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ORIGINAL ARTICLE

Detection and characterization of palm lethal decline phytoplasmas, subgroups 16SrIV-A and -D, in *Phoenix canariensis* and *Syagrus romanzoffiana* in Puebla, Mexico

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Abstract

Phytoplasma subgroups 16SrIV-A and -D are the agents associated with two diseases that significantly threaten palm cultivation in the Americas, namely lethal yellowing (LY) and Texas Phoenix palm decline (TPPD), respectively. Recently, in Puebla State, Mexico, several *Phoenix canariensis* Chabaud and *Syagrus romanzoffiana* (Cham.) Glassman palms used as ornamentals began to show symptoms resembling those of TPPD and LY. Therefore, the present study aimed to demonstrate the spread of group 16SrIV phytoplasmas to Puebla, Mexico. Ten symptomatic individuals of both palms were sampled and a nested PCR assay with primer pair P1/P7 followed by LY16Sf/LY16Sr was performed to detect phytoplasma presence. A fragment of about 1.4 kb was amplified in six palms, three (of four) *P. canariensis* and three (of six) *S. romanzoffiana*. Sequence analysis of the amplicons revealed that the phytoplasma isolates from Puebla were members of group 16SrIV, subgroups – A (one isolate from *P. canariensis*) and -D (rest of isolates). This study reports the first occurrence of TPPD and LY on ornamental palm species in the state of Puebla, Mexico.

Keywords: lethal bronzing, lethal yellowing, ornamental palms, phytoplasma diseases, Texas Phoenix palm decline

Introduction

Palms (Arecaceae) are some of the most important plants for humans as they can be exploited for food, oil, and a variety of different products, in addition to their ornamental value. However, they are susceptible to many pests and pathogens (Howard *et al*. 2001; Elliott *et al*. 2004), including the 16SrIV group of phytoplasmas, which are associated with two diseases widely distributed in North America and the Caribbean that significantly threaten palm cultivation: Texas Phoenix palm decline (TPPD) and lethal yellowing (LY) (Ntushelo *et al*. 2013).

TPPD, also called lethal bronzing, affects at least 22 different palm species (Table 1), and is associated with phytoplasma subgroup 16SrIV-D. On the other hand, LY is known to affect 45 palm species and is associated with subgroup 16SrIV-A (Palma-Cancino *et al*. 2023). Both diseases are fatal to palms and cause similar symptoms, which include premature fruit drop (i), inflorescence necrosis (ii), chlorosis of the foliage, starting from the oldest leaves (iii), death of the spear leaf (iv) and death of the entire foliage (v) (Ntushelo *et al*. 2013). They also share a common

Table 1. Documented host range of the 16SrIV-D phytoplasma, associated with TPPD

Palm species	Common name	Reference
Adonidia merrillii (Becc.) Becc.	Manila palm	Bahder *et al*. (2019)
Arenga engleri Becc.	Dwarf sugar palm	Mou *et al*. (2022)
Attalea butyracea (Mutis ex L. f.) Wess. Boer	Corozo	Ramos Hernández *et al*. (2020)
Bismarckia nobilis Hildebrandt & H. Wendl.	Bismarck palm	Dey *et al*. (2018)
Brahea brandegeei (Purpus) H.E. Moore	San Jose Hesper palm	Poghosyan *et al*. (2019)
Butia capitata (Mart.) Becc.	Jelly palm	Bahder *et al*. (2019)
Carpentaria acuminata (H. Wendl. & Drude) Becc.	Carpentaria palm	Bahder *et al*. (2019)
Caryota mitis Lour.	Clustering fishtail palm	Ntushelo *et al*. (2013)
Cocos nucifera L.	Coconut	Bahder *et al*. (2019)
Livistona chinensis (Jacq.) R. Br. ex Mart.	Chinese fan palm	Bahder *et al*. (2019)
Phoenix canariensis Chabaud	Canary Island date palm	Palma-Cancino *et al*. (2020)
Phoenix dactylifera L.	Date palm	Ferguson *et al*. (2020)
Phoenix roebelenii O'Brien	Pygmy date palm	Bahder *et al*. (2019)
Phoenix sylvestris (L.) Roxb.	Silver date palm	Bahder *et al*. (2019)
Pritchardia pacifica Seem. & H. Wendl.	Fiji fan palm	Narváez *et al*. (2017)
Pseudophoenix sargentii H. Wendl. ex Sarg.	Buccaneer palm	Vázquez-Euán *et al*. (2011)
Rhapidophyllum hystrix (Pursh) H. Wendl. & Drude	Needle palm	Mou *et al*. (2022)
Sabal mexicana Mart.	Mexican palmetto	Vázquez-Euán *et al*. (2011)
Sabal palmetto (Walter) Lodd. ex Schult. & Schult. f.	Cabbage palm	Ferguson *et al*. (2020)
Syagrus romanzoffiana (Cham.) Glassman	Queen palm	Bahder *et al*. (2019)
Thrinax radiata Lodd. ex Schult. & Schult. f.	Florida thatch palm	Vázquez-Euán *et al*. (2011)
Trachycarpus fortunei (Hook.) H. Wendl.	Chinese windmill palm	Singh and Ferguson (2017)

