

## Full Length Research Paper

# Microbial diversity in leaves, trunk and rhizosphere of coconut palms (*Cocos nucifera* L.) associated with the coconut lethal yellowing phytoplasma in Grand-Lahou, Côte d'Ivoire

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The diversity of culturable bacterial and fungal communities was assessed from leaves, trunk and rhizosphere of coconut palms infected and non-infected by the Côte d'Ivoire lethal yellowing (CILY) phytoplasma. Bacterial and fungal microbes were isolated from leaves, trunk and rhizosphere samples collected from two villages of Grand-Lahou, Braffedon and Yaokro, by using a high-throughput 'dilution-to-extinction' cultivation method coupled with PCR and sequencing with primers that amplified both the 16S ribosomal RNA and intergenic transcribed spacer (ITS) genes. The relative abundance was higher for *Bacillus* and *Candida* in Braffedon, and *Burkholderia* and *Neodeightonia* in Yaokro. Commonly genera identified from rhizosphere included *Bacillus*, *Burkholderia*, *Pseudomonas*, *Streptomyces*, *Cryptococcus*, *Penicillium*, *Purpureocillium* and *Trichoderma*. The most abundant endophytes identified were *Pantoea*, *Candida*, *Cryptococcus*, *Bacillus*, *Pseudomonas*, *Penicillium*, *Aspergillus* and *Rhodotorula*. Genera limited to symptomless palms included *Arthrimum*, *Chaetomium*, *Phialemonium*, *Fusarium*, *Klebsiella* and *Candida*. Results indicate that the CILY phytoplasma may be a factor determining the level of diversity of a microbial community in a given location. Our research provides the basis to investigate the possible effect of endophytic and rhizosphere microbes against the CILY phytoplasma to further effectively improve the management of CILY in Grand-Lahou.

**Key words:** Côte d'Ivoire lethal yellowing phytoplasma, endophytes, biocontrol, coconut.

## INTRODUCTION

Grown in tropical and subtropical regions, the versatile coconut (*Cocos nucifera* L.) is an important source of

food, shelter, and household income for smallholder farmers around the globe (Batugal et al., 2005). Côte d'Ivoire is among the top 20 coconut producers in the world, with an annual production of 182,170 tonnes (FAOSTAT, 2015), and it is the top African exporter of coconut oil from copra to Europe and West Africa. However, the Ivorian coconut industry is currently impacted by the Côte d'Ivoire lethal yellowing disease (CILY) (Konan Konan et al., 2013a). Lethal yellowing-like diseases are considered a serious world threat for coconut-producing countries in Central America and the Caribbean, West and East Africa, and Mozambique (Danyo, 2011).

The disease CILY has been associated with a phytoplasma of the group 16SrXXII, subgroup B, '*Candidatus* Phytoplasma palmicola'-related strains (Harrison et al., 2014). It is widespread throughout the south coast littoral of Grand-Lahou, where it has caused the destruction of more than 400 ha within the last ten years, and losses of 12,000 tonnes of copra per year (Kra et al., 2017).

Phytoplasmas are bacteria-like phloem-inhabiting pathogens of the class *Mollicutes* transmitted by Hemiptera insect vectors that cause diseases in over a thousand plant species, including crops, fruit trees, and ornamental plants (Maejima et al., 2014). Management and control of phytoplasma diseases have been based on the removal of infected plants that can act as an inoculum source (Romanazzi et al., 2009), the use of resistant varieties, and the control of insect vectors; however, no effective disease control has been achieved so far. No genotypes are available that show reliable resistance to phytoplasma diseases, and particularly for coconut lethal yellowing, the sort of unexplained resistance breakdown seen in some widely used hybrids is a global concern for replanting programs (Baudoin et al., 2008). Phytoplasma vector control has been focused on insecticidal treatments against natural vectors such as *Macrostelus quadripunctulatus*, the vector of chrysanthemum yellows phytoplasma (Saracco et al., 2008), and *Cacopsylla pyri*, the vector of pome fruit phytoplasmas (Bangels et al., 2010).

Some other strategies tested to control phytoplasma diseases include transgenic plants as in the case of tobacco plants expressing antibodies against the stolbur phytoplasma (Malembic-Maher et al., 2005); the use of elicitors such as indole-3-acetic acid/butyric acid (Perica, 2008; Lherminier et al., 2003); organic fertilizers or algal extracts; natural and synthetic peptides and essential oils; or recovery promoted by abiotic stress in grapevines affected by 'bois noir' (Romanazzi et al., 2009). Previous studies have shown that applying the arbuscular mycorrhizal fungus *Glomus intraradices* can improve

tolerance to pear decline phytoplasma (Garcia-Chapa et al., 2004). Similarly, it has been shown that plant-growth promoting bacteria like *Pseudomonas*, isolated from the rhizosphere of daisy plants, when applied to phytoplasma-infected daisies, are able to extend the daisy's life span (D'Amelio et al., 2007).

