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Molecular and biological characterization of phytoplasmas from coconut palms affected by the lethal yellowing disease in Africa

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ABSTRACT

Côte d'Ivoire lethal yellowing (CILY) is a devastating disease associated with phytoplasmas and has recently rapidly spread to several coconut-growing areas in the Country. Phytoplasmas are phloem-restricted bacteria that affect plant species worldwide. These bacteria are transmitted by plant sap-feeding insects, and their cultivation was recently achieved in complex artificial media. In this study, phytoplasmas were isolated for the first time from coconut palm trunk borings in both solid and liquid media from CILY symptom-bearing and symptomless coconut palms. The colony morphology, PCR and sequencing analyses indicated the presence of phytoplasmas from different ribosomal groups. This study reports the first biochemical characterization of two of these phytoplasma isolates. Moreover, a disc-diffusion antibiotic susceptibility assay revealed that these bacteria exhibit tobramycin susceptibility and cephalexin hydrate and rifampicin resistance. Urea and arginine hydrolysis, and glucose fermentation tests that were performed on colonies of phytoplasmas and *Acholeplasma laidlawii* indicated that both phytoplasmas tested were negative for urea and positive for glucose and arginine, whereas *A. laidlawii* was positive for glucose and negative for urea and arginine. The growth of coconut phytoplasmas in both solid and liquid artificial media and the biological characterization of these isolates are novel and important advancements in the field of disease management and containment measures for the CILY disease. The characterization of isolated phytoplasmas will allow for more efficient management strategies in both the prevention of a coconut phytoplasma epidemics and the reduction of the economic impact of the disease in the affected areas.

1. Introduction

Phytoplasmas are phloem-restricted bacteria from the class *Mollicutes* that affect more than one thousand crop and plant species worldwide; these bacteria are transmitted by plant sap-feeding insect species (Weintraub and Beanland, 2006). Since their discovery (Doi et al., 1967) and until recently, phytoplasmas were long reported to be unculturable in cell-free media; thus, they have been characterized only from the genetic perspective. However, the cultivation of phytoplasmas was recently achieved using micropropagated periwinkle infected shoots as a source of phytoplasmas (Contaldo et al., 2012). Later, phytoplasma colonies from naturally phytoplasma-infected grapevine plants were obtained in artificial media (Contaldo et al., 2016).

Côte d'Ivoire lethal yellowing phytoplasma disease (CILY) was shown to be associated with a phytoplasma in 2013 in Grand-Lahou, Côte d'Ivoire (Konan Konan et al., 2014); since then, this disease has rapidly spread to several coconut-growing villages where circa 400 ha were destroyed, and an additional 7000 ha are under threat (Arocha-Rosete et al., 2014). Lethal yellowing (LY)-like diseases of palms have been associated with several phytoplasmas worldwide and have killed millions of palms in the last 40 years (Sullivan and Harrison, 2013).

The CILY phytoplasma was recently classified as a member of group 16SrXXII, subgroup –B, '*Candidatus* Phytoplasma palmicola'–related strain (Harrison et al., 2014); this group also includes the Cape St. Paul

Abbreviations: '*Ca.* P.', '*Candidatus* Phytoplasma'; CFU, colony forming unit; CILY, Côte d'Ivoire lethal yellowing; CSPWD, Cape St. Paul Wilt Disease; CTAB, hexadecyltrimethylammonium bromide; DDSW, distilled, deionize, sterile water; DNA, deoxyribonucleic acid; dNTP, deoxynucleotide triphosphate; ICSB, International Committee for Systematic Bacteriology; LY, Lethal yellowing; MIC, minimal inhibitory concentration; PCR, polymerase chain reaction; rDNA, ribosomal DNA; RFLP, restriction fragment length polymorphism; RNA, ribonucleic acid; rRNA, ribosomal RNA; UK, United Kingdom; UV, ultra violet

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Wilt Disease phytoplasma (CSPWD) strain from Ghana, which collapsed the Ghanaian coconut industry in the last 20 years (Danyo, 2011).

Bila et al. (2015) identified three phytoplasma strains that were associated with the LY in Mozambique, which included '*Ca.* P. palmicola' (16SrXXII-A), the Tanzanian LD strain (16SrIV-C), and a '*Ca.* P. pini' – related strain (16SrXXI-A) found in co-infection with a '*Ca.* P. palmicola' strain. Phytoplasmas of the 16SrI group, '*Ca.* P. asteris'-related, were recently identified from CILY phytoplasma-infected coconut palms from villages in Grand-Lahou (Kwadjo et al., 2017), indicating that mixed phytoplasma infection occurs in coconut palms affected by CILY along the south coastal littoral of Côte d'Ivoire.

The present work was conducted to elucidate some of the biological properties of phytoplasmas isolated from coconut palms affected by the CILY disease. Samples from fifteen coconut palms were collected during 2015 and 2016 and used for the isolation and cultivation of phytoplasmas in artificial media. Data were collected on the colony morphology and the biochemical performance of the phytoplasma strains isolated from these coconut palms, which were previously confirmed to be CILY phytoplasma-infected. These results represent the first steps of phytoplasma species description, following the ICSB minimal standard criteria (1995).

2. Material and methods

2.1. Plant material

During 2015 and 2016, surveys were carried out in the following seven coconut-growing villages of Grand-Lahou: Baffredon, Badadon, Palmindustrie V1, Yaokro, Doudougbauzou, Adjadon and Amanikro (Fig. 1).

