Detection and incidence of pineapple heart rot disease caused by *Phytophthora nicotianae* in commercial farms of Puerto Rico[1,2]

Luz M. Serrato-Diaz[3], Lorena L. Simbaña-Carrera[4], Yesenia Vélez-Negrón[5], and Lydia I. Rivera-Vargas[6]

J. Agric. Univ. P.R. 106(2):233-246 (2022)

ABSTRACT

Pineapple heart rot disease (PHRD) caused by *Phytophthora* spp. is one of the most important diseases of pineapple. This disease is characterized by soft rot and water-soaked lesions. In severe cases plant mortality is 100%. During a one-year survey, conducted from March 2018 to March 2019, a total of 29 pineapple hectares were evaluated from five commercial pineapple fields located in the Puerto Rican municipalities of Guánica, Lajas, Manatí and Santa Isabel, and at the Agricultural Experiment Substation of the University of Puerto Rico in Isabela. Symptoms of PHRD were observed in all fields evaluated, except at the Agricultural Experiment Substation in Isabela. Diseased plant tissue was surface disinfected and plated on PARPH-V8 selective media for *Phytophthora* spp. Aerial photography using a DJI Phantom 3 drone was used to corroborate PHRD incidence in the field. Approximately eight hectares were infected by *P. nicotianae* with an average of PHRD disease incidence of 28.82%. The highest percentage of PHRD incidence was observed in fields located at Guánica, Lajas and Manatí with 40%, 40% and 30%, respectively. Eleven isolates of *Phytophthora nicotianae* were collected from pineapple fields in Guánica, Lajas, Manatí and Santa Isabel and identified using morphology and phylogeny of sequences of the mitochondrial cytochrome oxidase subunit I region (COI). The estimation of the incidence of *P. nicotianae* as the causal agent of PHRD is important as a first step in developing specific control measures in the pineapple fields of Puerto Rico.

[1]Manuscript submitted to Editorial Board 9 February 2022.

[2]This research was supported by project ECaFSS, Encouraging Careers in Food Security and Safety: A multi-institutional approach for success in Puerto Rico (USDA NIFA-HIS Award no. 2016-38422-25541; Accession no. 1009896). Authors would like to thank agronomists Víctor González and Luis Collazo for their collaboration on this research. We thank Jose R. Almodóvar, specialist in scientific instrumentation for the aerial photographs; the UPR-Extension Service personnel, Aníbal Ruiz from Lajas; and pineapple farmers, agronomist Lourdes Fernández from Manatí, Jesús Flecha from Santa Isabel, and Jaime Acevedo from Guánica

[3]Plant Pathologist at USDA-ARS Tropical Agriculture Research Station, Mayagüez, PR 00680-5470.

[4]Former Research Associate, Department of Agro-Environmental Sciences, University of Puerto Rico-Mayagüez.

[5]Former undergraduate student, Department of Agro-Environmental Sciences, University of Puerto Rico-Mayagüez.

[6]Professor (retired), Department of Agro-Environmental Sciences, University of Puerto Rico-Mayagüez, Mayagüez, P.R. 00680; e-mail: lydiai.rivera@upr.edu.

Key words: ***Phytophthora nicotianae,*** **disease incidence, drone, pineapple heart rot disease**

RESUMEN

Detección e incidencia de *Phytophthora nicotianae* causante de la pudrición del cogollo de la piña en fincas comerciales de Puerto Rico

La pudrición del cogollo de la piña (PCP) causada por *Phytophthora nicotianae* es una de las enfermedades más importantes de la piña. Se caracteriza por pudriciones blandas y acuosas que en casos severos ocasiona una mortalidad del 100%. Durante un año de muestreo realizado de marzo de 2018 a marzo de 2019, se evaluaron un total de 29 hectáreas de piña en cinco campos comerciales localizados en los municipios de Guánica, Lajas, Manatí y Santa Isabel, y en la Subestación Experimental Agrícola de la Universidad de Puerto Rico en Isabela. Los síntomas de PCP fueron observados en todos los campos evaluados, excepto en la Subestación Experimental Agrícola de la Universidad de Puerto Rico en Isabela. El tejido enfermo fue desinfectado y sembrado en un medio selectivo PARPH-V8 específico para *Phytophthora* spp. La incidencia de PCP fue estimada en el campo y corroborada con fotografías aéreas tomadas con el dron DJI Phantom 3. Aproximadamente ocho hectáreas estaban infectadas con *P. nicotianae* con una incidencia de la enfermedad de 28.82%. Se observó una mayor incidencia de la enfermedad en campos localizados en Guánica, Lajas y Manatí con un 40%, 40% y 30%, respectivamente. Se recolectaron once aislados de *Phytophthora nicotianae* provenientes de campos de piña en Guánica, Lajas, Manatí y Santa Isabel y se identificaron usando morfología y filogenia de secuencias de la región de citocromo oxidasa sub-unidad I. La estimación de la incidencia de *P. nicotianae* como agente causal de PCP es importante como un primer paso para desarrollar medidas específicas de control en campos de Puerto Rico.