The plant rhizosphere harbors numerous bacteria capable of stimulating and aiding plant growth and are termed plant growth promoting rhizobacteria (Lugtenberg and Kamilova, 2009). They exert their beneficial effects through biofertilization, stimulation of root growth, rhizo-remediation, plant stress control, or biological control including antibiosis, induction of systemic resistance and competition for nutrition and niches, besides they have become the new inoculants for biofertilizers. Studies on the bacterial population isolated from the rhizosphere of coconut palms resistant and tolerant to Kerala wilt disease phytoplasma in India (Gopal et al., 2005) have suggested that rhizosphere microflora could play a role in evading phytoplasma infection.

Over the last few years, there has been an increasing interest in the use of endophytes to control plant pathogens (Romanazzi et al., 2009). Indeed, one of the latest trends is the identification of fungal or bacterial endophytes with biocontrol potential against phytoplasmas (Compant et al., 2013; Martini et al., 2009; Romanazzi et al., 2009). Endophytes refer to endosymbionts that colonise the inside of the plants without causing any disease to the plant host (Schulz and Boyle, 2006; Wilson, 1995). They can inhabit different plant parts such as tubers, stems, leaves and roots, and can enter the plant via insect sucking or by passive diffusion or active selection from the adjacent rhizosphere (Romanazzi et al., 2009).

Endophytes establish mutualistic relationships with plants and produce important secondary metabolites and compounds that can act as plant growth promoters or enhancers of plant resilience to certain pests and plant pathogens, as well as to abiotic factors such as environmental stress and drought (Golinska et al., 2015; Rodriguez et al., 2009).

The interaction of endophytic bacteria and fungi in phytoplasma-affected plants has been poorly studied, and limited to a few crops like grapevine infected with the "flavescence dorée" and "bois noir" phytoplasmas (Bulgari et al., 2011; Grisan et al., 2011), and apple infected with the apple proliferation phytoplasma (Bulgari et al., 2012; Musetti et al., 2011). Recently, endophytic communities associated with healthy and phytoplasma-infected plants have been characterised (Bulgari et al., 2009; Martini et al., 2009) to find possible biocontrol agents. Martini et al. (2009) identified *Aureobasidium pullulans* and *Epicoccum nigrum* as fungal endophytes

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associated with grapevine varieties that spontaneously recovered from phytoplasma disease. Musetti et al. (2011) showed that periwinkle plants, *Catharanthus roseus* L. G. Don, grafted with 'Ca. Phytoplasma mali'-infected apple scions, and treated with *E. nigrum* exhibited reduced symptoms, and lower concentration of the phytoplasma when compared with untreated control plants. Grisan et al. (2011) identified *A. pullulans* and other fungal species when characterising the fungal endophytic bio-diversity in both healthy and phytoplasma-infected grapevines. Other species included *Alternaria* sp., *Phoma* sp., *Cladosporium* sp., *Pestalotiopsis* sp., and *Pestalotia* sp.

A number of endophytes have been studied for their potential as biocontrol in coconut palms. A member of the genus *Cordyceps* has been reported as a biocontrol against *Lecopholis coneophora*, the causal agent of coconut root grub (Kumar and Aparna, 2014). Endophytic bacteria such as *Pseudomonas*, *Bacillus*, and *Enterobacter* have been commonly isolated from a wide range of monocots and dicots including coconut (Rajendran et al., 2008). Rajendran et al. (2015) used endophytic bacteria *Bacillus subtilis* and *Pseudomonas fluorescens* in a bioconsortium with *Trichoderma viride* as a soil application against *Ganoderma lucidum* (Leys) Karst, the causal agent of basal stem rot on coconut palm, and showed a higher induction of defense related enzymes.

High-throughput cultivation – 'dilution-to-extinction' cultivation – has proven to be an effective method to assess endophytic diversity (Unterseher and Schnittler, 2009). The method increases the diversity of cultivable species as inoculum density decreases (Collado et al., 2007). It has been primarily used to lower species richness so functional ability can be correlated with biodiversity.

A multi-well plate is used to provide spatial separation and reduce interspecific interactions. This increases the possibility of detecting slow-growing and weak competitors, while, at the same time, allows the isolation of ubiquitous and dominant taxa. The 'dilution-to-extinction' cultivation method has been widely used to study the modified microbial diversity in different materials such as mineral soils (Wertz et al., 2006, 2007; Griffiths et al., 2001), peat (Dimitriu et al., 2010) and sewage (Franklin and Mills, 2006). It has been applied to assess the fungal endophytic community of leaf-litter fungi in beech, *Fagus sylvatica* L. (Unterseher and Schnittler, 2009), and to isolate ammonia-oxidizing bacteria from arable and lead-contaminated soil (Aakra et al., 1999).

