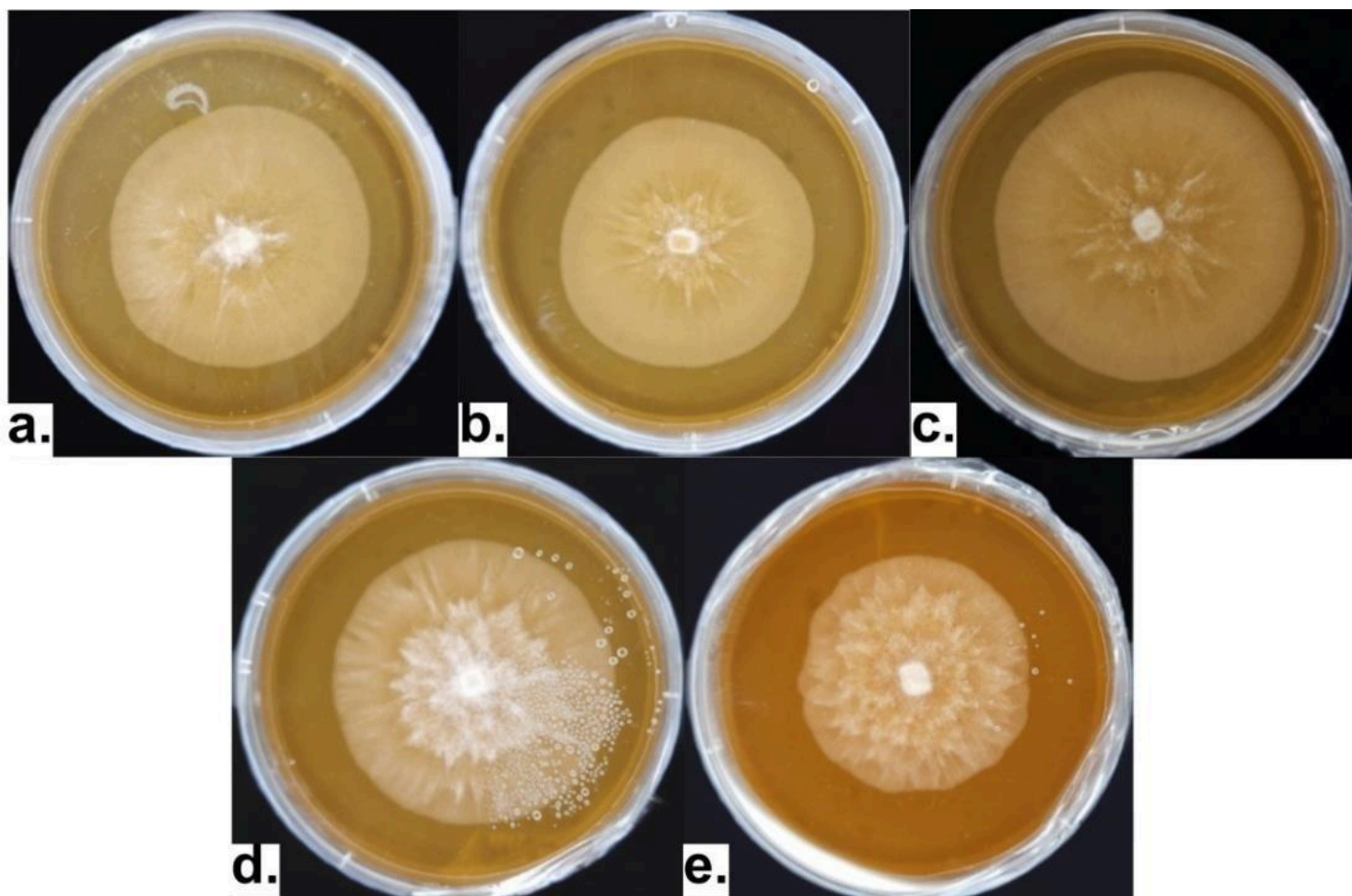


Figure 1

Varying colony morphology of the *P. palmivora* isolates. Stellate striated pattern: CP1 (a), CP2 (b), CP3 (c). CP4 is cottony with a central rosette/chrysanthemum pattern and stellate radiate edge (d). CP5 with rosette/chrysanthemum pattern (e).