Palabras claves: ***Phytophthora nicotianae,*** **incidencia, drone, pudrición del cogollo de la piña**

INTRODUCTION

Pineapple (*Ananas comosus*), known as the king of fruits, belongs to the Bromeliaceae family. It is native to the Amazon rainforest in Brazil where it was domesticated by indigenous people and later spread through Central America to Mexico and the Caribbean islands (Wright, 2017). Columbus introduced this fruit to the European aristocracy, and it quickly became a symbol of the upper classes (Beauman, 2005).

Pineapple is the most commercialized bromeliad. In 2019 the major producers were Costa Rica, Philippines, and Brazil with 3.3, 2.74 and 2.42 million metric tons, respectively (Statista, 2021). In Puerto Rico, pineapple was grown in Pre-Columbian times by the Arawaks and Caribe indians (Ramírez et al., 1970). Currently, production basically supplies local consumption (Wright, 2017). In the 1970s, Puerto Rico's pineapple industry was based on two cultivars: 'Red Spanish'

and 'Smooth Cayenne'. Pineapple was the first crop produced and exported to the United States (Ramírez et al., 1970). According to the Puerto Rico Department of Agriculture, the pineapple sector planted approximately 200 hectares, mainly in the municipalities of Manatí and Lajas with production totaling 2,186 tons and market value of $5 million (USDA-NASS, 2012). Pineapple cultivars planted are 'MD-2', 'Pan de azúcar' and 'Cabezona' (Wright, 2017).

Pineapple heart rot disease (PHRD) caused by *Phytophthora* spp. is one of the most important diseases affecting both young and mature plants. During the initial growth of pineapple plants, the oomycete causes young leaves to stop growing and eventually turn yellow. Water-soaked lesions with soft rot at the base of the plant cause the leaves to detach easily. In severe cases, plant mortality reaches 100% (Ratii et al., 2018). In mature plants, infection moves up through the peduncle rotting the fruit. *Phytophthora nicotianae* (syn. *P. parasitica*) is the most common pathogen of PHRD in tropical countries including Puerto Rico (Shen et al., 2012; Espinosa-Rodríguez et al., 2015; Estévez de Jensen et al., 2008; Ratii et al., 2018). *Phytophthora nicotianae* was first discovered in pineapple fields in Santa Isabel and associated with a discoloration of the basal leaf tissue, root necrosis and stem rot, but to date pathogenicity tests have not been conducted (Estévez de Jensen et al., 2008). This study has two objectives: 1) to determine disease incidence of PHRD at five locations throughout Puerto Rico's pineapple growing areas, and 2) to conduct pathogenicity tests of *P. nicotianae* on pineapple plantlets to confirm the oomycete as the causal agent of PHRD in Puerto Rico.

MATERIALS AND METHODS

Collection of plant material and field incidence of Phytophthora

During a one-year survey conducted from March 2018 to March 2019, we evaluated symptomatic pineapple plants showing leaf discoloration, general chlorosis, rotting of the apical meristem, tip blight in detached leaves and plant decline, from five commercial fields in Guánica, Santa Isabel, Lajas and Manatí and at the Agricultural Experiment Substation of the University of Puerto Rico in Isabela (Figure 1). Aerial photographs taken from a DJI Phantom 3[7] drone were used to confirm