vector, the planthopper *Haplaxius crudus* Van Duzee (Dzido *et al*. 2020). However, they differ in distribution and host range, and molecular analyses of several genes provide evidence that they are associated with genetically distinct phytoplasmas (Soto *et al*. 2021).

In the USA, TPPD is currently causing significant economic losses to the nursery and landscaping industries, particularly in Florida (Bahder *et al*. 2019), though a large outbreak is also affecting the state of Louisiana (Ferguson *et al*. 2020). Moreover, a similar situation is developing in Mexico, with reports of unusually high mortality of ornamental palms, presumably due to TPPD, dating back to the early 2010s in the states of Guanajuato (Aviña-Padilla *et al*. 2011) and Michoacán (Rojas Martínez *et al*. 2013). More recently, the TPPD phytoplasma was detected in the northern state of Coahuila (Palma-Cancino *et al*. 2020) and in Mexico City (Ortiz-García *et al*. 2024), causing the death of thousands of palms, mostly Canary Island dates (*Phoenix canariensis* Chabaud). These recent outbreaks indicate an increase in the geographic distribution of the phytoplasma subgroup 16SrIV-D. In the case of LY, in both the USA and Mexico, there are no reports of this disease occurring outside of states that border the Gulf of Mexico, and active spread of this disease is apparently limited to the Lesser Antilles (Palma-Cancino *et al*. 2023).

In Puebla State, Mexico, both *P. canariensis* and the queen palm, *Syagrus romanzoffiana* (Cham.) Glassman), are representative ornamental species of the urban landscape. Populations of these palms were in overall good health until 2017–2020 when several *P. canariensis* showed symptoms of decline resembling those of TPPD and LY. In this study, symptomatic individuals from both palms were sampled, and phytoplasma detection was performed using nested PCR followed by *in silico* characterization to demonstrate the spread of group 16SrIV phytoplasmas to Puebla State, Mexico.

Materials and Methods

Study sites and sampling

The sampling was done between April 19 and 20, 2023, at three sites within the municipality of Puebla, Puebla State, Mexico (Fig. 1). Site one was located in the vicinity of the Ovando Bridge, in the Analco neighborhood (19°2'25.6"N, 98°11'35.2"W), city of Puebla; site two was located on a campus of the Benemérita

Fig. 1. Location of the state of Puebla (red) in Mexico – A; Location of the municipality of Puebla (red) in Puebla State – B

Universidad Autónoma de Puebla (BUAP), known as "Ecocampus" (18°56'07"N, 98°09'17"W), in the town of San Pedro Zacachimalpa; site 3 was on BUAP's main campus, known as "CU" (19°0'2.67"N, 98°12'1.84"W), which is also located in the city of Puebla.

