Full Length Research Paper

Changes observed in *Pritchardia pacifica* palms affected by a lethal yellowing-type disease in Mexico

María Narváez[1,2], Elizabeth Ortíz[2], Carmen Silverio[1], Jorge M. Santamaría[1], Francisco Espadas[1] and Carlos Oropeza[1*]

[1]Centro de Investigación Científica de Yucatán, Mérida, Yucatán, México.
[2]Instituto Tecnológico de Mérida, Mérida, Yucatán, México.

Lethal yellowing (LY) is a devastating disease, affecting more than 35 palm species. One of them, *Pritchardia pacifica*, was found to be very susceptible in Florida and it is affected by a LY-type syndrome in Yucatan, Mexico. In this study, plants of *P. pacifica* were exposed naturally to feral insects in an area affected by LY in Mexico. All the plants exposed died showing symptoms of leaf decay with death occurring within approximately four months after first symptom. Real-time (polymerase chain reaction) PCR analysis of DNA extracts for phytoplasma detection and *in silico* sequence analysis of nested-PCR amplicons showed an association with 16SrIV-D phytoplasmas in plants studied. Time-course changes were studied for the detection of phytoplasmas, stomatal conductance and leaf temperature by thermography. One month before appearance of the first symptom, changes in all the parameters were observed: positive detection of phytoplasma DNA, a reduction in stomatal conductance that is complete and irreversible, and the appearance of a peak in leaf temperature. These results provide insights into a better understanding of this disease in *P. pacifica*. Moreover, from a practical point of view, the changes described could be useful for developing methods for early detection of the disease.

Key words: Lethal yellowing-type disease, phytoplasmas, *Pritchardia pacifica*, stomatal conductance, thermography.

INTRODUCTION

Lethal yellowing (LY) is a devastating disease associated with phytoplasmas, identified as the causal agent when they were found in phloem cells of coconut plants (*Cocos nucifera*) with symptoms of LY, through observations using transmission electron microscopy (Beakbane et al., 1972; Heinze et al., 1972; Plavsic-

Banjac et al., 1972) and observation of differential remission of symptoms in symptomatic plants treated with oxytetracycline antibiotics and penicillin (McCoy, 1972; Hunt et al., 1974). Actually, LY affects more than 35 species of palms (Harrison and Oropeza, 2008). According to McCoy et al. (1983), the most susceptible

are coconut, *Phoenix dactylifera* and *Pritchardia* species, including *Pritchardia pacifica*. In studies carried out in Florida (USA) using insect-proof cages, the transmission of LY was achieved with the insect *Haplaxius crudus* (previously known as *Myndus crudus*) to young palms of different species selected for their susceptibility, including coconut and *P. pacifica* (McCoy et al., 1983; Howard, 1984; Harrison and Oropeza, 2008). When these studies were carried out, the molecular techniques to identify taxonomically, the phytoplasmas causing the disease in different palms were not available. At present, the phytoplasmas of 16SrIV group are accepted as the causal agent of LY and similar diseases, based on the detection and identification of their DNA with PCR and amplicon sequencing (Ntushelo et al., 2013). In Mexico, the presence of three sub-groups of 16SrIV were reported: 16SrIV-A in coconut, *Thrinax radiata, Coccothrinax readii* (Narvaez et al., 2006), *Roystonea regia* and *Acrocomia mexicana* (Narvaez et al., 2015); 16SrV-B in coconut (Harrison and Oropeza, 1997); and 16SrV-D in *Carludovica palmata* (Cordova et al., 2000), *Pseudophoenix sargentii* and *Sabal mexicana* (Vázquez et al., 2011).

Since coconut is the most economically important palm species affected by LY, it is also the palm species in which LY studies have been more extensive. The LY symptoms observed in coconut are: first all nuts drop within days, followed by necrosis of inflorescences, then yellowing of leaves starts with the oldest leaves progressing to younger leaves, and finally loss of all leaves leaving the bare trunk (McCoy et al., 1983). During the development of these visual symptoms, related physiological and biochemical changes occur, such as the reduction of stomatal conductance before the appearance of the first symptom which becomes complete and irreversible (León et al., 1996; Maust et al., 2003). These authors proposed that LY-induced stomatal closure is central to the development of LY symptoms in coconut palms and leaf yellowing is part of a leaf senescence process, only that instead of one leaf senescing approximately each month in non-infected palms, this rate is about ten times faster in LY affected palms (Leon et al., 1996). Also, with the closure of the stomata, transpiration and its refreshing effect is reduced, thus, an increase in the temperature of the leaf would be expected, which could be detected by thermography as reported in other species (Jones and Schofield, 2008; Oerke and Steiner, 2010).

Other species have also been important for the study of LY, as it is the case of *P. pacifica* young palms that were used as a model palm for research on LY transmission given its high susceptibility (Howard et al., 1984). Casual observations at the gardens of the Centro de Investigación Científica de Yucatán (CICY) in Merida, Yucatan, Mexico, showed that *P. pacifica* plants could develop an LY-type syndrome and die (CICY, unpublished results). Also, a preliminary analysis of DNA

from one of these plants by BLAST suggested that a 16SrIV-D phytoplasma could be associated with this occurrence (Cordova et al., 2014).

The present study was carried out with the objective of extending the understanding of this association, including the evaluation of changes in physiological parameters during the development of LY-type syndrome in *P. pacifica* plants, and show if these could be useful for early detection of infection by 16SrIV phytoplasmas in palms.