The present work aimed at characterizing the culturable bacterial and fungal communities residing in leaves, trunk, and the rhizosphere of CILY phytoplasma-infected and CILY phytoplasma-free coconut palms by using the 'dilution-to-extinction' cultivation method coupled with PCR and sequencing approaches.

## MATERIALS AND METHODS

### Sampling from leaves, trunks and rhizosphere from coconut palms

Samples were collected in 2016 from Braffedon during April, and from Yaokro in June. Braffedon and Yaokro are two coconut-growing villages of the Grand-Lahou village area, located at 18 and 43 Km from Grand-Lahou downtown, respectively (Arocha Rosete et al., 2017). Samples included leaves, trunk borings and rhizosphere from PB121 coconut palm ecotypes. Two symptomless coconut palms were sampled per village. Coconut palms exhibiting CILY-like symptoms were also surveyed. Samples were collected from two palms with symptoms resembling those of each disease stage 1, 2 and 3 for a total of six symptom-bearing coconut palms per village. Symptoms of disease stage 1 (S1) corresponded to palms with initial yellowing in the older leaves and initial blackening of the inflorescences. Symptoms of disease stage 2 (S2) were related to the progress of yellowing from the older to the younger leaves, and increase of the inflorescence blackening. In palms showing symptoms similar to those of disease stage 3 (S3), the older yellowed leaves turned brown and desiccate, and in some cases hanged down forming a skirt around the trunk. This latter sign along with full blackening of the inflorescences were only observed in coconut palms from Yaokro.

Rhizosphere samples were collected from clay-rich sandy tertiary soils, characteristic of the Grand-Lahou coastal littoral zone at 10 cm from the trunk, and 26 cm deep in the soil with a stainless steel soil-probe sampler (Konan Konan et al., 2013b). Three young leaves emerging from the coconut heart were collected per palm, then individually surface sterilised with both 0.5% sodium hypochlorite and 70% ethanol, and rinsed in Sigma-graded sterile deionized water (SDW). Small plastic bags were used to collect around 2 g of trunk borings by boring into the trunk at one meter from the trunk base using an eight cm long drill bit sterilised with 70% ethanol before collecting each sample (Harrison et al., 2013). For the trunk borings, 1 g of each sample was weighed and placed in a 15 mL Falcon™ tube, and sterilized with 10 mL of 0.5% sodium hypochlorite, followed by 10 mL of 70% ethanol, and rinsed in 10 mL of Sigma-graded SDW. UV-sterilized Whatman paper was used to dry up the wet trunk borings after the final rinse. The trunk borings were transferred to a clean sterile 1.5 mL microtube. All samples were kept at 4°C until further analysis.

### Culture isolation of bacterial and fungal organisms

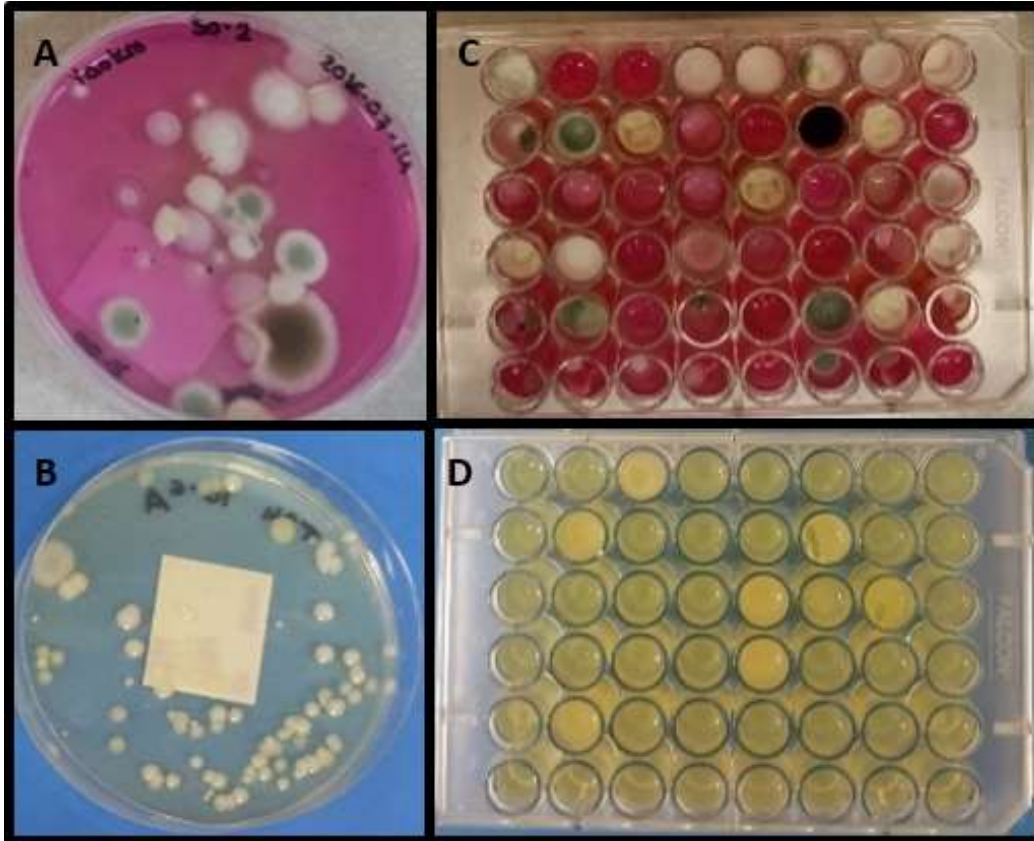
Bacterial and fungal organisms were isolated by using a modified 'dilution-to-extinction' cultivation method (Collado et al., 2007) with some modifications explained below and illustrated in Figure 1.