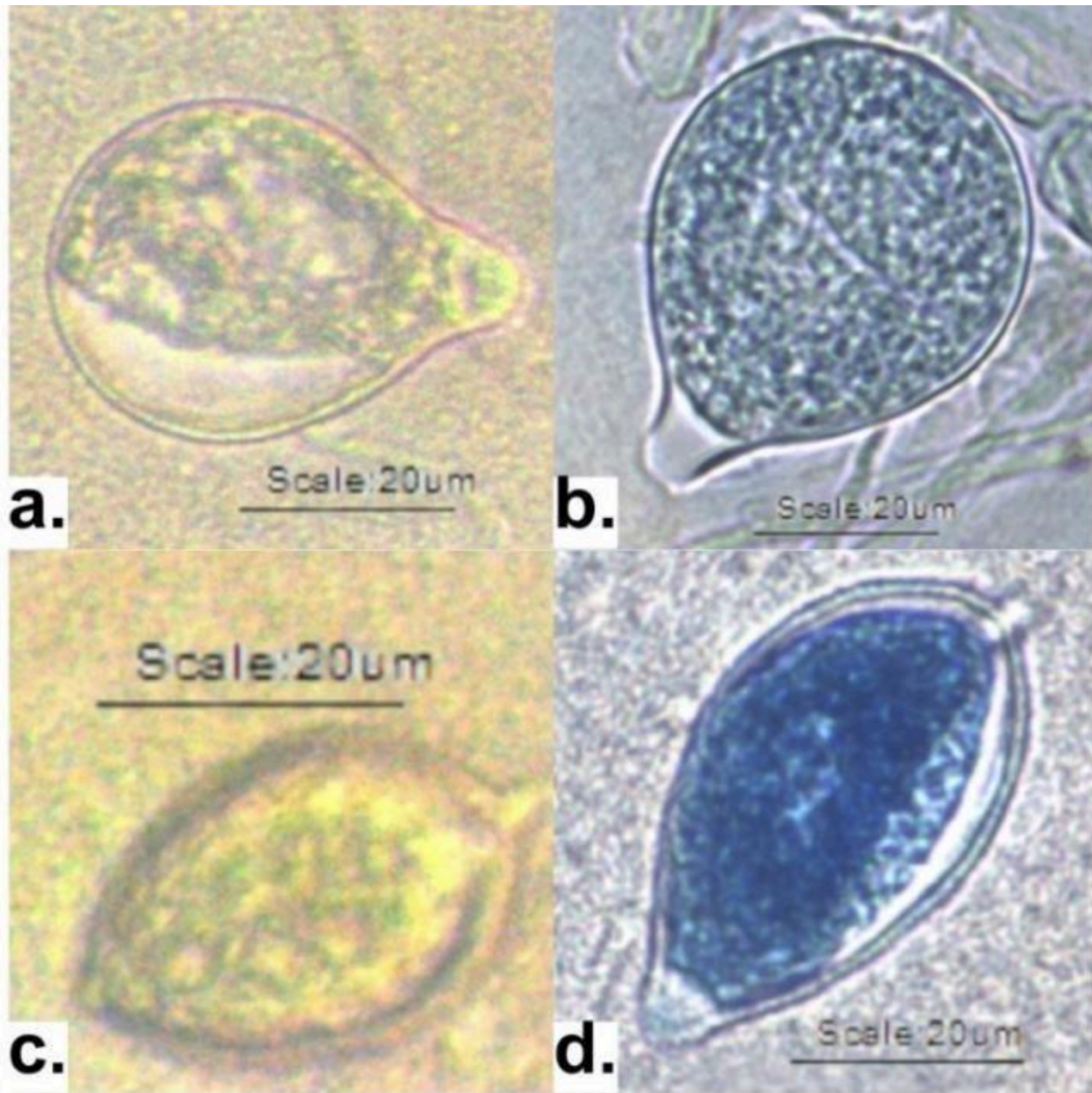


Figure 2

Variation of sporangia shape in *P. palmivora* isolates. Different shapes include ovoid (a), globose (b), limoniform (c), and ellipsoid (d) shapes.