Thirty-nine samples of leaves, inflorescences, and trunk borings were collected from 15 coconut palms of West African Tall (WAT) or PB121 (Malayan Yellow Dwarf × WAT) ecotypes that were approximately 30 years old and exhibited CILY symptoms corresponding to the disease stages 1, 2 and 3 (Table 1) (Arocha Rosete et al., 2017); these ecotypes were representative of those cultivated in the area. A sample from one symptomless palm was also collected from each village to serve as a negative control. Trunk borings were obtained using an 8-cm drill bit sterilised in 70% ethanol prior to each sample collection. The trunk was also surface-sterilized at approximately 1.20 m from the soil, where it was drilled, and the sawdust was collected in a sterile plastic bag that was placed in a cool container until it reached the laboratory. The sawdust was then transferred in a sterile manner to sterile polycarbonate tubes (50 mL) and maintained under cool conditions (6 to 8 °C) until further processing.

2.2. PCR and RFLP analyses

Total DNA was extracted from 1 g of each sample type using the CTAB (Cetyl trimethylammonium bromide) method (Angelini et al., 2001). Phytoplasma DNA was amplified by PCR using the 16S rDNA universal primer pairs R16F2n/R2 (Gundersen and Lee, 1996) nested with U5/U3 primers (Lorenz et al., 1995). Deionized distilled sterile water (DDSW) (Sigma-Aldrich, St Louis, USA) was used as a non-template control for each PCR reaction, performed in a total volume of 25 µL, containing 2.5 µL of the 10× buffer, 200 µM dNTPs, 0.625 U of *Taq* polymerase (Sigma-Aldrich, St Louis, USA), and 0.4 µM of each primer. The PCR cycling conditions were set as previously described (Schaff et al., 1995). Six microliters of the PCR products were separated by electrophoresis on 1% agarose gels, stained with ethidium bromide, and photographed under UV light. The preliminary classification of the phytoplasmas at the group/subgroup level was performed with RFLP analyses, as previously reported (Gundersen and Lee, 1996).

2.3. Isolation in artificial media

A previously described phytoplasma isolation method (Contaldo et al., 2016) was employed for the symptom-bearing and symptomless palms, using leaves, inflorescences and trunk boring tissues. The samples tested were from four out of the seven villages surveyed; samples that were more representative of the various stages of the disease were selected (Table 1).

The leaf and inflorescence pieces were soaked in 5 mL of CBl (liquid medium CB) (Contaldo et al., 2016), which was examined daily for an acidification-induced colour change from orange to yellow.

For the trunk borings, approximately 100 mg from each plant was placed in two separate sterile mortars and mixed with 1 mL of CBl using a sterile pestle. Immediately afterward, the mixtures were transferred into 1.5-mL sterile tubesand 100 µL of the mixtures were transferred into 8-mL Monovette Urine tubes (monovette, Sigma-Aldrich, St Louis, USA) and incubated at 25 ± 1 °C. The medium was examined daily for an acidification-induced colour change from orange to yellow. Non-inoculated tubes were prepared following the same procedure.

After the colour change, 100 µL of medium per sample was pipetted from each tube and used to inoculate plates containing 8 mL of CBs solid medium (Contaldo et al., 2016).

2.4. Strain purification and molecular identification

Single phytoplasma colonies (Contaldo et al., 2012, 2016) grown for 24 to 36 h were picked with sterile toothpicks and transferred into 8-mL monovette containing 2 mL of CBl for the subsequent purification steps.

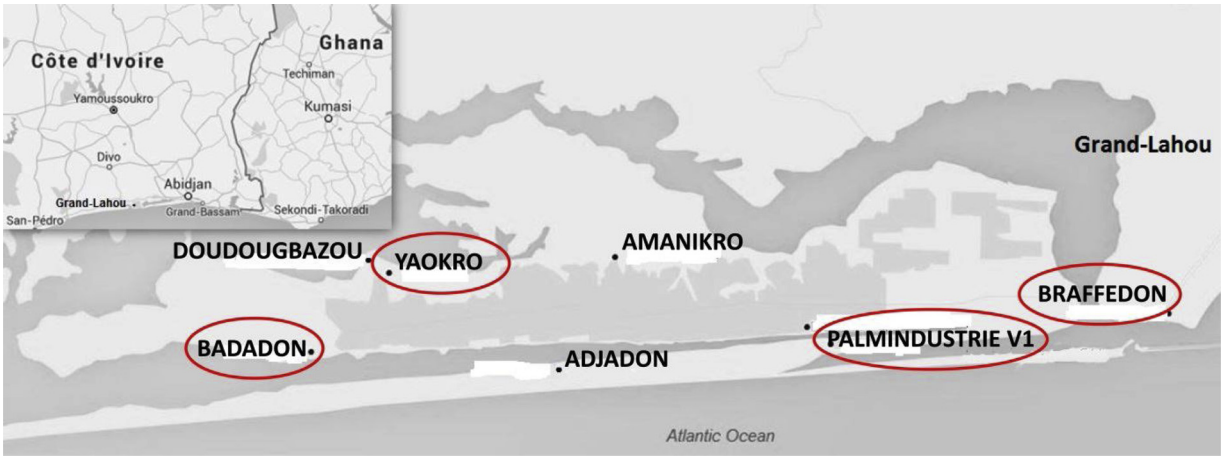

Fig. 1. A map showing the villages of Grand-Lahou that were surveyed for the presence of CILY phytoplasma in Côte d'Ivoire; red circles indicate the villages from which phytoplasma isolation was performed.