#### Rhizosphere culture sample preparation

One gram of rhizosphere was weighed from each palm, and the weighed amounts from each two replicates representing each disease stage (1, 2 and 3) were pooled in a 15 mL Falcon™ tube, and mixed by a 10 sec vortexing. A total of 0.1 g of each mixture was taken with a small spatula and added to 900 µL of SDW in individual 2 mL microtubes resulting in a 10<sup>-1</sup> dilution.

#### Trunk culture sample preparation

A total of 0.5 g of trunk borings collected was weighed from each tree, and the weighed amounts from each two replicates representing each disease stage (1, 2, and 3) were pooled in a 15 mL Falcon™ tube, and mixed by a 10 sec vortexing. A total of 0.1 g



**Figure 1.** Conventional isolation method in 35 mm Petri dishes with 100  $\mu$ L of fungal (A) or bacterial (B) suspensions. Fungal (C) and bacterial (D) isolations obtained by 'dilution-to-extinction' cultivation method in 48-well plates. Fungal (A and C) isolates were grown on Rose Bengal Agar with chloramphenicol. Bacterial (B and D) isolates were grown on Tryptic Soy Agar with amphotericin B.

of each pool was taken with a small spatula and added to 900  $\mu$ L of SDW in individual 2 mL microtubes resulting in a  $10^{-1}$  dilution.

#### Leaf culture sample preparation

Since three leaves were collected per palm, 0.5 g of leaf midrib from each leaf sample were finely cut into 0.5 cm square pieces with a sterile scalpel, and pooled together in a 15 mL Falcon™ tube, and briefly vortexed. A total of 1 g from each pool representing each disease stage was weighed, placed in a sterile porcelain mortar and macerated with a sterile pestle in 2 mL of SDW. The homogenate was collected per sample in 2 mL microtubes and diluted six-fold ( $10^6$ ) in SDW.

#### Sample plating

An aliquot of 100  $\mu$ L of each dilution series was plated in duplicate in 35 mm Petri dishes with Tryptic Soy Agar (TSA) medium (BD Difco™) supplemented with amphotericin B (4  $\mu$ g/mL; TSAA) for bacterial isolation. Plates were incubated for 2 days at 35°C. For fungal isolation, fresh 100  $\mu$ L aliquots were plated in 35 mm Petri dishes with Rose Bengal agar (RBA medium; Oxoid) supplemented with chloramphenicol (50 mL; RBAC). Plates were incubated at 25°C for 4 to 6 days.

#### Endophytic sterility check

For the verification of the effectiveness of the disinfection procedure and the confirmation that microbial growth was endophytic and not from leaf surface or trunk contamination, sterility checks were carried out for each leaf and trunk sample. For these checks, 0.1 mL from the final SDW rinse were plated out on TSAA and RBAC plates. Plates were incubated as described above. The effectiveness of the sterility checks was measured based on the absence of bacterial or fungal growth in both TSAA and RBAC plates after the corresponding incubation period.

#### Colony counts

Colony forming units (CFU) were counted for each duplicate sample and averaged to approximate the dilution factor in SDW to 100 CFU/mL. Duplicates of 48-wells of RBAC and TSAA Falcon™ Polystyrene Microplates (Fisher) were used per sample and inoculated with 10  $\mu$ L of the final dilution resulting in approximately 1 colony per well. TSAA and RBAC microplates were incubated for 2 days at 35°C, and for 4 to 6 days at 25°C, respectively.

#### Colony morphology identification

Bacterial and fungal colonies were morphologically identified based

on the colony shape and appearance, texture and pigmentation. Colonies of similar morphologies were assigned the same number. A colony representative of each number was subcultured into corresponding TSA or RBAC Petri dishes. TSA plates were incubated for 2 days at 35°C, while RBAC plates were incubated for 4 to 6 days at 25°C.

### Total DNA extraction

After incubation, plates were used for total DNA extraction using the FastDNA® SPIN Kit (MP Biomedicals, USA) following manufacturer's recommendations. Total DNA was also extracted directly from leaf and trunk boring samples (Harrison et al., 2013).