Trunk shavings from four *P. canariensis* and six *S. romanzoffiana* palms with LY-type symptoms (Fig. 2A–B) were obtained with an electric drill following the procedure described by Harrison *et al.* (2002). Additionally, three visibly healthy palms, two *P. canariensis* (Fig. 2C) and a *Washingtonia robusta* H. Wendl., were sampled for comparative purposes.

DNA extraction, phytoplasma detection by nested PCR and sequencing

Total DNA was extracted from 1 g of the collected sawdust with a standard CTAB protocol (Doyle and Doyle 1990), re-suspending the DNA pellet in 50 µl of nuclease-free water. Subsequently, phytoplasmas were detected by nested PCR following a previously described assay (Harrison *et al.* 2002). Briefly, phytoplasma DNA (16S rRNA gene) was first amplified with primer pair P1 and P7 (Schneider *et al.* 1995). The P1/P7 product was then re-amplified using group-specific (16SrIV) primers LY16Sf and LY16Sr (Harrison *et al.* 2002). Amplifications were performed in a C1000™ thermal cycler (Bio-Rad), in 25 µL reaction volumes, each containing 2 µL of DNA template (a 1:5 dilution of total DNA for the first reaction and a 1:40 dilution of the P1/P7 product for the second reaction), 0.8 µM of each primer, 12.5 µl of DreamTaq™ Hot Start PCR Master Mix 2X (Thermo Scientific) and nuclease-free water. Thermal cycling parameters were the same as previously reported (Harrison *et al.* 2002). Positive and negative controls were DNA from a group 16SrIV phytoplasma-positive palm and nuclease-free water, respectively.

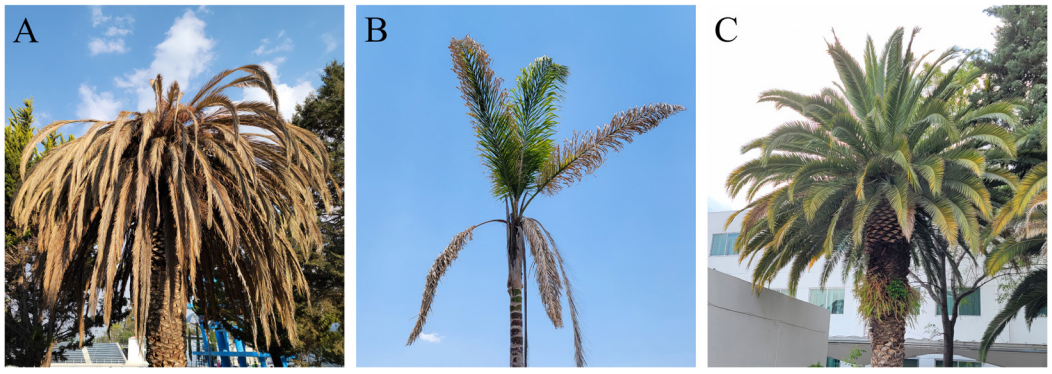

Fig. 2. Palms sampled in this study: *Phoenix canariensis* with late-stage LY-type symptoms (chlorosis of the entire foliage followed by collapse of the crown) – A; *Syagrus romanzoffiana* with intermediate symptoms (chlorosis of basal and intermediate leaves) – B; visibly healthy *P. canariensis* with no foliar or flower symptoms indicative of the presence of phytoplasma – C

The PCR products of the phytoplasma-positive samples were purified from 1.5% agarose gels with the QIAquick® Gel Extraction Kit (QIAGEN) following the manufacturer's instructions. Direct sequencing of the amplicons was then performed at the Laboratorio Nacional de Biotecnología Agrícola, Médica y Ambiental (LANBAMA), with the primers LY16Sf, LY16Sr and 503f (Harrison *et al.* 1999).

Sequence analysis

Sequences were viewed and assembled with Chromas software version 2.6.6 (Technelysium Pty Ltd.). Molecular identification of phytoplasma strains was achieved by comparing with other available phytoplasma sequences using the BLAST® algorithm of the National Center for Biotechnology Information (USA). Also, *i*PhyClassifier online program (Zhao *et al.* 2009) was used to confirm subgroup-level classification.