Table 1
Phytoplasma identification in coconut palm samples.

Sample code	Sample type	Disease stage	Village	Sampling month	Phytoplasma PCR/RFLP identification		
					From plant material	From CBl	From CBs[a]
2T-15	Trunk boring	1	Badadon	03/ 2015	16SrXXII-B	16SrXII-A	16SrXII-A
20T-15	Trunk boring	3	Badadon	03/ 2015	16SrXXII-B	16SrXXII-B	16SrI
40T-15	Trunk boring	1	Badadon	06/ 2015	16SrXXII-B	–	16SrI
41T-15	Trunk boring	2	Badadon	06/ 2015	16SrXXII-B	–	16SrI
11F-15	Inflorescence	2	Badadon	06/ 2015	16SrXXII-B	–	16SrI
15F-15	Inflorescence	2	Badadon	06/ 2015	16SrXXII-B	–	16SrXII-A
43T-15	Trunk boring	2-3	Badadon	06/ 2015	16SrXXII-B	–	16SrV-A
70L-15	Leaf midribs	2	Badadon	06/ 2015	16SrXXII-B	–	16SrXII-A
2T-16	Trunk boring	Asympt.	Baffredon	04/ 2016	–	16SrI	16SrI + 16SrXII-A
24T-16	Trunk boring	3	Yaokro	04/ 2016	16SrXXII-B	–	16SrI
20T-16	Trunk boring	3	Plamindustrie V1	04/ 2016	16SrXXII-B	16SrI	16SrI
L9-16	Leaf midribs	2	Baffredon	04/ 2016	16SrXXII-B	16SrI	16SrI
30T-16	Trunk boring	3	Palmindustrie V2	06/ 2016	16SrXXII-B + 16SrI	–	16SrI
9T-16	Trunk boring	3	Badadon	06/ 2016	16SrXXII-B + 16SrI	16SrXII-A	**16SrXII-A**
17T-16	Trunk boring	3	Palmindustrie V1	06/ 2016	16SrXXII-B	16SrI	**16SrXXII-B**

[a] Isolates in bold were used for biochemical and growth curve testing.

The colony purification was performed by gentle filtration of the entire liquid culture through 0.8-µm membrane filters (Whatman, Maidstone, UK) to avoid plugging, followed by $10^{-3}/10^{-4}$ serial dilutions in CBl. Both the undiluted filtrate and the filtrate dilutions were cultured on solid CBs plates and incubated at 25 ± 1 °C. This process, including the filtration, was repeated three times from single descending colonies. The final plates, which contained colonies that had grown for 72 h, were assessed and photographed under an optical bifocal microscope at 40 magnification (Nikon, Tokyo, Japan).

Single colonies from the exponential growth phase were marked, picked and dissolved in 100 µL of DDSW, and then subjected to nucleic acid extraction (Contaldo et al., 2016). Aliquots of CBl containing the isolation material were processed for DNA extraction using the alkaline lysis/phenol/chloroform method (Pourbakhs et al., 2013). The detection and identification of phytoplasmas were performed with specific PCR/RFLP assays on the 16S rRNA gene sequence. One µL of the total extracted DNA was used as template for PCR with the primers 758f (=M1)/B6, followed by nested amplification using the primers M1/1232 r (=M2) (Gibb et al., 1995; Padovan et al., 1995). Further PCR analyses were then performed using the primer pair R16F2n/R2 (Gundersen and Lee, 1996) nested with U5/U3 primers (Lorenz et al., 1995), as for the DNA from original plant materials. For each PCR reaction, DDSW and non-inoculated media were used as negative controls, and the samples were then processed as previously reported (Contaldo et al., 2016).

2.5. Sequencing and virtual RFLP analyses

The direct sequencing of selected M1/M2 and U5/U3 PCR products was performed as previously described (Contaldo et al., 2012, 2016). The sequences obtained from the coconut and colony amplicons were subjected, togheter with sequences available in GenBank, to virtual RFLP analyses on portions of the 16S ribosomal gene, using pDRAW32 (http://www.acaclone.com/). In particular, elm yellows (EY) '*Ca.* P. ulmi' (GenBank accession number, AC, AY197655), "stolbur" (STOL11),' *Ca.* P. solani' (AC, AF248959), Cape St. Paul wilt phytoplasma (CSPWB), '*Ca.* P. palmicola' (AC, JQ868442), coconut lethal yellowing (LYDM, Mozambique), '*Ca.* P. palmicola' (AC EU549768), aster yellows (MAY) '*Ca.* P. asteris' (AC M30790) were included.