### PCR amplification

All PCR reactions (25 µL) were performed with illustra PuReTaq Ready-To-Go PCR Beads (GE Healthcare, UK) with a starting concentration of 50 ng of genomic DNA and 0.4 µM final primer concentrations. The CILY phytoplasma was detected by nested PCR with universal 16S rRNA PCR primers P1 and P7 (Schneider et al., 1995; Deng and Hiruki, 1991) with an initial denaturation of the template at 94°C for 3 min, followed by 35 cycles: 94°C for 40 s, 54°C annealing for 40 s, 72°C extension for 1 min 40 s, and a final 10 min extension at 72°C. One microliter of the first PCR reaction was diluted 1:30 in SDW and used as a template for the nested PCR with primers G813f and AwkaSR (Tymon et al., 1998) specific for West African phytoplasma strains. The nested PCR protocol followed 35 cycles of initial denaturation of the template at 94°C for 3 min, 94°C for 40 s; 53°C annealing for 40 s; 72°C extension for 1 min, and a final 10 min extension at 72°C. P1 and P7 primers were nested with phytoplasma generic primers U5 and U3 (Lorenz et al., 1995) to confirm the detection of any other phytoplasma group in samples yielding no amplification with primers G813/AwkaSR.

For bacterial identification, the 16S rRNA gene was amplified with universal primers 27f and 907r (Lane, 1991) with an initial denaturation of the template at 94°C for 3 min, followed by 30 cycles of 94°C for 30 s, 50°C annealing for 30 s, 72°C extension for 1 min and 20 s, and a final 7 min extension at 72°C. For fungal identification, the ITS region of the rRNA gene was amplified using the universal primers ITS5 and ITS4 (White et al., 1990) with the following amplification protocol: initial template denaturation at 94°C for 3 min, followed by 35 cycles of 94°C for 30 sec, 55°C annealing for 30 sec, 72°C extension for 1 min and 15 s, and a final 5 min extension at 72°C.

### Sequencing and sequence analyses

All PCR amplicons were purified using the E.Z.N.A.® Cycle-Pure Kit (Omega Bio-tek, USA), and sequencing reactions were prepared using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA) following the manufacturer's recommendations. Samples were directly sequenced at the Centre for the Analysis of Genome Evolution and Function (CAGEF; University of Toronto). The sequences obtained were assembled into contigs using the Sequencher 4.9 software (Gene Codes Corporation, USA). Bacterial and phytoplasma contigs were compared to NCBI (Genbank <https://www.ncbi.nlm.nih.gov>), and in the case of bacteria, the sequences were also analyzed at the Ribosomal Database Project (RDP; <http://rdp.cme.msu.edu/>). Fungal contigs were compared to the CBS Fungal Biodiversity Centre (<http://www.cbs.knaw.nl/>) by pairwise sequence similarity search. A sequence similarity greater than 97% was used to assign a genus to the bacterial and fungal isolates. Multiple sequence alignments of the obtained contigs were made with Clustal W and

phylogenetic analyses were performed with MEGA version 7 (Kumar et al., 2016), using the maximum likelihood method based on the Tamura-Nei model (Tamura and Nei, 1993) with a bootstrap analysis of 1000 replicates.

### Diversity analyses

Shannon (H') and Simpson (D) diversity indices were calculated for the microbial communities using PAST version 3.14 (Hammer et al., 2001). A modified t-test for the above-mentioned diversity indices (Brower et al., 1998; Hutcheson, 1970) was used in order to assess the differences in diversity between the microbial communities identified. Relative abundance was calculated by dividing the number of species from one given group (bacterial or fungal) by the total number of species from leaves, trunk and rhizosphere.

## RESULTS

### Screening for the presence of CILY phytoplasma

Total DNA from leaf and trunk boring samples was subjected to nested PCR for the detection of the CILY phytoplasma. No amplification was obtained for any of the two symptomless palms collected in Braffedon or Yaokro with any of the primer combinations. The six coconut palms exhibiting S1, S2 and S3 CILY symptoms collected from Yaokro tested positive for the presence of the CILY phytoplasma with the specific primers G813/AwkaSR. Sequences were 99% identical to those of the CILY phytoplasma isolates from Grand-Lahou from previous studies (Harrison et al., 2014). Interestingly, no CILY phytoplasma was detected with primers G813/AwkaSR in any of the leaf or trunk samples collected in Braffedon regardless the presence of CILY-like symptoms. Only four out of the six palms exhibiting CILY-like symptoms collected in Braffedon: two palms with S3-like symptoms (from trunk and leaf, respectively), one palm with S2-like symptoms (from trunk), and one palm with S1-like symptoms (from trunk) yielded P1/P7 amplicons. After PCR purification and sequencing, the partial P1/P7 sequence was determined to belong to the Gram positive bacterium *Bacillus megaterium*. Besides, nested PCR with phytoplasma generic primers U5/U3 yielded no amplification for all the six coconut palms surveyed in Braffedon that exhibited CILY-like symptoms.