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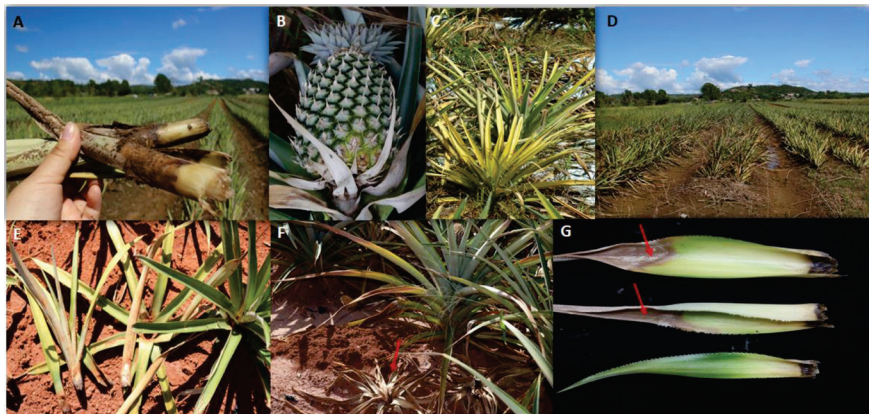

FIGURE 1. Symptoms of pineapple heart rot disease in commercial fields in Puerto Rico. A. Easy detachment of pineapple stalks B. Rotting of apical meristem. C and D. General chlorosis. E. Basal necrosis. F. Dead plant (arrow). G. Tip blight of detached leaves (arrows).

disease incidence in the field. Change in color of pineapple fields served as a parameter to measure disease incidence (Figure 2). Percentage of infected area was determined by visual examination of the total area of each field, divided by the portion of dead plants or those showing symptoms of PHRD. To confirm the presence of *Phytophthora,* diseased tissue collected in the field, showing symptoms described here, was brought to the plant pathology lab of the University of Puerto Rico (UPR), Mayagüez campus for morphological and molecular identification of the pathogen.

FIGURE 2. Aerial photograph taken by DJI Phantom 3 used in disease incidence calculations. Red rectangles represent infected pineapple heart rot disease area, while white rectangles represent healthy plants.

Isolation of Phytophthora

Diseased pineapple leaves brought from the fields were rinsed with distilled water for five minutes to remove any soil attached to plant tissue, and surface disinfected with 70% ethanol for one minute. A section of surface-sterilized plant tissue (10 cm) was transferred to a beaker, but only necrotic basal tissue was in contact with sterile-distilled-water (SDW). Plant sections were incubated for three days in darkness to promote the development of *Phytophthora* mycelium. After incubation, white mycelium was transferred to selective media for *Phytophthora* containing V8 juice, pimaricin, ampicillin, rifampicin, pentachloronitrobenzene and hymexazol (PARPH-V8) (Ivors, 2015). The mycelium was incubated at room temperature with a photoperiod of 12 h for eight days. *Phytophthora* pure mycelium or pineapple plant infected tissue was used to extract DNA. Pure isolates were identified using taxonomic keys of morphology of sexual and asexual reproductive structures such as shape of the sporangia, sporangia's papillae, antheridia and oogonia, oospores and morphology of the colony (Gallegly and Hong, 2008).

DNA extractions and PCR amplifications

Genomic DNA extraction was performed on both symptomatic plant tissue and pure cultures grown on PARPH-V8 using FastDNA SPIN kit for soil (MP Biomedicals, OH) following manufacturer's instructions. For plant tissue, 40 mg of symptomatic pineapple plant showing white cottony mycelia (Figure 5D) was used to extract DNA. For cultures, white mycelium from pure *Phytophthora* colonies was scraped from culture media and used to extract DNA. DNA was used to amplify the mitochondrial cytochrome c oxidase subunit I region (COI) using primer pair OomCoxI Levup (5'TCA WCW MGA TGG CTT TTT TCA AC 3' and OomCoxI-Levlo 5' CYT CHG GRT GWC CRA AAA ACC AAA 3') (Robideau et al., 2011). PCR using GoTaq® DNA Polymerase (Promega) was conducted according to Robideau et al. (2011). PCR products were visualized on 1% agarose gel and sent to Psomagen in Rockville, MD, for purification and sequencing. Sequences were edited using Sequencher (version 4.9, Gene codes corporation) and deposited in NCBI GenBank (Table 1).

Phylogenetic inference

Phylogenetic analysis included 50 ingroup taxa of *Phytophthora* clade 1 (Table 2), with *Pythium aphanidermatum* reference isolates CBS28779 and Lev3014 as outgroups (Abad et al., 2019). Sequences were aligned using SATé (Katoh and Standley, 2013) and MAFFT with RAxML for the tree estimator with 10 iterations. A maximum likelihood tree was constructed to infer the phylogeny of *Phytophthora* spp.