Additional molecular analyses were conducted in MEGA software version 11.0.13 (Tamura *et al.* 2021). A phylogeny reconstruction with representative 16S rRNA phytoplasma sequences was inferred by using the Maximum Likelihood method and the Hasegawa-Kishino-Yano model. Support for internal nodes was estimated with a bootstrap test of 1000 replicates. A sequence from *Acholeplasma palmae* was used as an external group to root the tree.

Results

Observed symptoms

In the present study, two palms were sampled from site one (Ovando Bridge), a *P. canariensis* and a *S. romanzoffiana*, both showing late-stage chlorosis of the entire foliage. A collapsed central frond was evident in the case of the *S. romanzoffiana* (Fig. 3A). From site two (BUAP Ecocampus), five *S. romanzoffiana* were sampled showing mid-stage chlorosis. The basal leaves appeared brown and desiccated, while yellowing was evident in the upward-facing leaves (Fig. 3B). Finally, from site three (BUAP CU), three *P. canariensis* were sampled with bronzing of the entire foliage, necrotic inflorescences and collapse of the upper crown (Fig. 2A).

Phytoplasma detection

The nested PCR assay revealed the presence of group 16SrIV phytoplasma DNA in three *P. canariensis* (one from Ovando Bridge and two from BUAP CU) and three *S. romanzoffiana* (one from Ovando Bridge and two from BUAP Ecocampus) palms with LY-type symptoms. A fragment of about 1.4 kb – expected size for primer pair LY16Sf/LY16Sr – was observed in phytoplasma-positive samples and the positive control, while no amplification was observed in the negative

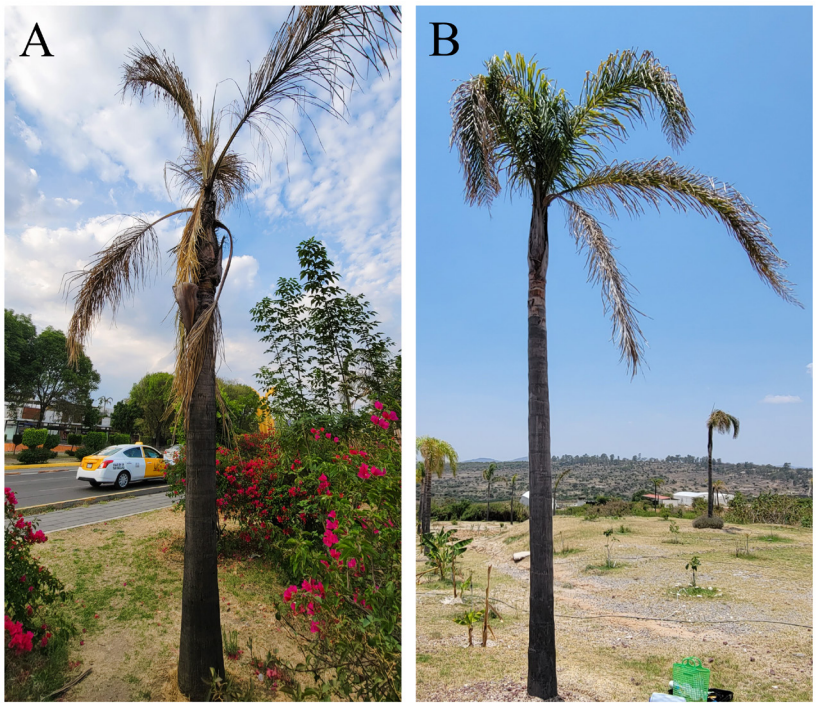

Fig. 3. Symptoms observed during sampling: *Syagrus romanzoffiana* at Ovando Bridge with late-stage chlorosis of the foliage and a collapsed central frond – A; *S. romanzoffiana* at BUAP Ecocampus with mid-stage chlorosis of the foliage (bronzed basal leaves and yellowing of the upper crown) – B

control and the three visibly healthy palms used for comparison (data not shown).