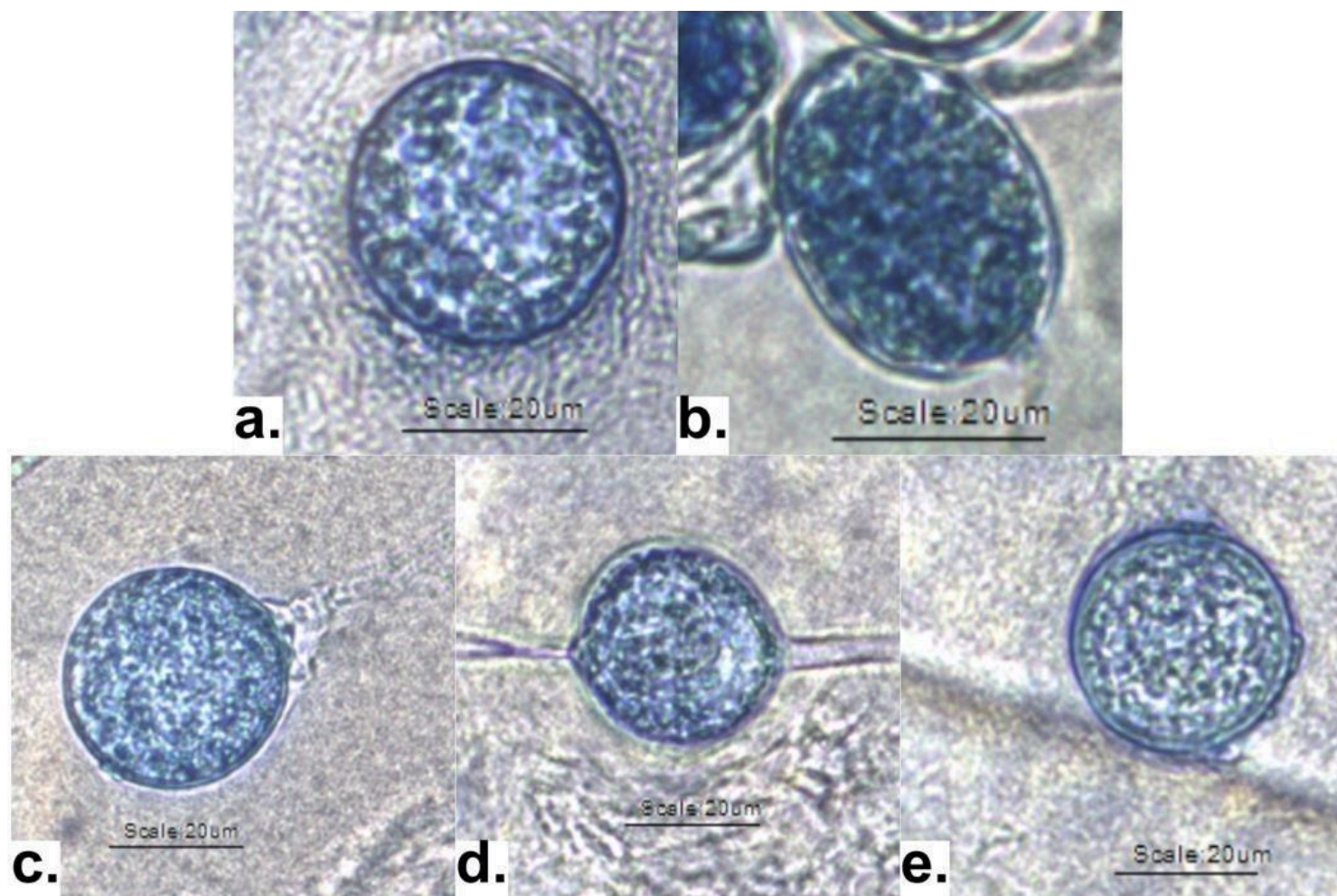


Figure 3

Variation of chlamydospore shapes and location in *P. palmivora* isolates. Different shapes include globose (a) and semiglobose (b). The locations of these chlamydospores may vary from being terminal (c), intercalary (d), or lateral (e).