TABLE 1.—*Location and accession numbers of nucleotide sequences of mitochondrial cytochrome c oxidase subunit I (COI) of* Phytophthora nicotianae *isolates from pineapple leaves in Puerto Rico.*

Isolates	Location	GenBank accession COI#
SI_4	Santa Isabel	MZ394772
SI_3	Santa Isabel	MZ394773
SI_2	Santa Isabel	MZ394774
SI_1	Santa Isabel	MZ394775
M3	Manatí	MZ394776
M2	Manatí	MZ394777
M1	Manatí	MZ394778
L4	Lajas	MZ394779
L2	Lajas	MZ394780
L1	Lajas	MZ394781
G1	Guánica	MZ394782

using RAxML with GTRCAT as the default model, 25 gamma categories and the automatic bootstrap MRE implemented in CIPRES Science Gateway portal (Miller et al., 2010; Stamatakis et al., 2006). Trees were visualized and edited in FigTree V.1.4.0 (Rambaut, 2013).

Pathogenicity tests and in vitro propagation

Apical buds (explants) from healthy pineapple plants were surface disinfected with 70% ethanol for 10 minutes and cultured on liquid Murashige-Skoog (MS) medium. One month after root formation, disease-free pineapple plantlets were transferred to autoclaved topsoil and acclimatized at UPR greenhouses. After three months, acclimatized pineapple plants were used to conduct pathogenicity tests. Four pineapple plants were inoculated in the center (pineapple 'heart') with 1.5 ml of a solution of 4 x 10^6 zoospores/ml. Control plants were inoculated with SDW only. For zoospore formation and liberation, *Phytophthora* mycelia grown on clarified liquid V8 was transferred to SDW and incubated for one hour at 4° C. Symptoms of PHRD such as leaf detachment and basal necrosis were evaluated three days after inoculation.

RESULTS

Identification of Phytophthora nicotianae

Eleven isolates of *P. nicotianae* were identified using taxonomic keys and phylogeny of COI gene. Three isolates of *P. nicotianae* were obtained from Lajas, three from Manatí, one from Guánica and four from Santa Isabel (Table 1). No isolates were obtained from fields lo-

TABLE 2.—*Origin of isolates of* Phytophthora *species and related genera used in this study to construct the phylogenetic tree and accession numbers for sequences of mitochondrial cytochrome c oxidase subunit I (COI) obtained from GenBank.*

Species	Isolate ID	Origin	GenBank accession # COI
Phytophthora nicotianae	Ex-type CPHST BL44	Indonesia	MH136943
P. nicotianae	116-0008	United States	MF441674
P. nicotianae	PHY1	Italy	MH011396
P. nicotianae	P10381	China	HQ261378
P. nicotianae	CBS30429	Indonesia	HQ708351
P. nicotianae	CBS30329	Puerto Rico	HQ708352
P. nicotianae	BER11	United States	GU945494
P. nicotianae	CBS101655	Netherlands	HQ708354
P. nicotianae	CBS31062	No country	HQ708350
P. nicotianae	P10297	United States	HQ261379
P. nicotianae	P6915	Germany	HQ261377
P. nicotianae	UM301	Australia	MT981124
P. nicotianae	NP-2	China	MG880698
P. nicotianae	CPHSTL BL162	Indonesia	MH477752
P. nicotianae	P7146	Mexico	HQ261376
P. idaei	Ex-type CPHST BL38	Scotland	MH136903
P. tentaculata	Ex-type CPHST BL29	Germany	MH136983
P. tentaculata	CBS100411	Netherlands	HQ708415
P. pseudotsugae	Ex-type CPHST BL51	United States	MH136967
P. pseudotsugae	CBS44484	United States	HQ708381
P. mirabilis	Ex-type CPHST BL25	Mexico	MH136934
P. mirabilis	CBS67885	Mexico	HQ708339
P. iranica	Ex-type CPHST BL40	Iran	MH136913
P. iranica	CBS37472	Iran	HQ708314
P. ipomoeae	Ex-type CPHST BL21	Mexico	MH136912
P. ipomoeae	P10227	Mexico	HQ261342
P. infestans	CPHST BL143	No country	MH136907
P. infestans	CPHST BL142	Netherlands	MH136906
P. hedraiandra	Ex-type CPHST BL4	Netherlands	MH136898
P. hedraiandra	CBS118732	Australia	HQ708300
P. clandestine	Ex-type CPHST BL15	Australia	MH136873
P. clandestine	P3943	Australia	HQ261284
P. cactorum	CPHST BL9	Netherlands	MH136858
P. andina	Ex-type CPHST BL32	Ecuador	MH136846
P. andina	PRI814	Ecuador	HQ708398
P. cactorum	CBS110121	Belgium	HQ708238
Pythium aphanidermatum	CBS28779	Bulgaria	HQ708486
Pythium aphanidermatum	Lev3014	Oman	HQ708487