2.6. Antimicrobial susceptibility

To evaluate the in vitro antimicrobial activity, seven antibiotics were tested against two phytoplasma isolates from trunk borings, CILY-XII-A (9T-16) and CILY-XXII-B (17T-16). The standard disc diffusion method was employed to determine the level of antibiotic susceptibility following both, the guidelines of the Clinical and Laboratory Standard Institute and Hannan's protocols (Hannan, 2000; CLSI, 2002). The assays were performed using rifampicin (2.5 mg/mL in DDSW), 5-fluorouracil [0.5 mg/mL in C_2H_6O, ethanol (Sigma-Aldrich, St Louis, USA)], tetracycline (0.5 mg/mL in C_2H_6O), tobramycin (0.5 mg/mL in DDSW), polymyxin (0.5 mg/mL in DDSW) and cephalexin hydrate (0.5 mg/mL in C_2H_6O). All antibiotics were obtained from Sigma-Aldrich (St Louis, USA). Standard sterile 6-mm paper discs (Whatman, Maidstone, UK) were gently placed on the surface of the agar plates, which were previously inoculated with 10^8 CFU/mL of phytoplasma isolates and 25 µL of each antibiotic at the indicated concentrations. The plates were then incubated at 25 ± 1 °C for 24 h in triplicate. Diffusion distances were calculated by subtracting half of the disc diameter from the inhibition zone diameter (measured with a centimetre ruler). For comparison, a negative control disc containing DDSW without any added antibiotic was included in the experiment.

2.7. Biochemical properties

The biochemical tests that were previously described for *Mollicutes* were employed (Aluotto et al., 1970) to assess the specific properties of the above mentioned two isolates. These tests included analyses of the breakdown of glucose, arginine and urea. Three types of media were prepared, each containing 10 mL of the 10% w/v test substrate (either arginine, urea or glucose) (Merck, Darmstadt, Germany). All media included 10 mL of horse serum (Oxoid, Basingstoke, Hampshire, UK), 5 mL of 10% w/v yeast extract stock solution (Oxoid, Basingstoke, Hampshire, UK), and 1 mL of 0.5% phenol red (Sigma-Aldrich, St Louis, USA) in 74 mL of CBl medium. The pH values were adjusted to 7.6 (for the glucose medium), 7.0 (for the arginine medium), and 7.0 (for the urea medium). The test tubes were inoculated with 500 µL of overnight CBl culture and incubated at 25 ± 1 °C. The following two sets of controls were incubated at the same time: one set of three media, each containing either glucose, arginine or urea was inoculated with DDSW; and a second set of CBl media, adjusted to the indicated pH values with 5 N NaOH (Sigma-Aldrich, St Louis, USA) or 5 N HCl (Sigma-Aldrich,St Louis, USA) was inoculated with the CBl cultures. The pH values for each tube were measured daily with pH strips (Macherey-Nagel, Düren, Germany). The growth was measured by a decrease of 0.5 pH units or more in the glucose-containing tubes compared to the negative control, or an increase of 0.5 pH units or more in the arginine- and urea-containing tubes. The same biochemical tests were performed on a pure culture of *Acholeplasma laidlawii* strain PG8 (ATCC# 1039) that was kindly provided by Dr. M. Kube (Thünen-Institute of Forest Genetics,

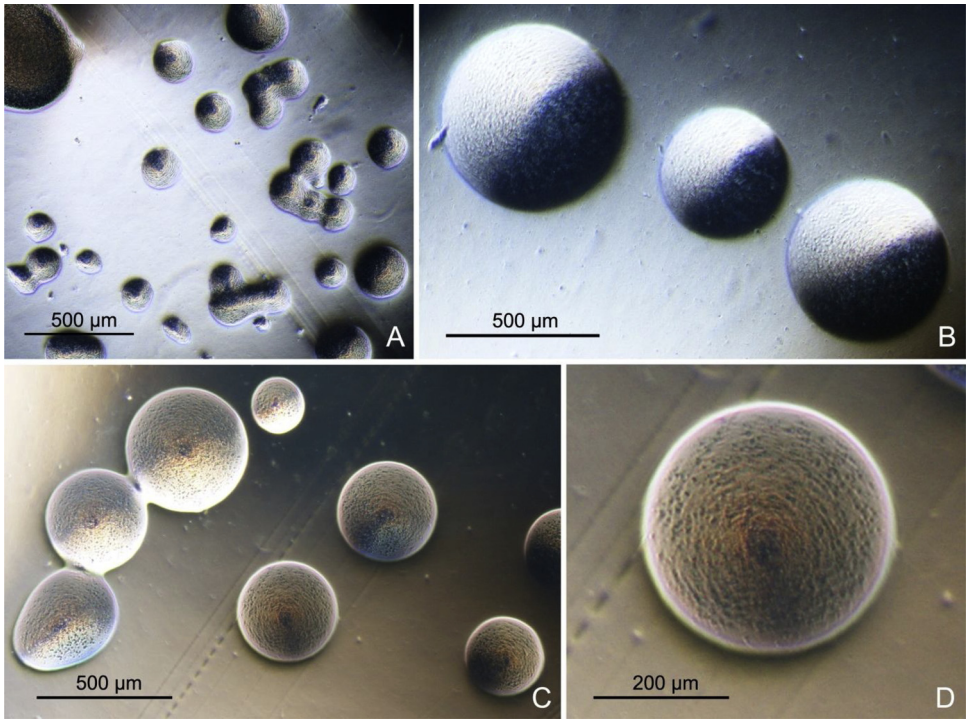

Fig. 2. Phytoplasma colonies. A: 16SrV-A phytoplasma, B: 16SrXXII-B phytoplasma, C: 16SrI (left) and 16SrXII-A (right) phytoplasmas, D: close up of a single phytoplasma colony.

Großhansdorf, Germany) and used as reference control.