MATERIALS AND METHODS

Biological material

Two batches of *P. pacifica* plants were used. The first batch consisted of nine plants in pots (soil/compost 60/40 w:w) of approximately one year of age, located in the nursery "Palma Real" (Temozon Norte Merida, Yucatan, Mexico, 21°3'48.5"N, 89°36'39.7"W), all of which showed symptoms similar to those of LY. These plants remained in the nursery and were used only for taking leaf samples to determine the presence of phytoplasma DNA by real-time PCR and identification of phytoplasmas found. The second batch consisting of 22 asymptomatic plants in pots (soil/compost 60/40 w:w) of approximately one year of age was obtained from another nursery "Xochimilco" (Santa Gertrudis Copo, Merida, Yucatan, Mexico, 21°1'46.8"N, 89°34'28.8"W) and analyzed by real-time PCR to determine if they were infected or not. Once confirmed that they were negative, free of phytoplasma infection, a set of 17 plants were transferred to a single site on the lawns of the gardens of CICY in Yucatan, Mexico, where LY is present. They were kept under natural conditions without a cover and irrigated with tap water twice a week. This way, plants were exposed naturally to feral insects to facilitate phytoplasma infection. These plants were then evaluated every month during the period of September 2013 to February 2014, for symptom development, analysis by real-time PCR, stomatal conductance and thermography. The other five plants of this second batch were placed within an insect-proof cage 1 to avoid contact with insects and kept free of phytoplasma infection. Another set of five plants, those from the first batch that were infected with phytoplasmas and symptomatic were placed within an insect-proof cage 2, with the purpose of keeping them under the same conditions of those plants within cage 1. The cages were kept under natural conditions and plants were irrigated with tap water twice a week. These two sets of caged plants were used for comparative diurnal evaluation of stomatal conductance.

Symptom development

In order to describe the development of symptoms in affected palms in the second batch, visual observations were carried out continuously during the period of the study to define the changes observed and the chronology of appearance.

Extraction of DNA and detection of phytoplasma by real-time PCR

Leaf tissue samples were taken from symptomatic and asymptomatic plants. DNA was extracted from 1 g of each leaf tissue sample using the CTAB protocol (Doyle and Doyle, 1990). Detection of DNA phytoplasma was done using the real-time

PCR/Taqman LY16S assay reported by Cordova et al. (2014) with modifications. The reaction mix was 20 µl of final volume containing 1 µl of DNA sample (50 ng/µl), 1 µl of TaqMan Probe 503Sr (250 nM) and 2 µl of the Universal PCR Master Mix with AmpErase Uracil N-glycosylase (UNG) (Applied Biosystems USA), 1 µl of primer mix containing 900 nM of each primer. The equipment used for the detection was a Rotor-Gene Q - QIAGEN thermal cycler. Program conditions were 50°C for 2 min (1 cycle), 95°C for 10 min (1 cycle), 95°C for 15 s and 61°C for 1 min (40 cycles). All DNA samples including controls were assessed in duplicate. The threshold cycle (Ct) value of each PCR reaction was set manually intersecting the exponential phase of the amplification curves and the baseline was automatically set by Rotor-Gene Q manager software (QIAGEN). The Ct cut-off value was determined by preparing a standard curve by sequential 10-fold dilutions of control DNA sample (extracts positive to Real-time detection), each dilution was tested in triplicate and the curve was carried out six times. Negative controls included were DNA samples (extracts negative to real-time detection) and water.

Identification of phytoplasma strain

DNA was taken from 7 plants (2 from first batch and 5 from the second batch) that were positive to the detection of phytoplasmas by real-time PCR and used to determine the type of phytoplasma present. Corresponding amplicons were obtained by nested-PCR. In the first reaction, the primers P1 (Deng and Hiruki, 1991) and P7 (Smart et al., 1996) were used and 2 µL of the DNA extract (50 ng) product, was used as template in the second reaction and the primers used were LY16Sf/LY1623Sr (Harrison et al., 2002), finally obtaining a product of approximately 1.7 Kb. Amplification conditions for the first and second reactions for nested-PCR were as previously described by Harrison et al. (2001, 2002). The amplified products were cloned in the PGEM-T Easy Vector (Promega) and *E. coli* top 10 (Invitrogen), and sent for sequencing to the University of California (The College of Biological Sciences UCDNA Sequencing Facility). The sequences obtained were compared in the database of the NCBI-BLAST. F2n/R2 partial sequences (Gundersen and Lee, 1996) from the 1.7 Kb sequences of two *P. pacifica* plants (Acc. KY508691, KY508693) were subjected to virtual RFLP analysis, using iPhyclassifier software and *in silico* digestion with 17 restriction enzymes (AluI, BamHI, BfaI, BstUI, DraI, EcoRI, HaeIII, HhaI, Hinfl, Hpal, HpaII, KpnI, Sau3AI, MseI, RsaI, SspI and TaqI). The F2N/R2 sequences obtained in the present study and other equivalent sequences of phytoplasmas associated with LY type diseases in palms were used to construct a phylogenetic tree by the method of neighbor-joining and a bootstrap value of 2000, using MEGA5.05 (Tamura et al., 2011) software. *Acholeplasma palmae* was used as external group.

Stomatal conductance and thermography

Stomatal conductance (mmol m^{-2} s^{-1}) was calculated as the inverse of diffusive stomatal resistance, measured *in situ* with a LI-COR LI-1600 porometer on the surface of the leaves or parts of leaves that were not affected. Two assays were carried out. For the first assay, measurements were taken throughout the day (at 6:00, 9:00,13:00, 18:00 and 20:00 h) in five asymptomatic plants (real-time PCR negative) and five plants with symptoms (real-time PCR positive). These two sets of palms were kept separated, each within insect-proof cages 1 and 2, respectively. For the second assay, the measurements were taken in the morning between 10:00 and 12:00 am, and in monthly determinations throughout the development of the disease in 17 plants. At the beginning of the assay, all the plants were asymptomatic (real-time PCR negative). In this second

assay, the temperature of the surface of the canopy foliage was also recorded in the 17 plants with a thermographic camera (ICI Prodigy 640, thermal resolution of 640 x 480) and the environmental temperature was measured independently. For the analysis, the difference between both temperatures was taken into consideration (ΔT = temperature by thermography – environmental temperature). Each ΔT value of the thermography reported for a plant was the average of five points, each one taken from a different leaf. For analysis of the different parameters, the statistical method used to calculate the differences between means of each point in the graph was Holm-Sidak with a test of ANOVA statistical package of SigmaPlot. The set of five in cage 1 (asymptomatic and real-time PCR negative) used for the first assay were kept as control plants (not exposed to feral insects) for the second assay.