cated at Isabela. For morphological identification, colonies of *P. nicotianae* grown on PARPH-V8 produced white semi-immersed mycelia

with coenocytic hyphae. Papillated and bipapillated sporangia were ovoid measuring 32 x 29 µm on average. Chlamydospores were terminal with hyphal swellings. Oogonia containing aplerotic oospores with amphigynous antheridia were observed (Figure 3).

For molecular characterization, around 700 bp amplicons were obtained from the COI gene. Phylogenetic analysis confirmed the morphological characterization of isolates of *P. nicotianae* from Puerto Rico, showing clustering of eleven isolates from Lajas, Manatí, Santa Isabel and Guánica with ex-type CPHST BL44 of *P. nicotianae* (Bootstrap BS=99) (Figure 4).

Disease incidence of pineapple heart rot

A total of 29 hectares were evaluated from five commercial farms and from the Agricultural Experiment Substation in Isabela. Approximately 8 ha were infected with *P. nicotianae* with an average of PHRD incidence of 28.82%. The highest incidence observed was on farms in Guánica, Lajas and Manatí with 40%, 40% and 30%, respectively. No PHRD was observed at the Agricultural Experiment Substation at Isabela. Most of the fields (6 ha) infected by *P. nicotianae* were located on a commercial farm in Manatí (Table 3).

Pathogenicity test on in vitro plants

Three days after inoculations (DAI), detachment of pineapple heart leaves and soft rot were observed in all of the in vitro plants. At 8 DAI, white mycelia were growing on pineapple heart leaves, and basal

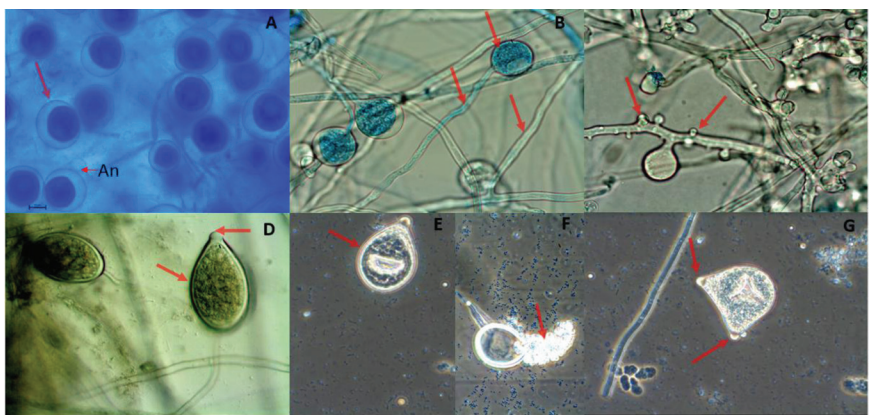

FIGURE 3. Morphology of *Phytophthora nicotianae* isolates obtained from pineapple fields in Puerto Rico. A. Oogonia with aplerotic oospores (arrow) and amphigynous antheridia (An). B. Terminal chlamydospores. C. Hyphal swellings (arrows). D and E. Ovoid papillated sporangia. F. Zoospores discharge (arrow). G. Sporangia with two papillae (arrows).