2.8. Growth curves

Two flasks containing 20 mL of CBl medium were inoculated with 2 mL of 9T-16 and 17T-16 culture suspensions (ca. 10^8 CFU/mL) that were measured with a previously calibrated Beckman Coulter DU730 spectrophotometer (Brea, USA) (0.1 $OD_{600\ nm}$). The flasks were then incubated at 24 °C on a KS10 rotational shaker (Edmund Buhler, Tubingen, Germany) set at 80 oscillations per min for 2–3 weeks. Individual suspensions corresponding to the two isolates were six-fold serially diluted in the CBl medium. Two 100-µL aliquots of each dilution were plated onto three CBs plates and incubated at 24°C for 2–3 days before the colonies were counted. Two independent replicates for each phytoplasma strain were tested 30 days apart. For each growth curve, during the exponential phase, the number of phytoplasma generations (n) and the time elapsed between generations (duplication time) were calculated with the Pedersen formula (Panikov, 1995).

$$n = (\log Nt - \log N0) / \log 2 = (\log Nt - \log N0) / 0{,}301$$

- $N(t)$ = the number of cells at time t
- $N(0)$ = the number of cells at time 0

2.9. qPCR analysis

The phytoplasma presence and titre were verified in 9T-16 and 17T-16 culture suspensions at the inoculation time (T0) and after 18 h (T18) following DNA extraction (Pourbakhs et al., 2013) by qPCR analysis in an ABI PRISM®7000 cycler (Applied Biosystems, Waltham, USA). The primer pair 16S(RT)F1/16S(RT)R1 was used, and a previously reported protocol was followed (Saccardo et al., 2012). The assays were carried out in duplicate, each experiment was repeated three times and the mean comparisons were evaluated. An additional sample was added to each plate as a DNA-free negative control.

For the quantification of phytoplasma genes, a standard curve was produced using the amplicons obtained from 10-fold serial dilutions ranging from 10 to 1×10^{-7} ng / µL of the phytoplasma strain STOL (16SrXII-A) (http://www.ipwgnet.org/collection). The standard curve was constructed by the interpolation of the log10 of the copy number of each standard dilution with the respective threshold cycle (Ct) values. Relative quantification of gene expression in the phytoplasma samples was estimated from the extrapolation of the standard curve values.

3. Results

3.1. Phytoplasma identification

Prior to the isolation and cultivation trials, PCR/RFLP testing yielded amplicons for all the samples from CILY symptom-bearing coconut palms corresponding to the 16SrXXII-B phytoplasma. Two of the palms showed mixed infection with 16SrI phytoplasmas. PCR testing yielded no amplicons for any of the symptomless palms or the negative controls (Table 1).

A total of 39 samples corresponding to trunk borings, leaves and inflorescences collected from 15 coconut palms were used for the isolation trials. Inflorescences were not collected from palms exhibiting CILY symptoms of stage 3, as they were rotten before reaching the laboratory. The CBl changed colour from orange to yellow at varying times after inoculation, depending on the plant tissue. Particularly for trunk borings, the colour change was visible from 24 to 36 h post-inoculation. For leaves, medium acidification was not visible until 1 week post-inoculation. For inflorescences, the colour change times post-inoculation were extremely variable due to the contamination of the medium by several other microorganisms, primarily bacteria. This contamination rendered the inflorescences unsuitable for phytoplasma isolation under the current procedures. The non-inoculated tubes did not exhibit colour changes or colony growth throughout the experiments. The plating from tubes, immediately after the medium acidification, produced phytoplasma colonies 1 to 2 days post-inoculation (Fig. 2). The single colony purification method was used to rule out contamination with larger bacteria and to prepare the isolates for further analyses.

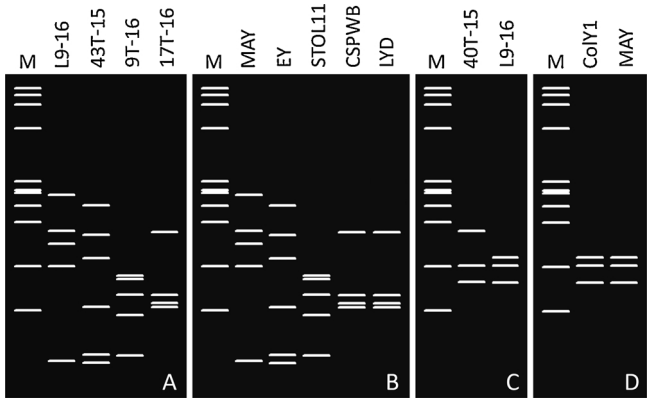

Fig. 3. Virtual RFLP on 3% gels showing the polymorphisms of the '*Ca.* Phytoplasmas' detected in coconut and culture samples obtained in this work and retrieved from GenBank. The enzyme used was *Mse*I. In A and B the sequences used are from U5/U3 amplicons, while in C and D the sequences are from M1/M2 amplicons. The acronyms are reported in materials and methods and Table 1. P, marker phiX174 *Hae*III digested with fragment sizes in base pairs from top to bottom of 1353; 1078; 872; 603; 310; 281; 271; 234; 194; 118 and 72.

Phytoplasma DNA amplification was performed on a total of 18 samples from liquid and/or solid media (Table 1). The results revealed that 7 of the tested tubes with isolation materials from symptom-bearing palms were positive; of these, 6 were from trunk borings, and 1 was from a leaf midrib. An inoculated CBl tube corresponding to a trunk boring sample from one symptomless coconut palm was positive for 16SrI phytoplasmas ('*Ca.* P. asteris'), whereas the trunk borings from the three remaining symptomless plants did not exhibit colour changes or colony growth in the medium. Phytoplasma DNA was also amplified from the colonies obtained from the 14 inoculated tubes at the exponential phase. The nested PCR was necessary since the yield of the recovered DNA with the kit used was about 10 ng/µl.