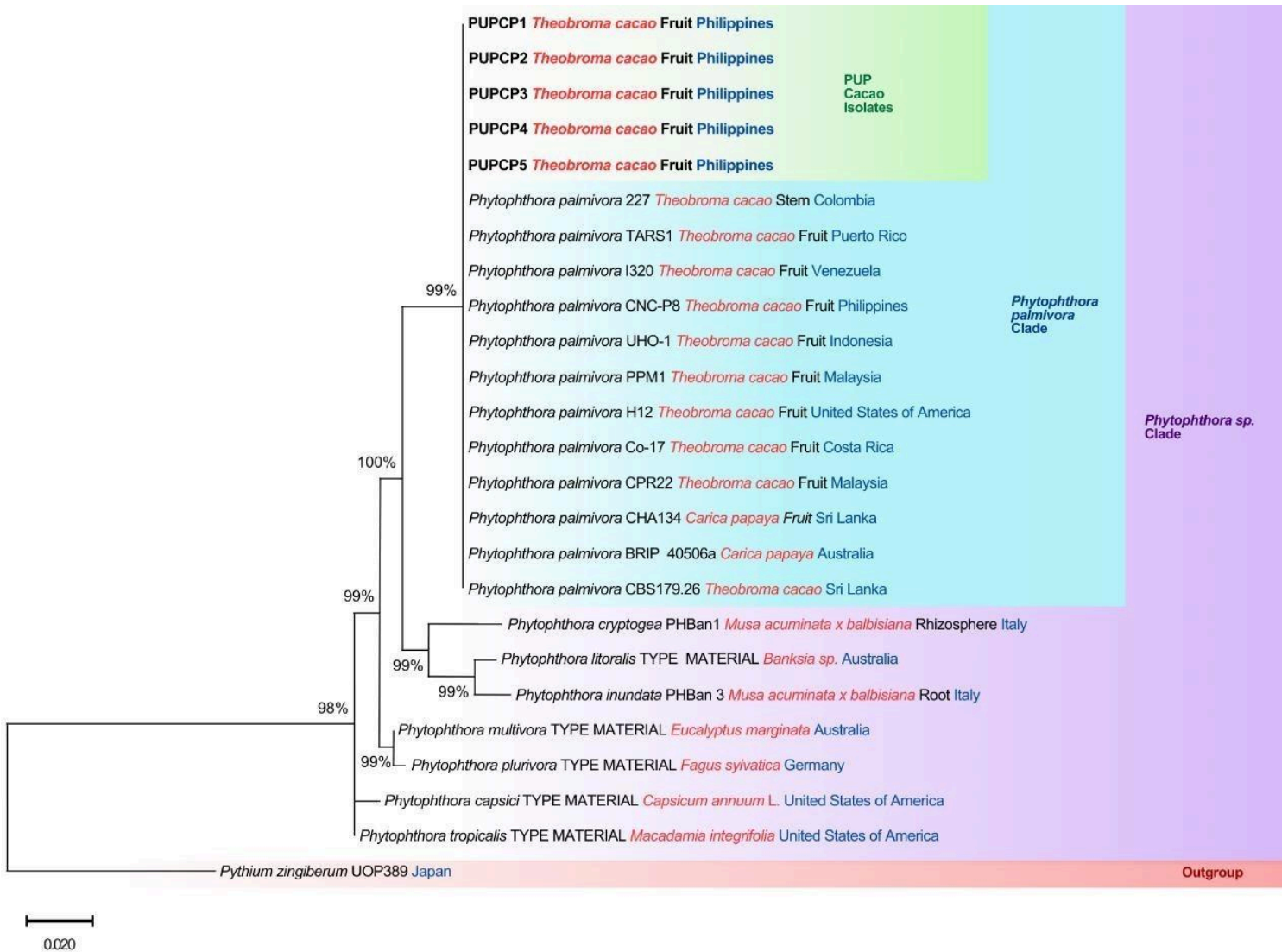


Figure 4

Phylogenetic Tree featuring the PUP *Phytophthora palmivora* isolates alongside various *Phytophthora* species of different origins and *Pythium zingiberum*. The evolutionary history was inferred using the Maximum Likelihood method and Kimura 2-parameter model (Kimura, 1980). With this, a discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.2286)).