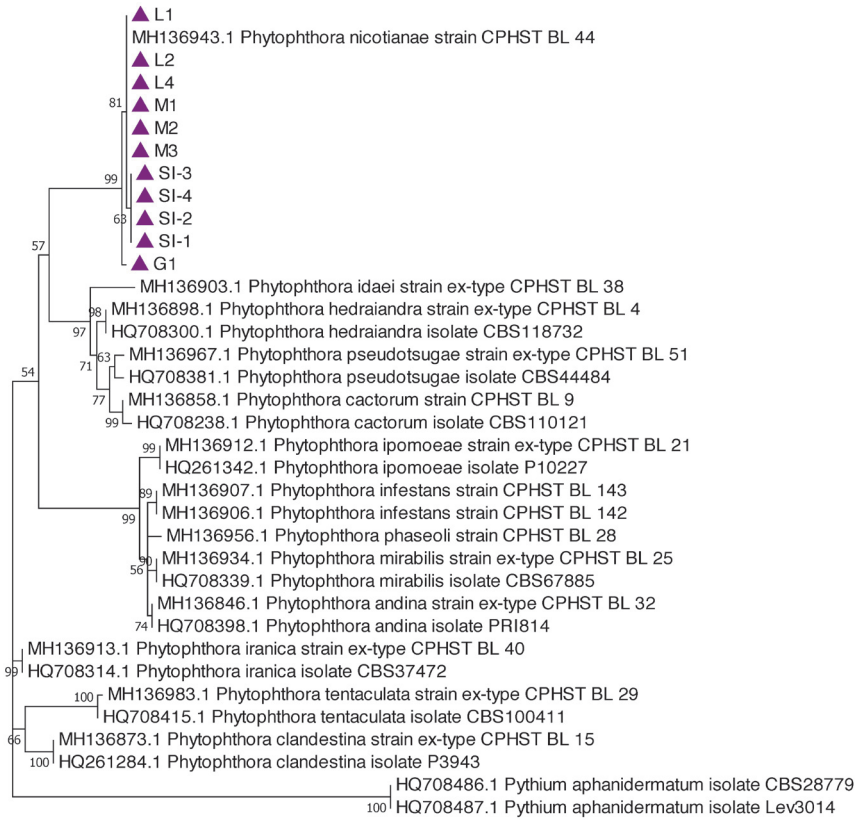

FIGURE 4. Maximum Likelihood best tree obtained from sequences of cytochrome c oxidase subunit I region of *Phytophthora* spp. Isolates obtained in this study are shown at the top of the phylogenetic tree (see triangles). Bootstrap support values are shown at the nodes. The tree was rooted to *Pythium aphanidermatum* isolates (CBS28779 and Lev3014).

necrosis and leaf discoloration were observed (Figure 5). Only control plants treated with SDW showed no symptoms of pineapple heart disease. *Phytophthora nicotianae* was re-isolated and identified from diseased tissue, fulfilling Koch's postulates.

DISCUSSION

This study confirms the existence of PHRD caused by *P. nicotianae* in five commercial fields planted with pineapple, in addition to the field in Santa Isabel previously reported by Estévez de Jensen et al. (2008). We report the presence of the PHRD in pineapple fields of Guánica,

TABLE 3.—*Disease incidence of Pineapple Heart Rot Disease (PHRD) caused by* Phytophthora nicotianae *at six locales in Puerto Rico.*

Localities	Hectares planted with pineapple	Hectares affected with PHRD	Incidence %
Isabela (Agricultural Experiment Substa.)	0.40	0	0
Guánica	1.21	0.48	39.66
Santa Isabel	2.00	0.2	10
Lajas farm 1	3.23	1.3	40.24
Lajas farm 2	2.00	0.40	20
Manatí	20.23	6	29.65
Total hectares surveyed	29.07	8.38	28.82

Lajas (two farms) and Manatí for the first time. Although pathogenicity tests were conducted on only four in vitro pineapple plants, *P. nicotianae* was able to cause PHRD symptoms on pineapple plantlets. A limitation on the number of plants used in the study is due to the availability of producing disease free pineapple plants from tissue culture. Prevalence of *P. nicotianae* in pineapple fields in Santa Isabel ten years after the first report by Estévez de Jensen et al. (2008) probably is due to the presence of oospores and chlamydospores in soil and plant debris. Chlamydospores and oospores are the primary survival struc-

FIGURE 5. Pathogenicity test on in vitro acclimatized pineapple cv. MD-2 plants. A. Detachment of pineapple heart leaves (arrow). B. White mycelia (arrow) of *Phytophthora nicotianae* growing on pineapple leaves. C. Soft rot and leaf discoloration (arrow). D. White mycelia (arrow) of *P. nicotianae* growing on basal pineapple leaves collected from field. E. Isolate of *P. nicotianae* used in pathogenicity tests.

tures of *P. nicotianae* (Erwin and Ribeiro, 1996). They can survive in soil for long periods of time and are spread between fields in the soil attached to farm tools (Green and Nelson, 2015). Chlamydospores and oospores of *P. nicotianae* may survive up to six years in the absence of its host plants and when weather conditions are not favorable for disease initiation and development (Erwin and Ribeiro, 1996). Our isolates collected from fields in Puerto Rico produced both oospores and chlamydospores (Figures 3A and 3B); most probably the production of these structures allowed *P. nicotianae* to survive for all those years in pineapple fields in Santa Isabel.