Comparative analysis of results of plants in the second batch

Plants were analyzed for different parameters (real-time PCR detection, stomata conductance and thermography) monthly, and once the symptom appeared, follow up continued further for three months. Due to the fact that symptoms started at a different time for each plant, for the purpose of comparative analysis of the parameters studied between all plants, the monthly data obtained for each plant was equated based on alignment of the timeline at the moment of appearance of first symptom. Therefore, the results are presented in the following chronology three months before appearance of first symptom and three months later.

RESULTS

Symptoms observed in the plants

The *P. pacifica* plants exposed naturally to feral insects in the gardens of CICY showed no immediate LY-type symptoms (Figure 1A). The appearance of the first symptom after exposure, took between one and three months depending on the plant, and this was followed by a progressive development of symptoms of leaf decay syndrome. The time lapse between the appearance of the first symptom and death of the plants was from 3 to 4 months. Development of symptoms was in the following manner: the first visual symptom was damage on the spear leaf, hindering subsequent aperture of this leaf in some cases, depending on the degree of damage (Figure 1B), this was followed by yellowing of mature leaves (Figure 1C) and emission of new leaves stopped in the palm, resulting in reduction of foliage (Figure 1 D). In most cases, yellowing occurs first in only one part of the leaf, the area becomes brown and this pattern extends to the rest of the leaf, which subsequently dries up and dies. Eventually, all the leaves are affected and the plant dies (Figure 1E). In some cases, yellowing occurs simultaneously in several leaves, affecting the whole leaf; the leaves then turn brown and die. In the case of plants in cage 1 (asymptomatic and real-time PCR negative) that were unexposed to feral insects throughout the whole study, no symptom was developed.

Detection by PCR of phytoplasma DNA

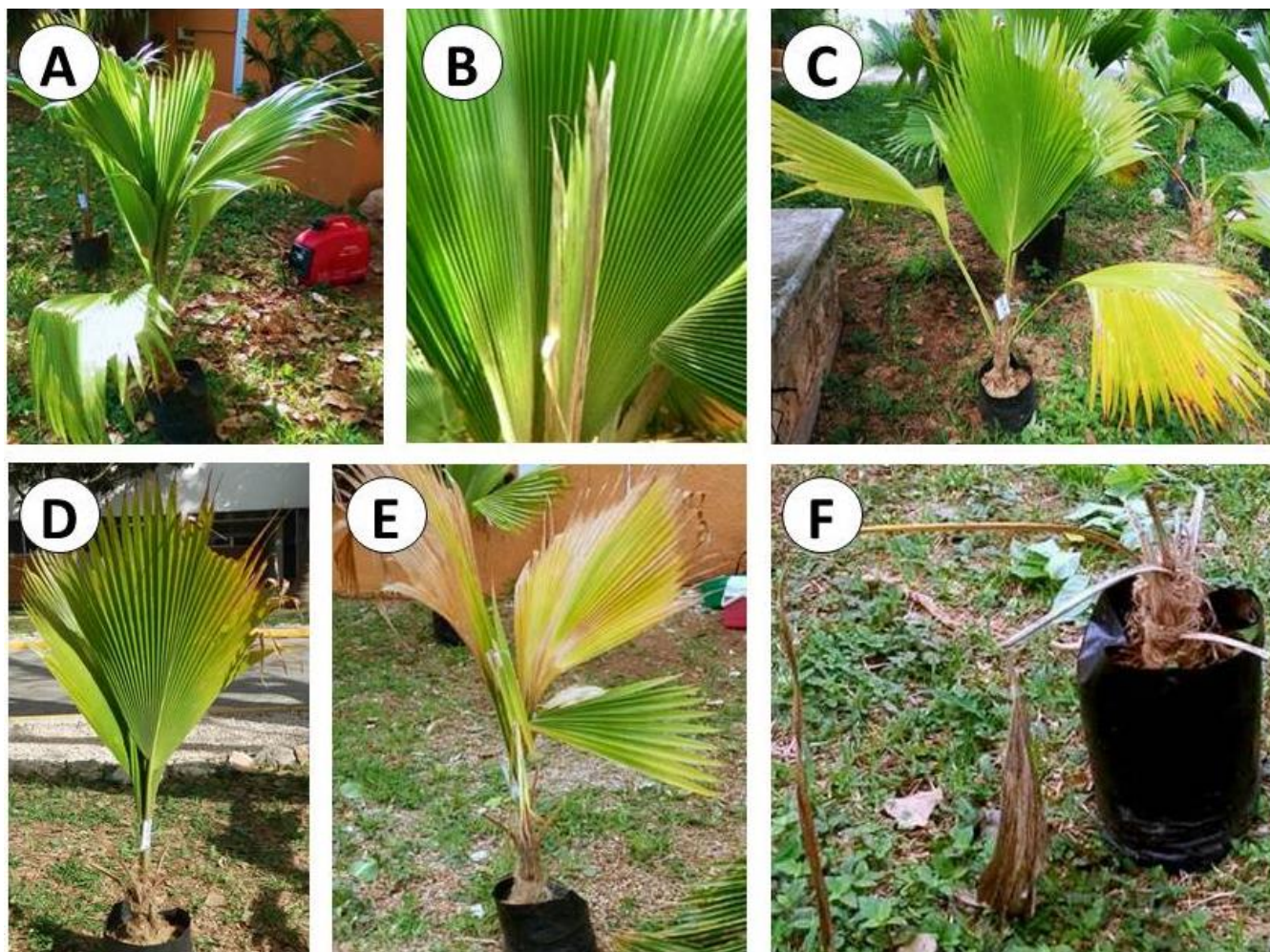For the detection of phytoplasma DNA by real-time PCR,

Figure 1. Development of symptoms of a lethal yellowing-type syndrome as observed in *P. pacifica* plants. **(A)** Asymptomatic plant. **(B)** appearance of the first symptom, damage in the spear leaf. **(C)** Yellowing of the older leaves. **(D)** Loss of the older leaves. **(E)** Yellowing of younger leaves. **(F)** Loss of young leaves and death of the palm.