The results of blast analyses of U5/U3 and/or M1/M2 sequences, deposited in GenBank under accession numbers MF419688 to MF419695, confirmed the presence of '*Ca.* P. asteris', '*Ca.* P. ulmi', '*Ca.* P. solani,' which had 99% identity with the corresponding '*Candidatus* Phytoplasma' strains; the identity to the corresponding '*Candidatus* Phytoplasma' was 100% only for '*Ca.* P. palmicola'. The results of the virtual RFLP analyses further confirmed the phytoplasma classification in groups 16SrI, 16SrV, 16SrXII and 16SrXXII (Fig. 3). The isolation of one '*Ca.* P. asteris' strain with SNPs to strains enclosed in subgroups 16SrI-B was revealed by *Mse*I restriction analyses (Fig. 3) and sequence comparison (data not shown).

3.2. Antimicrobial susceptibility and biochemical properties of two phytoplasma isolates

The results of the antibiotic susceptibility tests revealed that tobramycin exhibited the maximum activity against the two phytoplasma isolates, followed by polymyxin and tetracycline. The two isolates displayed an intermediate susceptibility to 5-fluorouracil but were completely resistant to cephalexin hydrate and rifampicin (Table 2).

The results of the biochemical tests for urea and arginine hydrolysis and glucose fermentation confirmed that both phytoplasma strains were negative for urea and positive for glucose and arginine (Fig. 4). For both phytoplasma strains, a decrease in the pH from 7.6 to 6.0 was observed in the glucose test, and a decrease in the pH from 7.0 to 5.5 was observed in the urea tests, whereas an increase in the pH of 1.5 (from 7.0 to 8.5) was estimated for the arginine test. On the other hand, *A. laidlawii* was positive for glucose and negative for urea and arginine, as previously reported (Tully et al., 1994) (Fig. 4).

Table 2
Phytoplasma antibiotic susceptibility measured by the disc diffusion method.

Antibiotic	Diameter (mm)	± standard error (mm)
5-fluorouracil	13	0.43
Cephalexin hydrate	2.5	0.45
Polymyxin	21	0.45
Rifampicine	2	0.2
Tetracycline	20	0.35
Tobramycin	23	0.71

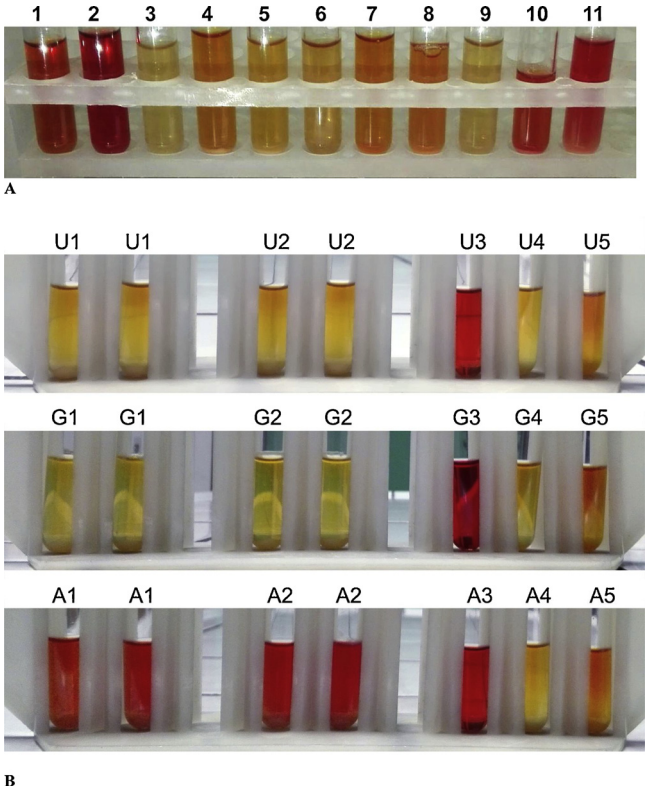

Fig. 4. Biochemical tests and pH variation at 48h. **A.** Numbers 1–2, liquid medium inoculated with DDSW and adjusted to a pH of 7.0. and 7.6, respectively. Numbers 3-4-5, glucose fermentation: 3 (*A. laidlawii*), 4 (isolate 9T-16), 5 (isolate 17T-16), pH from 7.6 to 6.0. Numbers 6-7-8, urea hydrolysis: 6 (*A. laidlawii*), 7 (isolate 9T-16), 8 (isolate 17T-16). Numbers 9-10-11, pH from 7.6 to 6.0; arginine hydrolysis: 9 (*A. laidlawii*), 10 (isolate 9T-16), 11 (isolate 17T-16), pH variation only in tubes 10 and 11 from 7.0 to 8.5. **B.** Tubes U1, G1 and A1, two replicas of the isolate 9T-16 and U2, G2 and A2 two replicas of the isolate 17T-16 both inoculated in media with the three different substrates (urea, glucose and arginine, respectively). Tubes U3, G3 and A3, liquid medium containing either urea (U) glucose (G) or arginine (A) inoculated with DDSW. Tubes U4, G4 and A4, media adjusted to a pH of 7.0 and inoculated with the isolate 17T-16. Tubes U5, G5 and A5, media adjusted to a pH of 7.6 and inoculated with the isolate 17T-16.