Figure 5

Pathogenicity Test in Cacao Pods. The figure shows the gradual growth of lesions in the inoculated cacao pods along with the control for comparison.

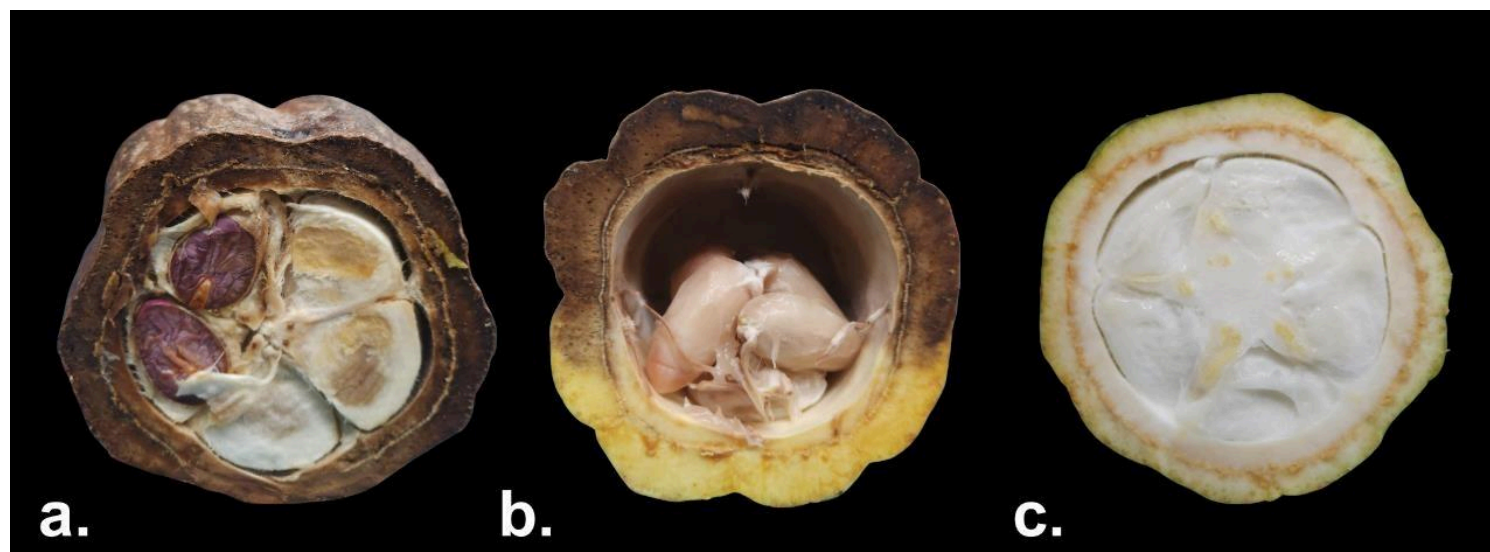


Figure 6

Cross-section of Cacao Pods after Pathogenicity Testing. The figure shows and compares the cross-section of infected pods: C1T2 (a) and C1T3 (b), along with a control pod: CTT1 (c).