a Ct cut-off value of 32 ± 0.13 was determined and values above were negative. In the first batch of nine plants at Palma Real Nursery, when monitoring started, four were already symptomatic and five were asymptomatic; the latter began to show symptoms within a month, and within four months, all of them died. The symptoms observed were also leaf decay syndrome as described earlier, and all the plants were positive to the detection of phytoplasma DNA by real-time PCR. In the case of the second batch of plants, all were real-time PCR negative and symptomless at the beginning of the study, and were exposed naturally to feral insects in the gardens of CICY. Each of them developed symptoms of leaf decay syndrome. The data presented here is reported within the following time frame: zero time denotes the moment of appearance of the first symptom (to chronologically equating development of symptoms and corresponding measurements); -3, -2 and -1 are

months before the appearance of the first symptom and +1, +2 and +3 are months after the appearance of the first symptom. Regarding real-time PCR in month -1, there was a positive detection of LY 16SrIV phytoplasmas with an average Ct value of 29.6 (Figure 2A). This initial detection took place because presumably, the titre of phytoplasmas increases with time to become detectable, and continues increasing as shown by a decrease of Ct value to 25.3 at time zero (time of appearance of first symptom). CT value remained with very little change afterwards (Figure 2A). The difference between average negative Ct values and average positive Ct values was significant.

Identification of the phytoplasma affecting *P. pacifica*

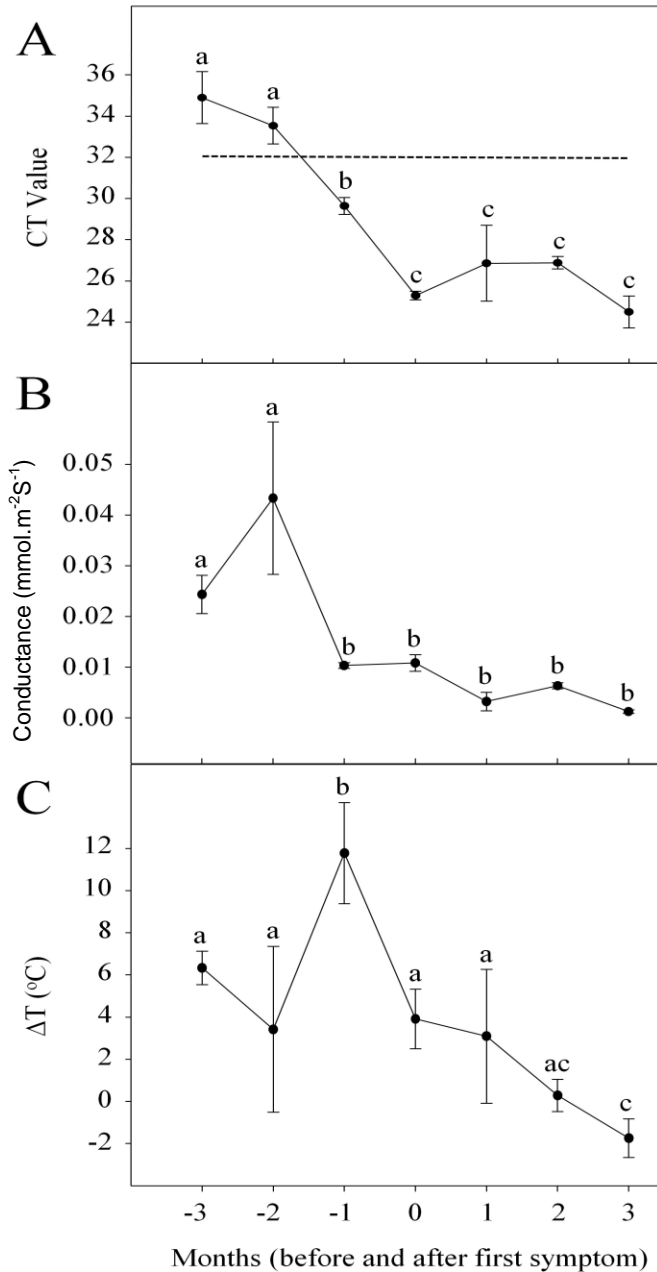Seven sequences were obtained from the products of the

Figure 2. Analysis of three parameters in *Pritchardia pacifica* plants exposed to *Haplaxius crudus* in a site with LY incidence during the different developmental stages of the disease. **(A)** LY detection by real-time PCR. Broken line denotes detection threshold, above CT 32 is negative, and below is positive. **(B)** Leaf stomatal conductance. **(C)** Thermography. Time zero denotes when the first visual symptom was observed. Similar letters have no significant differences, different letters have significant differences (P = 0.001).

amplification of seven DNA extracts from diseased plants (Accessions KY508690, KY508691, KY508692, KY508693, KY508694, KY508695 and KY508696). These sequences were compared in the NCBI-BLAST database and high similarity was obtained (99.56 to 99.94%) with the sequences of the phytoplasmas in *S. palmeto* (Accession HQ613895.1) and *P. dactylifera* (Accession AF434989.1) classified within the subgroup 16Sr-IV-D. Virtual RFLP analysis was carried out and the pattern (Accession KY508691, Figure 3B) was very
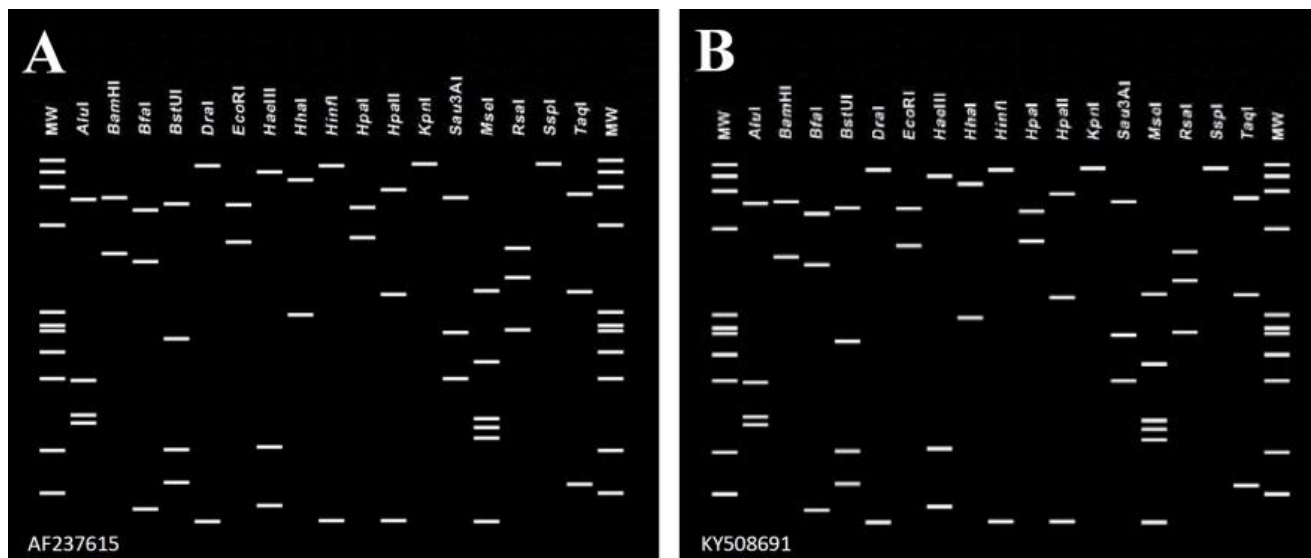
Figure 3. Virtual RFLP patterns derived from *in silico* digestions, using iPhyclassifier software. **(A)** Reference pattern of 16Sr group IV, subgroup D, accession: AF237615. **(B)** Pattern of *P. pacifica*, accession: KY508691.