### Characterization of isolated microbial communities

Six palms per location and a total of 24 leaf, 40 rhizosphere (24 from Braffedon and 16 from Yaokro), and 32 trunk (24 from Braffedon and 8 from Yaokro) samples were processed. Eighty-seven bacterial and 97 fungal isolates were taxonomically identified and their respective 16S rDNA and ITS sequences compared. The resulting 95 operational taxonomic units (OTUs) represented 3 phyla (Actinobacteria, Firmicutes, and Proteobacteria), 5 classes, and 26 genera for the bacterial isolates (Table 1

and Figure 2), and 3 phyla (Ascomycota, Basidiomycota, and Zygomycota), 7 classes, and 27 genera for the fungal isolates (Table 1 and Figure 3). One representative sequence of each OTU was deposited in GenBank (Accession numbers shown in Figures 2 and 3).

### Diversity of bacterial and fungal communities isolated per geolocation

There was no significant difference with regards to the whole microbial community diversity (combined analysis of bacterial and fungal genera/species) identified from Yaokro and Braffedon (Shannon and Simpson's indexes 3.0975 and 0.068594, respectively, for Braffedon and 3.1489 and 0.066162, respectively for Yaokro, Table 2). However, statistical differences were observed among diversity levels when the bacterial and fungal microbial communities were analyzed independently by village.

The bacterial community in Braffedon was more diverse than that of Yaokro (Shannon and Simpson's indexes 2.7085 and 0.091545, respectively, for Braffedon; Shannon and Simpson's indexes 2.4027 and 0.147, respectively, for Yaokro, Table 2). Six bacterial genera were commonly identified in both locations, which included *Bacillus*, *Burkholderia*, *Enterobacter*, *Pseudomonas*, *Ralstonia* and *Streptomyces*. Eight bacterial genera were only found in Braffedon (Table 1 and Figure 4A, 5A and 5C): *Agrobacterium*, *Alcaligenes*, *Chryseobacterium*, *Curtobacterium*, *Leifsonia*, *Paenibacillus*, *Pantoea* and *Streptococcus*, while thirteen genera were only found in Yaokro: *Blastococcus*, *Dyella*, *Herbaspirillum*, *Kitasatospora*, *Herbaspirillum*, *Klebsiella*, *Lysinibacillus*, *Ochrobactrum*, *Rhodococcus*, *Serratia*, *Sinomonas*, *Stenotrophomonas*, and *Variovorax*. Although Braffedon showed a higher bacterial diversity level than Yaokro, the latter scored a higher number of genera limited to that particular location.

Conversely, the fungal community was more significantly diverse in Yaokro when compared to that observed in Braffedon (Shannon and Simpson's indexes 2.0339 and 0.20588, respectively, for Braffedon and Shannon and Simpson's indexes 2.5142 and 0.11985, respectively, for Yaokro). Seven fungal genera were isolated from both Braffedon and Yaokro: *Aspergillus*, *Candida*, *Cryptococcus*, *Fusarium*, *Penicillium*, *Purpureocillium*, and *Trichoderma*. Five genera were only found in Braffedon (Table 1 and Figure 4B, 5B and 5D): *Exophiala*, *Meira*, *Pestalotiopsis*, *Pseudozyma*, and *Rhodosporidium*, while fifteen genera were only found in Yaokro: *Arthrinium*, *Chaetomium*, *Cladophialophora*, *Clonostachys*, *Cunninghamella*, *Eupenicillium*, *Gibberella*, *Gliocladium*, *Neodeighonia*, *Nirograna*, *Phialemonium*, *Phialocephala*, *Pseudallescheria*, *Rhodotorula*, and *Triplosphaeria*.

In Braffedon, the largest number of bacteria belonged

to the genus *Bacillus*, representing 15% of all the bacterial isolates identified, while *Candida* was the most abundant fungal genus, representing 24% (Table 1). In Yaokro, *Burkholderia* was the most abundant bacterial genus, while *Neodeighonia* was the most abundant fungal genus, corresponding to 19 and 12% of all the bacterial and fungal isolates identified, respectively (Table 1).