3.3. Growth curves of two phytoplasma isolates

Growth curves were determined for the 9T-16 and 17T-16 phytoplasma isolates (Fig. 5a). The growth rate of the 17T-16 (16SrXXII-B) isolate was higher than that of the isolate 9T-16 (16SrXII-A) by a factor of 10 at 12 h/growth. Both phytoplasma isolates reached a final growth plateau between 48 and 72 h with a concentration of approximately 10^{10} CFU/mL, but showed also an intermediate growth plateau at about 24 h. Furthermore, the number of generations and the time elapsed between each generation were lower for the phytoplasma isolate 17T-16 compared with the isolate 9T-16 (Fig. 5b). The duplication time (time elapsed between two generations) of the two phytoplasma

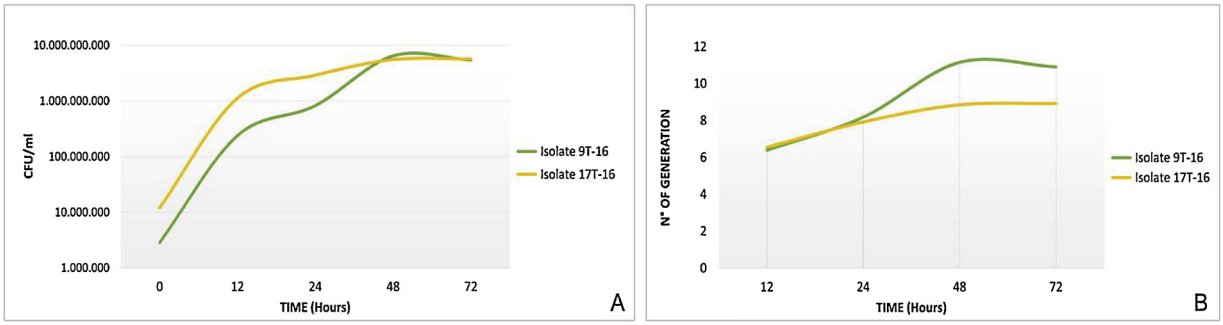

Fig. 5. Growth kinetics of phytoplasma isolates from coconut palms 9T-16 (16SrXII-A) and 17T-16 (16SrXXII-B). A, growth curves. B, number of generations and time elapsed between each generation (calculated with the Pedersen formula).

isolates, as estimated by colony count, was 250 and 500 min for 9T-16 (16SrXII-A) and 17T-16 (16SrXXII-B), respectively. In conclusion, the isolate 17T-16 was shown to multiply less efficiently in CBl or CBs media after 24 h of cultivation compared with the isolate9T-16.

3.4. qPCR analysis of two phytoplasma isolates

From the qPCR assay, an increase in the phytoplasma titre was detected for both isolates. The Tm of the standard curves exhibited a single peak at 83 ± 1 °C, and all the dissociation curves from phytoplasma DNA exhibited a single peak at the same temperature. The Ct values ranged from 6.5 to 35.0 for the 10-fold STOL dilutions and from 31.4 to 35 for the phytoplasma dilutions (Table 3). An increase of the phytoplasma titre was therefore detected between time 0 and 18 h; the Ct values were not determined in the following 24–48 considering the growth plateau registered at about 24 h.

4. Discussion

This is the first report of the isolation and growth of phytoplasmas from coconut palms in artificial media. The trunk borings, compared to tissues from leaves and inflorescences, proved to be the most suitable plant tissue for phytoplasma isolation, as the majority of phytoplasma colonies were obtained from these materials. This finding is in agreement with the fact that trunk borings have previously been shown to be the optimal plant tissue for phytoplasma sampling and detection in coconut and other palm species (Harrison et al., 2013). The 16SrXXII-B isolated phytoplasmas correspond to the prevalent subgroup associated with CILY in Côte d'Ivoire (Arocha Rosete et al., 2017). Furthermore, the detection of phytoplasmas of diverse ribosomal subgroups, such as 16SrI-B, 16SrV-A, 16SrXII-A and 16SrXXII-B from typical phytoplasma colonies, confirmed for the first time the natural occurrence of a mixed phytoplasma infection in coconut palms affected by CILY. The comparison between the phytoplasma subgroups found in the plant material prior to culture and those found in the liquid and solid media indicates the presence of different phytoplasmas in the tissues used for isolation. These somewhat contradictory results on phytoplasma identification obtained from plant material, CBl and CBs media confirm the presence of a mixed infection. While it is very likely that the prevalent strain in the plant material is the strain that determines the PCR/RFLP pattern in plants, the medium appears to favour other phytoplasma strains. Moreover, the differences in the identity of the phytoplasma that were detected from testing the DNA extracts from the CB liquid cultures and plate colonies further indicate the presence of mixtures of phytoplasmas. This finding was further confirmed by the detection of a mixed infection in both liquid and solid media. Moreover, the symptomless and PCR-negative sample 2T-16, exhibited no phytoplasma detection in the starting plant material. However, a single phytoplasma subgroup was detected in the liquid medium and a mixture of two subgroups on the solid medium. In particular, the presence of 16SrXII-A and 16SrI in the latter case confirms that the media used in this study are especially suitable for the growth of these phytoplasmas, as previously reported (Contaldo et al., 2016). Recently, the CILY phytoplasma was detected in a mixed infection with 16SrI phytoplasmas in both CILY-affected coconut palms and the potential leafhopper vector, *Nedotepa curta* Dimitriev, in coconut-growing villages of Grand-Lahou (Kwadjo et al., 2017); this finding partly confirms the results obtained from cultivation.