Molecular identification of phytoplasmas

Amplicons of positive samples were sequenced and six sequences of approximately 1300 bp were obtained and submitted to GenBank® with accession numbers PP657406, PP657407, PP657408, PP657409, PP657410 and PP657411. Both BLAST® and *i*PhyClassifier analyses identified the phytoplasma isolates from Puebla as members of group 16SrIV, subgroups -A (one isolate from a *P. canariensis* in the BUAP CU site, accession no. PP657410) and -D (the remaining isolates), based on sequence identity and RFLP similarity coefficient calculations, respectively (Table 2). No considerable differences in sequence identity between the Puebla isolates and the sequences of subgroups 16SrIV-A and -D used for reference in our study were found by the BLAST® algorithm (>99.9% in all instances). Likewise, similarity coefficients calculated by *i*PhyClassifier were 1.00 for all isolates, indicating virtual RFLP profiles identical to that of subgroups 16SrIV-A and -D. Furthermore, the resulting 16S rRNA gene phylogeny further supported this classification, as phytoplasma isolates from Puebla were not enclosed in the monophyletic clades formed by the other group 16SrIV subgroups, namely -B, -C and -F (Fig. 4). Thus, the phytoplasma-associated diseases that were found in Puebla State were TPPD and LY.

Discussion

Phytoplasma subgroups 16SrIV-A and -D are the agents associated with two severe diseases of palms, LY and TPPD, respectively. In the early 2010s, these diseases were viewed as 'tropical', since they had only occurred in the tropical climates of Central America, the Caribbean, south and southeast Mexico, and in coastal regions of Florida and Texas, USA (Ntushelo *et al.* 2013). Reports of *Phoenix* spp. palms infected with group 16SrIV phytoplasmas in non-coastal regions of