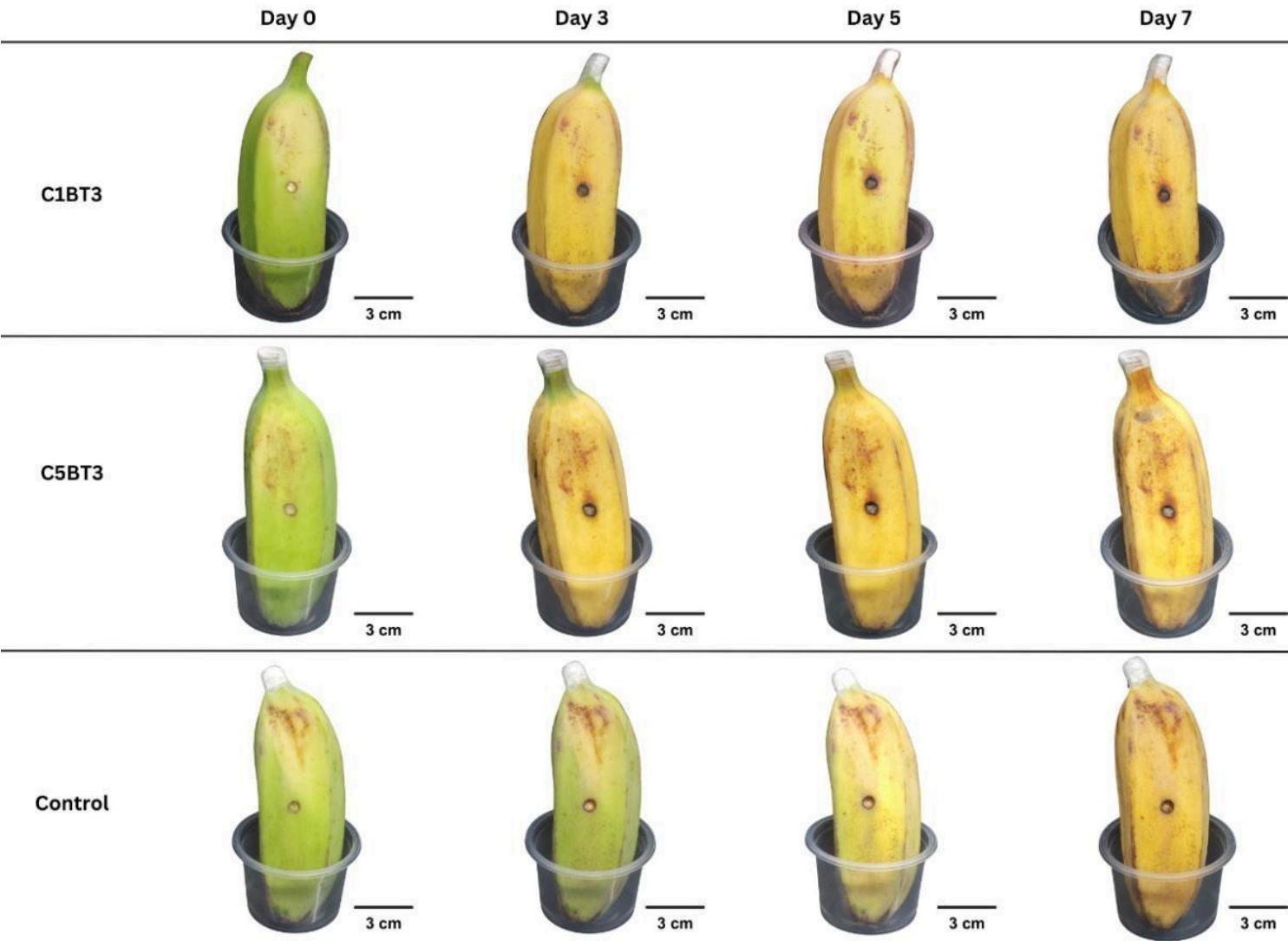


Figure 7

Pathogenicity Test in Banana Fruits. The figure shows the gradual growth of lesions in the inoculated banana fruits and the control for comparison.