The presence, dispersal, and infection of *Phytophthora* spp. in soils depend on water content and movement. *Phytophthora* spp. have been associated with soil textures ranging from loamy to silty or clay soils (Jung et al., 2000; Jönsson et al., 2005). Soils of pineapple fields located in Guánica, Lajas, Manatí and Santa Isabel are mainly Vertisols with a high content of expanding clays or Mollisols, which contain a thick dark surface horizon (mollic epidedon) compound of organic matter (Matos, 2018). Soils with high water-holding capacities, such as those with high content of organic matter, provide conditions conducive for *Phytophthora* inoculum to increase and sporulate (Gisi, 1983; Zentmyer, 1980; Erwin and Ribeiro, 1996). Soils with fine texture and thick A horizons infected by *Phytophthora* can be a great disadvantage to plant health due to higher levels of moisture retention, and because they favor root development, which increases the inoculum and stimulates root infection (Corcobado et al., 2013). Probably the excessive precipitation during the rainy season, the physical characteristics of the soils (Vertisols and Mollisols), and the susceptibility of pineapple cultivars used in producing areas facilitate the prevalence of *Phytophthora* inoculum and its efficiency to infect pineapple plants.

Species of *Phytophthora* can be dispersed by different methods such as 1) dispersal in soil; 2) inoculum dispersal in surface water; 3) splash dispersal from soil to plant tissue; 4) aerial dispersion from sporulating lesions; and 5) dispersal by human or invertebrate activity (Ristaino and Gumpertz, 2000). *Phytophthora* spp. can travel 1) in the soil from inoculum to roots and vice versa, and by root-to-root contact; 2) unidirectionally down the rows with surface water for long distances, up to 70 m in furrow-irrigation; 3) from soil to plant tissue due to splashes of single water drops of rainfall events; and 4) by human activity, including the movement of contaminated plant material or soil attached to field tools and boots (Ristiano and Gumpertz, 2000). Kurzawianka (1992) reported that planting spacing of 20 cm increases the infection of potato tubers with *P. infestans* due to root-to-root contact. In vegetable crops, there is a greater disease severity caused by *P. capsici*

in rainfall events and periodic soil flooding (Sanogo and Ji, 2013). In Puerto Rico, cultural practices used by pineapple farmers have contributed to the persistence, dispersal, incidence and severity of *P. nicotianae*. They include gravity or furrow irrigation, poor soil drainage, use of farmer-owned pineapple "disease-free" crowns, suckers or slips (without disease testing) to plant new fields; as well as planting distance between pineapple plants of 30 cm combined with adverse environmental conditions such as heavy rainfall during the hurricane season and poor drainage of clay soils.

Several control measures have been proposed against *Phytophthora* spp. such as decontamination of soils using solarization and fungicides, biocontrol using *Trichoderma* spp. and antagonistic bacteria such as *Streptomyces rochei* and *Bacillus subtilis*, and the use of salicylic acid nanoparticles to improve resistance (Cohen and Coffey, 1986; Coelho et al., 2000; Browning et al., 2008; Etxeberria et al., 2011; Lu et al., 2019; Meyer and Hausbeck, 2013; Cohen and Rubin, 2020; Lee et al., 2008; Segarra et al., 2013; Bhusal and Mmbaga, 2020). Common practices implemented by Puerto Rican farmers to control *Phytophthora* spp. in pineapple fields include applications of the fungicide fosetyl-aluminum and planting the pineapple hybrid cultivar MD-2. Cultivar MD-2 is a hybrid from 'Smooth Cayenne' parent which exhibits acceptable resistance when environmental conditions favor the pineapple plant, but not when conditions favor *Phytophthora*, according to Green and Nelson (2015). Various studies have shown insensitivity to fosetyl-aluminum in different oomycetes including *Phytophthora* spp. (Brown et al., 2004; Lozoya-Saldaña et al., 2017). Studies evaluating different control measures in pineapple fields with *Phytophthora* spp. must be conducted and cultural practices implemented to effectively manage PHRD on the island.

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