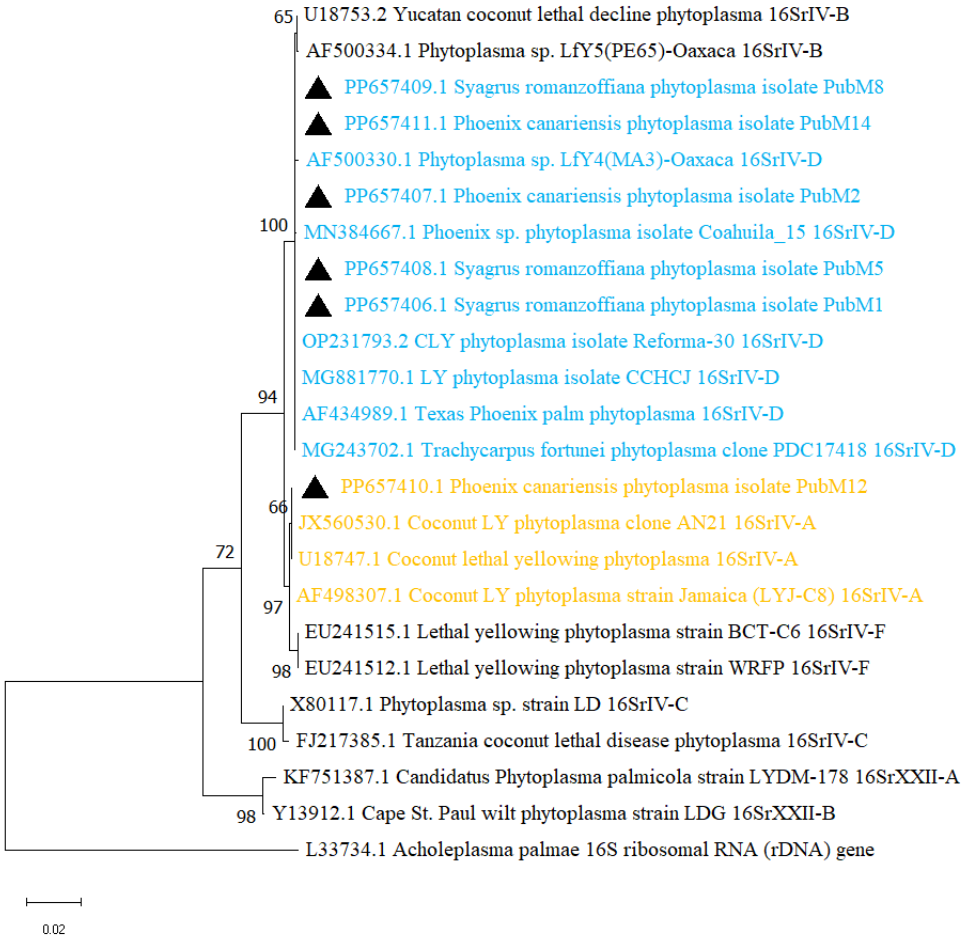

Fig. 4. Phylogeny of phytoplasma 16S rRNA gene sequences inferred by Maximum Likelihood. For the analysis, 24 representative sequences deposited in GenBank® and a total of 1248 base pairs were used. The sequences of phytoplasma isolates from Puebla are denoted with ▲. The trust level of the internal nodes is displayed next to the branches

Table 2. Molecular characterization of phytoplasma isolates from Puebla according to the BLAST® algorithm and the *i*PhyClassifier online tool

Phytoplasma isolate (GenBank Accession)	Host	Sampling site	Phytoplasma ID result	
			BLAST (sequence used for reference)	*i*PhyClassifier (Similarity coefficient)
PubM1 (PP657406)	*S. romanzoffiana*	Ovando Bridge	100% similar to subgroup 16SrIV-D (AF237615)	identical to subgroup 16SrIV-D (1.00)
PubM2 (PP657407)	*P. canariensis*	Ovando Bridge	100% similar to subgroup 16SrIV-D (AF237615)	identical to subgroup 16SrIV-D (1.00)
PubM5 (PP657408)	*S. romanzoffiana*	BUAP Ecocampus	100% similar to subgroup 16SrIV-D (AF237615)	identical to subgroup 16SrIV-D (1.00)
PubM8 (PP657409)	*S. romanzoffiana*	BUAP Ecocampus	100% similar to subgroup 16SrIV-D (AF237615)	identical to subgroup 16SrIV-D (1.00)
PubM12 (PP657410)	*P. canariensis*	BUAP CU	100% similar to subgroup 16SrIV-A (U18747)	identical to subgroup 16SrIV-A (1.00)
PubM14 (PP657411)	*P. canariensis*	BUAP CU	99.92% similar to subgroup 16SrIV-D (AF237615)	identical to subgroup 16SrIV-D (1.00)

Mexico, like the city of Morelia, Michoacán, started to increase at that time (Rojas Martínez *et al*. 2013), however, no molecular characterization beyond group level was performed. Some years later, a major outbreak of TPPD occurred in the city of Torreon, Coahuila, which has a hot desert climate and lies approximately 400 km from the nearest coast (Palma-Cancino *et al*. 2020). As these previous locations were considered atypical for TPPD or LY, an effort to survey palms in Mexico's central states in search of group 16SrIV phytoplasmas began.

In this study, 10 *P. canariensis* and *S. romanzoffiana* palms with LY-type symptoms in the state of Puebla were analyzed by nested PCR assays. Positive phytoplasma of the 16SrIV group was detected in six palms, three (of four) *P. canariensis* and three (of six) *S. romanzoffiana*. Though most palms tested positive for the presence of phytoplasma, a lower percentage of positive individuals was found in *S. romanzoffiana*. This could have resulted from a misinterpretation of symptoms since these palms were not yet flowering at the time of sampling. Lack of water and fertilization can also cause chlorosis of the foliage. In the case of *P. canariensis*, the age of all sampled palms allowed for a better assessment of symptoms associated with the presence of phytoplasma. Furthermore, the negative diagnosis of one of these palms may have resulted from insufficient phytoplasma titer.