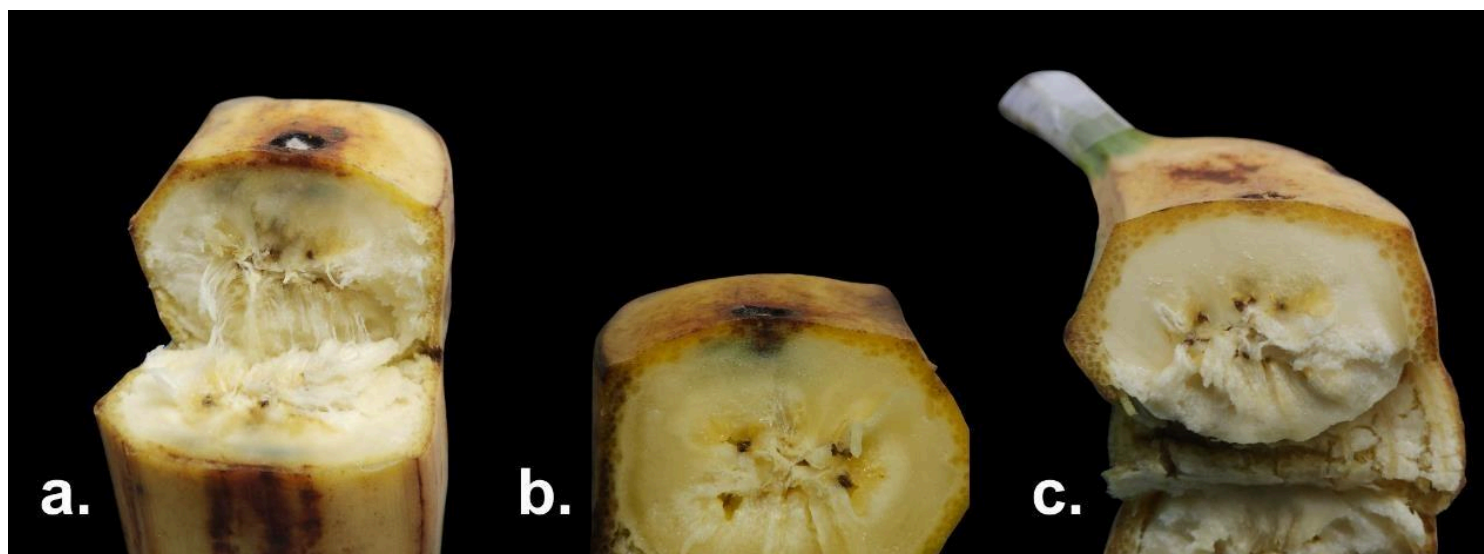


Figure 8

Cross-section of Banana Fruits after Cross-Infection Analysis. The figure shows and compares the cross-section of two infected banana fruits: C1BT2 (a) and C5BT1 (b), along with a control fruit: CTBT1 (c).