similar (similarity coefficients of 1.0) to reference pattern of *Carludovica palmata* 16Sr group IV, subgroup D (Acc. AF237615 (Figure 3A). Phylogenetic analysis carried out on the sequences obtained from *P. pacifica*, showed that these are grouped in the clade corresponding to the subgroup 16SrIV-D (Figure 4).

Stomatal conductance

First stomatal conductance was evaluated comparatively between healthy palms (asymptomatic, negative real-time PCR) and diseased palms (symptomatic, positive real-time PCR, one month after the appearance of the first symptom). In healthy palms, conductance showed a typical diurnal fluctuation throughout the day, at 6 am, it was 0.0146 mmol m^{-2} s^{-1}, at 13 pm, it increased to a maximum of 0.038 mmol m^{-2} s^{-1} and at 18 pm, it reduced to 0.00057 mmol m^{-2} s^{-1} (Figure 5). Conductance in diseased palms showed very small diurnal fluctuation with values ranging between 0.01 at (9 am) and 0.0028 mmol m^{-2} s^{-1} (18 pm) (Figure 5).

Also, stomatal conductance was evaluated during the development of the disease, (Figure 2B) and there was a reduction of about 80% from month -2 to month -1 (values dropped from 0.051 to 0.01 mmol m^{-2} s^{-1}). From then on, conductance values remained low (between 0.01 and 0.001 mmol m^{-2} s^{-1}) (Figure 2B). The differences between month -3 and -2 were not significant (P = < 0.001); however, between these and the following months (-1 to +3), they were significant (P = < 0.001).

Thermography

The measurements of ∆T throughout the development of

the disease showed a peak (∆T 11.78°C) at month -1, before the appearance of the first symptom, ∆T values after and before month -1 (0.28 to 6.33°C) showed no significant differences between them; in contrast, there was a significant difference with the value for month -1 (P=<0.001) (Figure 2C).

DISCUSSION

In casual observations, it was found that *P. pacifica* plants at CICY's gardens developed a LY-type syndrome and analysis of DNA of one of these plants by BLAST suggested that a 16SrIV-D phytoplasma could be associated with it (Cordova et al., 2014). Further studies to extend the understanding of this association were carried out and are reported in this study.