In silico molecular analyses of phytoplasma 16S rRNA gene sequences revealed the presence of two phytoplasma subgroups in Puebla, 16SrIV-A and -D, though the predominant subgroup was 16SrIV-D. This is similar to what has been reported in urban areas of Louisiana and Florida, USA, where both subgroups are also present but where recent surveys have shown that subgroup 16SrIV-D is responsible for most infections in palms (Ferguson *et al*. 2020; Mou *et al*. 2022). This is likely due to differences in susceptibility of the most common ornamental palms used in these landscapes towards both of these pathogens, since *P. canariensis*, *S. romanzoffiana* and *Sabal palmetto* (Walter) Lodd. ex Schult. & Schult. f. – a common palm in Florida and Louisiana – have only rarely been diagnosed with LY (Harrison *et al*. 2008; Mou *et al*. 2022). In contrast, LY is the predominant disease in the Caribbean and in coastal-rural regions of southeast Mexico (Palma-Cancino *et al*. 2023), where the coconut palm (*Cocos nucifera* L.) is more abundant.

In Puebla, evidence of the first arrival of these pathogens dates back to 2017, when a declining *P. canariensis* was noticed in Analco Park, next to the Ovando Bridge (sampling site one in this study). By 2021, all *P. canariensis* in the park (about 15 palms) had died after exhibiting LY-type symptoms. The presence of young, recently transplanted *S. romanzoffiana* palms in the vicinity of Analco park – one of which tested positive for subgroup 16SrIV-D in our study (Fig. 3A) – could have contributed to starting this outbreak, as will be discussed below. On the BUAP Ecocampus site, two of five *S. romanzoffiana* tested positive for subgroup 16SrIV-D; surprisingly, both of these palms were still alive after 1 year of being diagnosed. While TPPD is generally considered lethal, the work of Ramos Hernández *et al*. (2020) reported an *Attalea butyracea* (Mutis ex L. f.) Wess. Boer surviving for several years after testing positive for subgroup 16SrIV-D. Similarly, survival for years after a LY diagnosis has also been documented in a few coconut palms (Harries 1974). Though the mechanisms by which a palm can survive for long periods after infection are not exactly