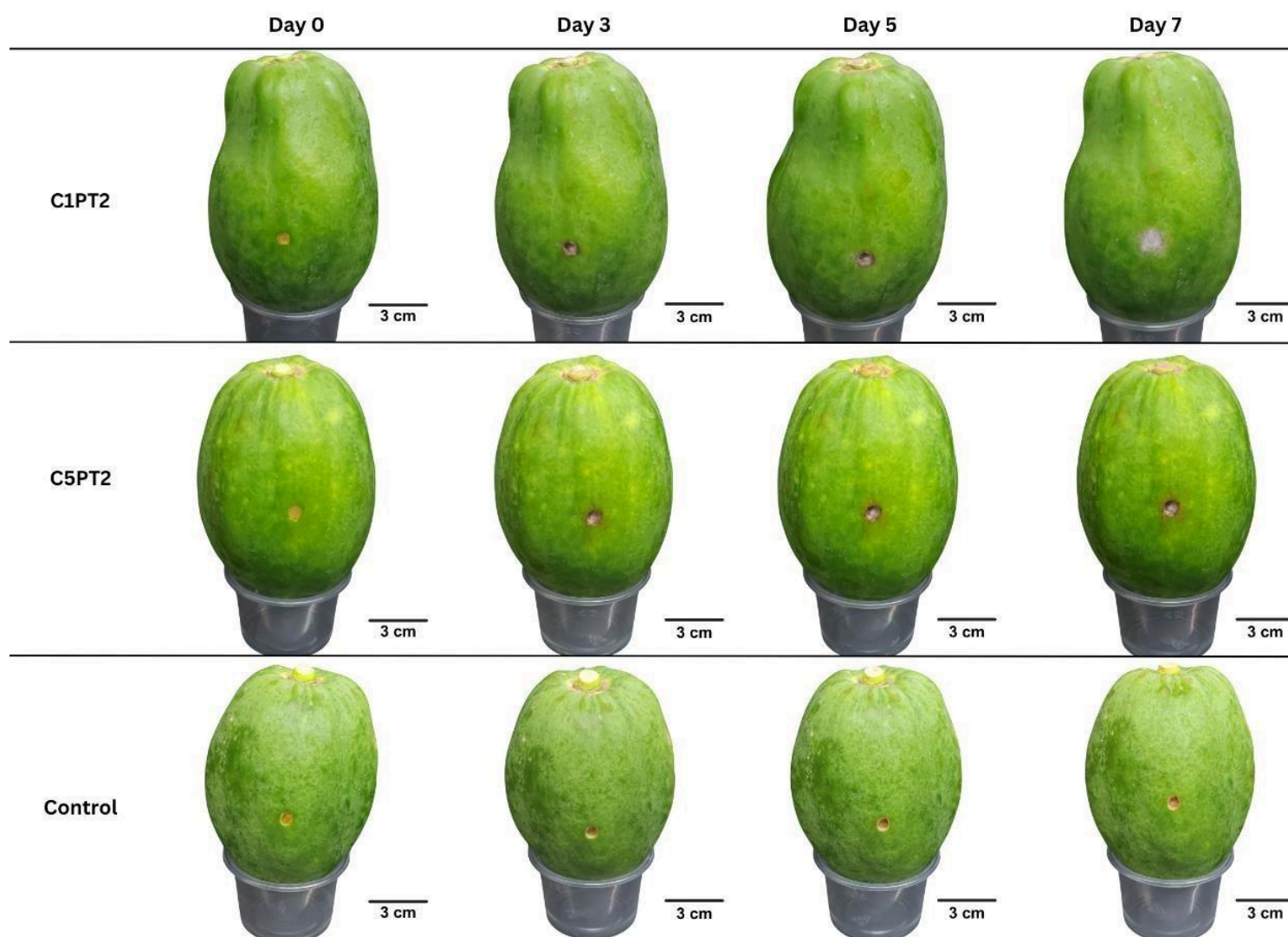


Figure 9

Pathogenicity Test in Papaya Fruit. The figure shows the gradual growth of lesions in the inoculated papaya fruits and the control for comparison.



Figure 10

Cross-section of Papaya Fruits after Cross-infection Analysis. The figure shows and compares the cross-section of infected papaya fruits C1PT3 (a) and C5PT2 (b), along with a control fruit, CTPT2 (c).

Supplementary Files

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