The first part of the study was on a batch of nine of *P. pacifica* plants in pots of approximately one year of age at Palma Real Nursery that was found to be already showing symptoms of leaf decay. DNA samples from the leaves were analyzed by real-time PCR specific for 16SrIV phytoplasmas and found positive. Then, another batch, in this case 17 *P. pacifica* plants from another site, (Xochimilco nursery) that were asymptomatic and negative for the detection of phytoplasma DNA by real-time PCR, were exposed naturally to feral insects in the gardens of CICY. They developed leaf decay symptoms similar to those of the first batch. Analysis by real-time PCR of DNA from these plants also showed positive detection of 16SrIV phytoplasmas. Amplicons obtained sequenced and after the sequences, were subjected to BLAST; high analogy was found for 16SrIV-D strains and this was further supported by virtual RFLP and phylogenetic analyses. All the sequenced amplicons from
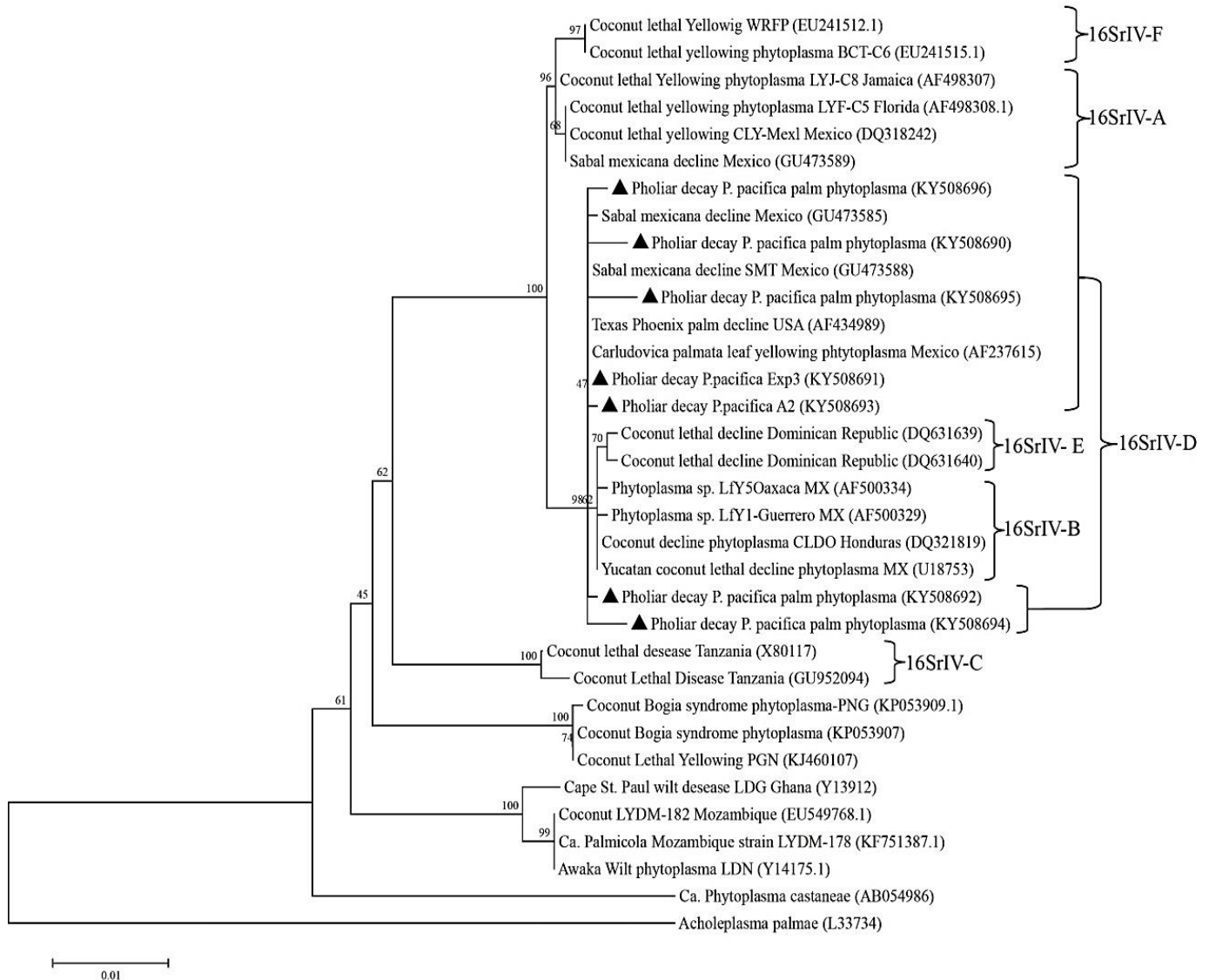
Figure 4. Phylogenetic tree constructed using the method of neighbour-joining (bootstrap: 2000) with 34 sequences of l6Sr RNA gene representative of the phytoplasmas associated with LY in coconut plants and other palm species, including the sequences of phytoplasmas isolated from *Pritchardia pacifica* plants in this study (▲) and from the root of *Acholeplasma palmae*.

DNA samples of plants from both sites (Palma Real Nursery and CICY) were all of the subgroup 16SrIV-D only. This is interesting since the sites are far away (8 km) from each other, and in CICY's gardens, 16SrIV phytoplasmas of subgroups 16SrIV-A and 16SrIV-D were found in different palm species (Vázquez et al., 2011). Thus, suggesting a possible selective interaction between *P. pacifica* plants and 16SrIV-D phytoplasmas, as found for LY-type syndromes in palm species studied in Yucatan, *T. radiata* was found to be associated specifically with 16SrIV-A and *S. mexicana* associated with 16SrIV-D phytoplasmas. However, this occurrence needs to be further studied, for instance, on samples from symptomatic *P. pacifica* plants from more sites and farther.

Follow-up of the development of symptoms of plants in the second batch exposed naturally to feral insects showed that they started appearing after three months of exposure. Chronologically, these symptoms included initial damage of the spear leaf, yellowing of mature leaves, yellowing of younger leaves, yellow leaves turning brown with reduction of foliage and finally, the palm death. As in the case of the first batch, all palms of this second batch also died, suggesting that they are very susceptible, as observed previously in Florida (McCoy et al., 1983; Howard, 1984; Harrison and Oropeza, 2008). According to the literature, the symptoms of palms affected by LY can vary according to the palm species
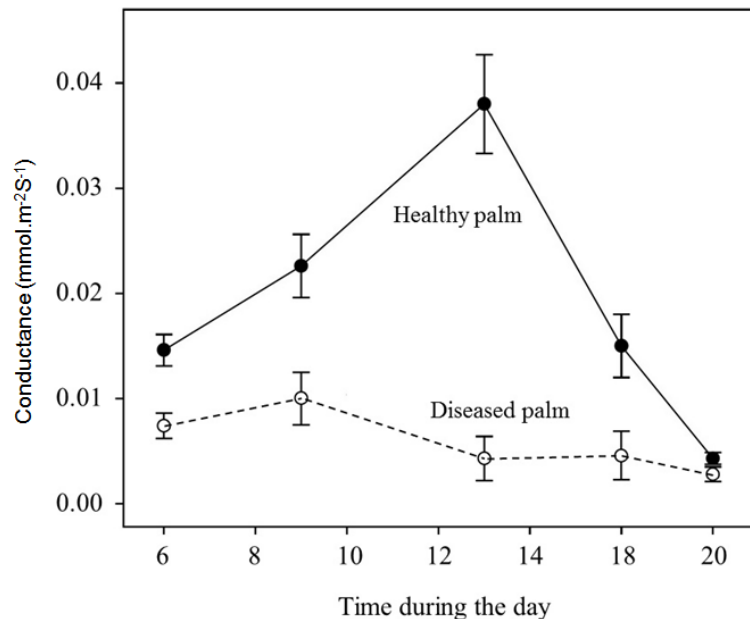
Figure 5. Daytime stomatal conductance in healthy *P. pacífica* plants (asymptomatic, negative real-time PCR) and diseased plants affected by LY (symptomatic, positive real-time PCR, at one month after the appearance of the first symptom).

and strain of phytoplasma affecting it (Harrison and Oropeza, 2008). The leaf decay symptoms observed in the *P. pacifica* plants in the present case, were similar to those reported for *P. dactylifera* associated with 16SrIV-D phytoplasmas (Harrison et al., 1995; Harrison and Oropeza, 2008) and *Washingtonia robusta* associated with 16SrIV-F phytoplasmas (Harrison et al., 2008), in which collapse of the spear leaf preceded yellowing of the leaves. In the case of the leaf decay syndrome of *P. pacifica* in this study, the time lapse between the appearance of the first symptom and death of the plant was from 3 to 4 months, a slightly shorter period of time than that reported for mature coconut palms, 3 to 6 months (Arellano and Oropeza, 1995). Unfortunately, these observations cannot be compared with those of either leaf decay syndrome in adult *P. pacifica* palms, or LY syndrome on young coconut palms, because there are no reports available for such types of cases.