When comparing Braffedon and Yaokro, no difference was found on the microbial community diversity levels among the different CILY stages S1, S2 or S3. The commonly identified bacterial and fungal genera: *Bacillus*, *Burkholderia*, *Enterobacter*, *Pseudomonas*, *Aspergillus*, *Penicillium*, *Purpureocillium*, and *Trichoderma* previously found indistinctly across the symptomatic palms in Braffedon and Yaokro were also found in the symptomless palms surveyed in both locations (Figure 5A to 5D). The genus *Fusarium* although found in both locations was limited to symptomless palms in Braffedon, and palms showing S2-like symptoms in Yaokro. However, there were isolates identified solely in each specific location. In Braffedon, *Paenibacillus* was only isolated from palms showing CILY S3-like symptoms. In Yaokro, four bacterial genera were only associated with symptomatic palms such as *Pseudomonas* and *Herbaspirillum* in those with S1-like symptoms, *Blastococcus* in those with S2-like symptoms and *Ralstonia* in those with S3-like symptoms. The fungal genera *Cunninghamella* was limited to palms with S1-like symptoms, and *Cladophialophora* and *Gibberella* to those with S3-like symptoms, while six other fungal genera *Eupenicillium*, *Hypocrea/Trichoderma*, *Nirograna*, *Phialocephala*, *Pseudallescheria*, and *Triplosphaeria* were only found in palms with S2-like symptoms (Figure 5A to 5D).

### Microbial community in the rhizosphere

The most abundant genera found in the rhizosphere in Braffedon (Table 1) were *Bacillus* (9.9%) followed by *Penicillium* (3.3%) then *Candida* (2.3%), while in Yaokro, these were *Burkholderia* (19%) followed by *Neodeighonia* (12.5%), *Penicillium* (6.6%) then *Trichoderma* (4.4%). Commonly genera identified from rhizosphere samples in both locations included *Bacillus*, *Burkholderia*, *Pseudomonas*, *Streptomyces*, *Cryptococcus*, *Penicillium*, *Purpureocillium* and *Trichoderma*. Four fungal genera *Arthrinium*, *Candida*, *Chaetomium*, and *Phialemonium* were solely isolated from symptomless palms in Yaokro.

### Endophytic community in leaves and trunks

The most abundant genera found in leaves in Braffedon (Table 1) were *Pantoea* (9.9%) followed by *Cryptococcus* (6.8%) then *Bacillus* (4.7%). For trunk samples, the most

**Table 1.** Relative abundance of bacterial and fungal isolates identified from leaves, trunk and rhizosphere in Braffedon and Yaokro.

Bacteria	Braffedon			Yaokro	
	Leaves	Trunk	Rhizosphere	Trunk	Rhizosphere
<b><i>Agrobacterium</i> sp.</b>			<b>0.5070</b>		
<b><i>Alcaligenes faecalis</i></b>			<b>0.4225</b>		
<b><i>Bacillus</i></b>	<b>4.6895</b>		<b>9.8859</b>	<b>6.0558</b>	<b>3.2669</b>
<i>B. aquimaris</i>			1.2674		
<i>B. cereus</i>					1.5139
<i>B. indicus</i>			0.5492		
<i>B. megaterium</i>	4.6895		0.5915		
<i>B. mycoides</i>			0.6337		1.1952
<i>B. niacini</i>					0.3187
<i>B. pseudomycooides</i>			0.5915		
<i>B. pumilus</i>				0.0797	
<i>B. subtilis</i>				0.2390	0.0797
<i>B. vietnamensis</i>			1.2674		
<i>Bacillus</i> sp.			4.9852	5.7371	0.1594
<b><i>Blastococcus</i> sp.</b>					<b>0.0797</b>
<b><i>Burkholderia</i></b>		<b>0.3802</b>	<b>0.2535</b>		<b>18.7251</b>
<i>B. anthina</i>			0.2535		
<i>B. cepacia</i>		0.3802			0.3984
<i>B. glumae</i>					0.1594
<i>B. nodosa</i>					4.8606
<i>Burkholderia</i> sp.					13.3068
<b><i>Chryseobacterium</i> sp.</b>			<b>0.5915</b>		
<b><i>Curtobacterium</i></b>	<b>1.8166</b>	<b>0.8450</b>			
<i>C. flaccumfaciens</i>		0.8450			
<i>Curtobacterium</i> sp.	1.8166				
<b><i>Dyella</i></b>					<b>0.7171</b>
<i>D. japonica</i>					0.6375
<i>Dyella</i> sp.					0.0797
<b><i>Enterobacter</i></b>	<b>2.6193</b>		<b>0.6337</b>	<b>3.2669</b>	
<i>E. hormaechei</i>			0.6337		
<i>Enterobacter</i> sp.	2.6193			3.2669	
<b><i>Herbaspirillum</i> sp.</b>					<b>0.0797</b>
<b><i>Kitasatospora</i> sp.</b>					<b>1.1952</b>
<b><i>Klebsiella pneumoniae</i></b>				<b>0.1594</b>	
<b><i>Leifsonia</i> sp.</b>	<b>3.1263</b>		<b>1.8589</b>		
<b><i>Lysinibacillus sphaericus</i></b>					<b>0.2390</b>
<b><i>Ochrobactrum</i> sp.</b>				<b>0.9562</b>	
<b><i>Paenibacillus assamensis</i></b>			<b>0.0422</b>		
<b><i>Pantoea</i></b>	<b>9.8859</b>	<b>1.1407</b>			
<i>P. agglomerans</i>	1.2252				
<i>P. dispersa</i>	8.6608				
<i>Pantoea</i> sp.		1.1407			
<b><i>Pseudomonas</i></b>	<b>1.8166</b>	<b>8.7452</b>	<b>1.0984</b>		<b>0.0797</b>
<i>P. denitrificans</i>					0.0797
<i>P. plecoglossicida</i>		8.7452	1.0984		
<i>P. psychrotolerans</i>	1.8166				
<b><i>Ralstonia</i> sp.</b>		<b>0.5492</b>			<b>0.0797</b>
<b><i>Rhodococcus</i> sp.</b>					<b>0.2390</b>