The presence of multiple phytoplasmas in a single plant host may modulate the expression of disease symptoms, as it was suggested regarding the presence of multiple apple proliferation phytoplasma strains in apple trees or for other phytoplasma-associated diseases (Seemüller and Schneider, 2007; Rid et al., 2016).

The biochemical characterization of 16SrXII-A, '*Ca.* P. solani' and 16SrXXII-B '*Ca.* P. palmicola' isolates from coconut tissues revealed that both 9T-16 (16SrXII-A) and 17T-16 (16SrXXII-B) shared metabolic features with *Mycoplasma* species group III (glucose positive/arginine positive), which are represented by several mycoplasma species (i.e., *M. fermentans*) (Brown et al., 2007; Freundt et al., 1979). The ability to metabolize both glucose and arginine differentiates these phytoplasma isolates from *Acholeplasma* and *Mycoplasma* species, which were isolated from the surfaces of several tropical flower plants (Rose et al., 1990). From this study, both phytoplasma isolates, 9T-16 and 17T-16 were shown to be positive for arginine, which suggests that these isolates may possess either the arginine dehydrolase pathway or, at least, certain components of the pathway for the arginine hydrolysis. This finding may be an indicator that supports the differentiation of phytoplasmas from other *Mollicutes* species that inhabit the phloem of coconut palms, such as *A. palmae* (Tully et al., 1994). Previous biochemical tests performed on various palm cultivars grown in Florida revealed that arginine was readily detected in lethal yellowing susceptible palm ecotypes, but it was absent (or present at minimal

Table 3
Phytoplasma titre quantification by qPCR analysis.

Sample	Ct Mean	Quantity ng/µl	Quantity copy number
STOL- 10	6.5	10	1.54×10^{13}
STOL- 10^{-1}	12.2	10^{-1}	1.54×10^{11}
STOL- 10^{-2}	17.0	10^{-2}	1.54×10^{10}
STOL- 10^{-3}	21.5	10^{-3}	1.54×10^{9}
STOL- 10^{-4}	25.8	10^{-4}	1.54×10^{8}
STOL- 10^{-5}	29.4	10^{-5}	1.54×10^{7}
STOL- 10^{-6}	32.3	10^{-6}	1.54×10^{6}
STOL- 10^{-7}	35.0	10^{-7}	1.54×10^{5}
9T-16 T0[a]	**35.0**	$\mathbf{2.1 \times 10^{-7}}$	$\mathbf{3.24 \times 10^{5}}$
9T-16 T18[b]	**33.3**	$\mathbf{6.0 \times 10^{-7}}$	$\mathbf{9.26 \times 10^{5}}$
17T-16 T0[a]	**34.2**	$\mathbf{3.5 \times 10^{-7}}$	$\mathbf{5.44 \times 10^{5}}$
17T-16 T18[b]	**31.4**	$\mathbf{1.7 \times 10^{-6}}$	$\mathbf{2.62 \times 10^{6}}$

Isolates in bold were used for biochemical and growth curve testing.
[a] Strain at the inoculation time.
[b] Strain after 18 h.

detectable levels) in LY-resistant palms, which led to the hypothesis that LY phytoplasmas may hydrolyse arginine (Mc Coy, 1984).

On the other hand, the medium-dependent growth kinetics for each isolate (9T-16 and 17T-16) indicated their different performances in the used medium. This phenomenon was clearly evidenced by the longer growth timeframe for the 16SrXII-A (9T-16) isolate and partly verified by the qPCR assays at the initial and 18 h growing times. The 16SrXXII-B phytoplasma correspond to the prevalent subgroup associated with CILY in Côte d'Ivoire [13]; however, the isolate 17T-16 was shown to multiply less efficiently in CBl or CBs media after 24 h of cultivation. The shorter survival time in the artificial medium for isolate 17T-16 may be associated with a limiting factor of the CILY phytoplasma multiplication rate in cell-free media. Further biochemical comparative studies on CILY phytoplasma-free and CILY phytoplasma-infected trunk borings may lead to the identification of new chemicals or bio-compounds which may improve the media composition to support a better growth performance and survival rate of 16SrXXII-B phytoplasmas.

The assessment of the phytoplasma susceptibility to antibiotics performed in this study was the first performed on cultured phytoplasmas and provided an indication of the resistance/susceptibility of the two studied isolates. This work is also the first report of phytoplasma growth kinetics, clearly demonstrating that the various isolates exhibited different performances in the same growth medium, confirming that different phytoplasmas exhibit different biological behaviour and very likely possess different plant colonization abilities. This phenomenon was clearly evidenced and confirmed by the longer growth timeframe registered for the 16SrXII-A (9T-16) isolate, which was not detected in the original coconut tissues by nested-PCR.

5. Conclusions

The first assessment of the antibiotic susceptibility of 16SrXII-A and 16SrXXII-B phytoplasmas isolated from coconut palm and their biochemical characterization provided experimental evidence about certain metabolic features of these strains. The availability of phytoplasma colonies associated with CILY disease represents a novel and important tool for the further study of their biology, pathogenicity and epidemiology and to support their species classification. This newly achieved coconut phytoplasma growth in both solid and liquid artificial media and the biological characterization of two isolates are important advancements in the field of phytoplasma disease management and containment measures.

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