Phytoplasma diseases can affect the physiology of stomata and according to Matteoni et al. (1983), plants with phytoplasma diseases that are lethal show complete closure of stomata even at the time when stomata of healthy plants are open, and in plants with phytoplasma diseases that are not lethal, stomata closure was only partial. In coconut plants affected by LY, there is a reduction of stomatal conductance that starts before the appearance of the first symptom and is complete and irreversible (León et al., 1996; Maust et al., 2003). In the present study, *P. pacifica* plants affected by the leaf decay disease were evaluated to know whether there are

changes in stomatal conductance, and leaf temperature. Stomatal conductance in healthy plants was monitored throughout the day and a normal diurnal variation was observed, increasing during the day and decreasing in the afternoon and night, indicating that the stomata are functional. This stomatal behavior is similar to that reported for healthy coconut palms (León et al., 1996). In contrast, in diseased *P. pacifica* plants, no variation were observed in stomatal conductance; remained low throughout the whole day, which indicates a disease-related stomatal closure. Moreover, when stomatal conductance was monitored during the development of the disease symptoms, it decreased in affected plants in a complete and irreversible manner one month before the appearance of the first symptom. These changes are similar to those observed in coconut palms during the development of the LY syndrome, and (together with other biochemical and physiological changes) are believed to be the result of a hormonal imbalance caused by the phytoplasma infection (León et al., 1996; Martinez et al., 2000; Aguilar et al., 2009).

Since the closure of the stomata could reduce plant transpiration and its refreshing effect, leading to an increase in leaf temperature (Jones and Schofield, 2008; Oerke and Steiner, 2010), changes in leaf temperatures (ΔT) in *P. pacifica* plants were evaluated throughout the development of the disease. There was an increase (of about 5°C) one month before the appearance of the first symptom, but it peaked, and decreased again to the previous level by the time of appearance of the first

symptom. Similar changes have been observed in *Nicotiana tabacum* plants infected by the tobacco mosaic virus (Chaerle et al., 1999). The authors reported that the initial increase in temperature was as a result of reduced stomatal conductance and transpiration, but they associated the following decrease in temperature to water loss caused by cell death occurring as part of plant defense. In the case of *P. pacifica* plants infected by 16SrIV-D phytoplasmas, very fast leaf decay took place with noticeable damage in the leaf tissues and its integrity, and probably allowing evaporation to take place. These changes in stomatal conductance and leaf temperature of *P. pacifica* plants occurred before the appearance of the first visual symptom, and at the moment when there was already positive real-time PCR detection of phytoplasmas. Based on these results, it is suggested that changes in stomatal conductance and the resulting changes in leaf temperature in plants of *P. pacifica* with leaf decay syndrome, are result of the presence of pathogen in infected plants, as previously proposed for LY phytoplasmas in coconut (León et al., 1996) and tobacco mosaic virus in tobacco (Chaerle et al., 2007).

In conclusion, the results reported herein supported that the *P. pacifica* diseased plants showing leaf decay syndrome are associated with 16SrIV-D phytoplasmas, contributing in particular, to the understanding of this syndrome affecting *P. pacifica* plants in Mexico, and in general, the mode of action of 16SrIV phytoplasmas in palms. In addition, from a practical point of view, the fact that the changes in the parameters studied occur before the appearance of the first visual symptom, this could be useful for the development of methods for early detection of the disease in *P. pacifica* or 16SrIV phytoplasma diseases in other palm species. In particular, thermography can be helpful in registering increase in temperature in asymptomatic infected plants during surveillance of commercial plantations, for instance, by means of remote sensing equipment mounted on drones (Chen et al., 2015; Mahlein, 2012), and followed by immediate testing of the presence of phytoplasmas by real-time PCR and corresponding actions to stop spread of the disease. Finally, it is very important to extend the studies reported here to a larger population of plants and other palm species to determine how universal they can be.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

REFERENCES

Aguilar ML, Espadas F, Maust B, Sáenz L (2009). Endogenous cytokinin content in coconut palms affected by lethal yellowing. J. Plant Pathol. 91(1):141-146

Arellano J, Oropeza C (1995). Lethal yellowing. In *Lethal Yellowing: Research and Practical Aspects.* Springer Netherlands. pp. 1-15.

Beakbane B, Slater CH, Posnette, AF (1972). Mycoplasmas in the Phloem of Coconut, *Cocos Nucifera* L., with Lethal Yellowing Disease. J. Hortic. Sci. 47: 265-265.

Chaerle L, Leinonen I, Jones, HG, Van Der Straeten D (2007). Monitoring and screening plant populations with combined thermal and chlorophyll fluorescence imaging. J. Exp. Bot. 58:773-784.

Chaerle L, Van Caeneghem W, Messens E, Lambers H, Van Montagu M, Van Der Straeten D (1999). Presymptomatic visualization of plant–virus interactions by thermography. Nature Biotechnol. 17: 813-816.

Chen R, Crawford K, Davis C, Ehsani R, Vougioukas S, Upadhyaya S (2015). The drones are coming. Unmanned aerial vehicles for agriculture. Citrograph. Mag. 6:18-23.

Cordova I, Oropeza C, Almeyda H, Harrison NA (2000). First report of a phytoplasma-associated leaf yellowing syndrome of palma jipi plants in southern México. Plant Dis. 84:807.

Córdova I, Oropeza C, Puch-Hau C, Harrison N, Collí-Rodríguez A, Narvaez M, Sáenz L (2014). A real-time PCR assay for detection of coconut lethal yellowing phytoplasmas of group 16SrIV subgroups A, D and E found in the Americas. J. Plant Pathol. 96:343-352.