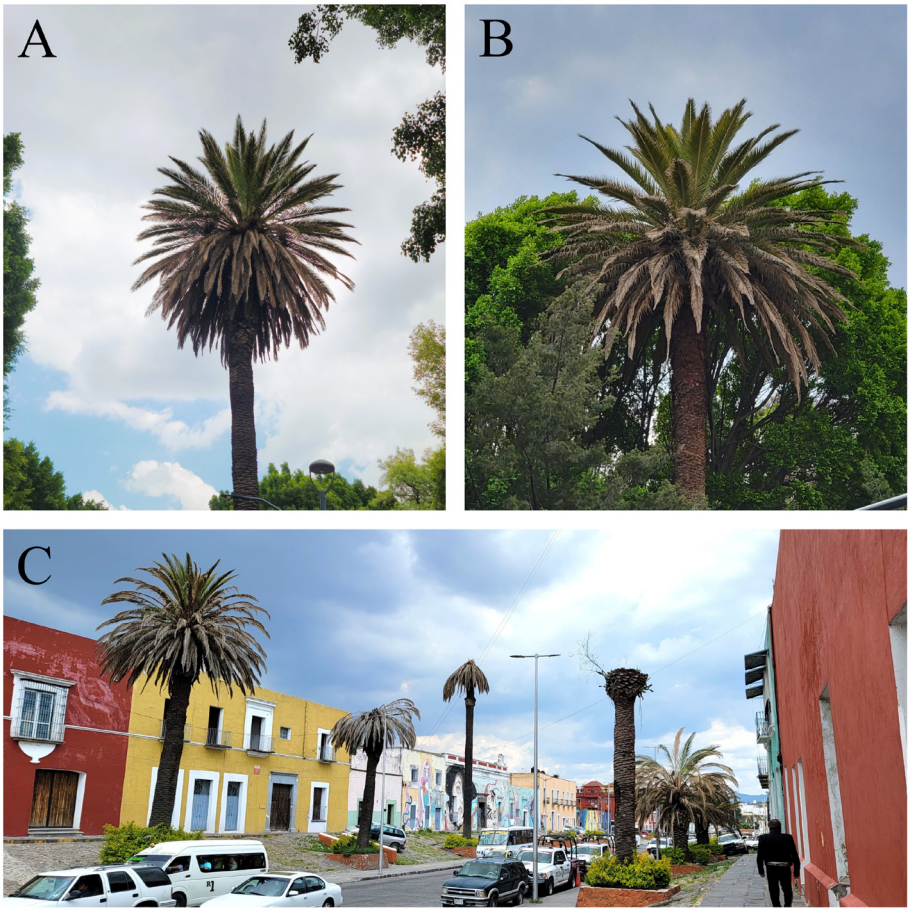

Fig. 5. Other locations in Puebla City with palms exhibiting TPPD-like symptoms: (A–B) *Phoenix canariensis* with flower necrosis and chlorosis of basal and intermediate leaves in Paseo Bravo Park; P. *canariensis* in various stages of decline in the 14 Oriente Ave site – C

known, it could indicate that, under some conditions, *S. romanzoffiana* palms can act as long-term inoculum sources for the 16SrIV-D phytoplasma. *S. romanzoffiana* palms in this site – a total of 98 individuals – were acquired from a nursery in the neighboring state of Morelos and transplanted in August 2017. Thus, considering the above, it is possible that some of these palms were already infected with subgroup 16SrIV-D while still at the nursery since these facilities are prone to harboring 16SrIV-D phytoplasmas as Bahder *et al.* (2019) demonstrated in Florida. Overall, it is possible that both phytoplasma subgroups could have been imported to Puebla from nearby states like Morelos and Veracruz, which are important *S. romanzoffiana* producers (Figueroa-Rodríguez *et al.* 2020), due to the rise in popularity of the queen palm as an ornamental plant in urban areas of central Mexico (authors' personal observations).

Management of outbreaks of these diseases is difficult as there are no cost-effective curative treatments for any of them to date. In Puebla, TPPD continued to spread from the initial disease focus in Analco Park to other areas in the city's historical center that contain numerous mature *P. canariensis*, such as Paseo Bravo Park and 14 Oriente Ave. (symptoms examined in August 2023; Fig. 5). Spread of this disease from palm to palm is indicative of the presence of a vector, however, very little is known about possible vector species for these phytoplasmas in Puebla and other cities in regions like Morelia and Mexico City, which have had similar outbreaks. Considering the above, a survey of palm-associated Auchenorrhyncha insects in the region – including, but not limited to *H. crudus* – should be prioritized in order to identify putative vectors of these phytoplasmas and develop better on-site control measures.

In conclusion, this study reports the first occurrence of TPPD and LY, in the state of Puebla, Mexico. The agents associated with these diseases have now been reported to be capable of subsisting in the subtropical highland climate (Köppen: *Cwb*) that predominates in the Megalopolis of Central Mexico, at altitudes averaging 2200 m (Ortiz-García *et al.* 2024; this study). Adding to these reports are other recent records of the

16SrIV-D phytoplasma infecting *P. canariensis* in the arid (*BWh*) and semi-arid (*BSh*) climates of the cities of Torreon (Palma-Cancino *et al*. 2020) and Queretaro (Vergara-Pineda *et al*. 2023), respectively. Therefore, these pathogens should be considered a threat to palm cultivation in all major climate types of Mexico, that is, tropical, arid and temperate climates. Appropriate quarantine regulations should be established for the interstate commerce of ornamental palms to prevent further outbreaks. Lastly, in light of all the evidence indicating an aggressive expansion of the geographical distribution of TPPD in Mexico and the USA in recent years (Bahder *et al*. 2019; Ferguson *et al*. 2020; Ramos Hernández *et al*. 2020; Ortiz-García *et al*. 2024; this study), it is suggested that TPPD should be considered an emergent disease that could potentially spread to other territories given the ample presence of *P. canariensis*, *S. romanzoffiana* and the insect vector *H. crudus* in most of the Americas.

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