Deng S, Hiruki C (1991). Amplification of 16S rRNA genes from culturable and non-culturable mollicutes. J. Microbiol. Meth. 14:53-61.

Doyle JJ, Doyle JL (1990). Isolation of plant DNA from fresh tissue. Focus 12:13-15.

Gundersen DE, Lee IM (1996) Ultrasensitive detection of phytoplasmas by nested-PCR assays using two universal primer pairs. Phytopathol. Med. 35:144-151.

Harrison N, Oropeza C (1997). Recent advances in diagnosis and detection of lethal yellowing in the Americas. In: Eden-Green, S.J. and Ofori, F. (Eds.) International Workshop on Lethal Yellowing-like Diseases of Coconut, Elmina, Ghana, November 1995. Chatham, UK, Natural Resources Institute. pp. 221-234.

Harrison NA, Griffiths HM, Carpio ML, Richardson PA (2001). Detection and characterization of an elm yellows (16SrV) group phytoplasma infecting Virginia creeper plants in southern Florida. Plant Dis. 85:1055-1062.

Harrison NA, Helmick EE, Elliott ML (2008). Lethal yellowing-type diseases of palms associated with phytoplasmas newly identified in Florida, USA. Ann. Appl. Biol. 153:85-94.

Harrison NA, Myrie W, Jones P, Carpio ML, Castillo M, Doyle MM, Oropeza C (2002). 16S rRNA interoperon sequence heterogeneity distinguishes strain populations of palm lethal yellowing phytoplasma in the Caribbean region. Ann. Appl. Biol. 141:183-193.

Harrison NA, Richardson P, Tsai JH (1995). Detection and diagnosis of lethal yellowing: conventional methods and molecular techniques. In: Lethal Yellowing: Research and Practical Aspects. C. Oropeza, F. W. Howard & G. R. Ashburner, (eds.), Kluwer Academic Publishers, Dordrecht. The Netherlands. pp. 79-91.

Harrison NA, Oropeza C (2008). Coconut lethal yellowing. In: NA Harrison, GP Rao and C Marcone (Eds.). Characterization, Diagnosis and Management of Phytoplasmas. Studium Press LLC, Houston, USA. pp. 219-248.

Heinze KG, Schuiling M, Romney DH (1972). The possible cause of lethal yellowing disease of coconut. FAO Plant Prot. Bull. 20: 58-68.

Howard FW, Williams DS, Norris RC (1984). Insect transmission of lethal yellowing to young palms. Int. J. Entomol. 26:331-338.

Hunt P, Dabek AJ, Schuiling M (1974). Remission of symptoms following tetracycline treatment of lethal yellowing infected coconut palms. Phytopathology 63:307-312.

Jones HG, Schofield P (2008). Thermal and other remote sensing of

plant stress. Gen. Appl. Plant Physiol. 34:19-32.

Leon R, Santamaria JM, Alpizar L, Escamilla JA, Oropeza C (1996). Physiological and biochemical changes in shoots of coconut palms affected by lethal yellowing. New Phytol. 134:227-234.

Mahlein AK, Oerke EC, Steiner U, Dehne HW (2012). Recent advances in sensing plant diseases for precision crop protection. Eur. J. Plant Pathol.133:197-209.

Matteoni JA, Sinclair WA (1983). Stomatal closure in plants infected with mycoplasma-like organisms. Phytopathology 73:378-402.

Martínez S, Cordova I, Maust B, Oropeza C, Santamaría J (2000). Is abscisic acid responsible for abnormal stomatal closure in lethal yellowing of coconut palms? J. Plant Physiol 156:319-323.

Maust BE, Espadas F, Talavera C, Aguilar M, Santamaría J, Oropeza C (2003). Changes in carbohydrate metabolism in coconut palms infected with the lethal yellowing phytoplasma. Phytopathology 93:976-981.

McCoy RE (1972). Remission of lethal yellowing in coconut palms treated with tetracycline antibiotics. Plant Dis. Rep. 56:1019-1021.

McCoy RE, Howard FW, Tsai JH, Donselman HM, Thomas DL, Basham HG, Atilano RA, Eskafi FM, Britt L, Collins ME (1983). Lethal yellowing of palms. University of Florida Agricultural Experiment Stations, Bulletin 834. Gainesville, Florida, USA. 100p.

Narvaez M, Cordova I, Orellana R, Harrison NA, Oropeza C (2006). First report of a lethal yellowing phytoplasma in Thrinax radiata and Coccothrinax readii palms in the Yucatan Peninsula of Mexico. Plant Pathol. 55:292-292.

Narvaez M, Córdova-Lara I, Reyes-Martínez C, Puch-Hau C, Mota-Narvaez L, Collí A, Camamal G, Harrison N, Oropeza C (2015). Occurrence of 16SrIV Subgroup A Phytoplasmas in *Roystonea regia* and *Acrocomia mexicana* Palms with Lethal Yellowing-like Syndromes in Yucatán, Mexico. J. Phytopathol. 164:1111-1115.

Ntushelo K, Harrison NA, Elliott M. (2013). Palm phytoplasmas in the Caribbean basin. Palms. 57:93-100.

Oerke EC, Steiner U (2010). Potential of digital thermography for disease control. In Precision Crop Protection-the Challenge and Use of Heterogeneity. Springer Netherlands. pp. 167-182.

Plavsic Banjac B, Hunt P, Maramorosch K (1972). Mycoplasmalike bodies associated with lethal yellowing disease of coconut palms. Phytopathology 62:298-299.

Smart CD, Schneider B, Morrer R, Blomquist DJ, Guerra LJ, Harrison NA, Ahrens U, Lorenze KH, Seemüller E, Kirkpatrick BC (1996). Phytoplasma-specific PCR primers based on sequences of the 16S-23S rRNA spacer region. App. Environ. Microbiol. 62:2988-2993

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S (2011). MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol. Biol. Evol. 10:2731-2739.

Vázquez-Euán R, Harrison N, Narvaez M, Oropeza C (2011). Occurrence of a 16SrIV group phytoplasma not previously associated with palm species in Yucatan, Mexico. Plant Dis. 95:256-262.