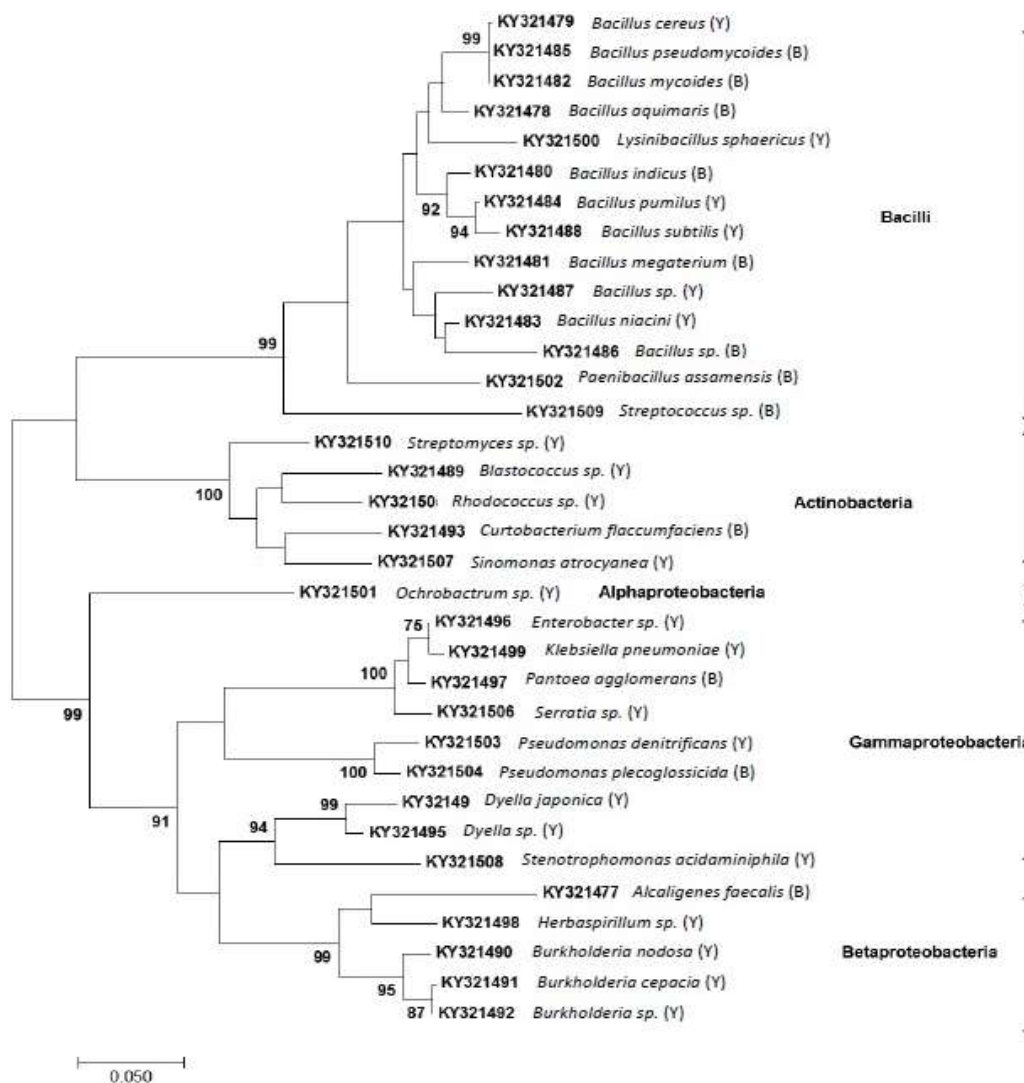
Table 1. Contd.

<b>Serratia sp.</b>				<b>2.4701</b>
<b>Sinomonas atrocyanea</b>				<b>2.3904</b>
<b>Stenotrophomonas</b>		<b>0.0797</b>		<b>0.1594</b>
<i>S. acidaminiphila</i>				0.1594
<i>Stenotrophomonas</i> sp.		0.0797		
<b>Streptococcus sp.</b>	<b>4.1403</b>			
<b>Streptomyces sp.</b>		<b>1.3519</b>		<b>0.7171</b>
<b>Variovorax paradoxus</b>				<b>1.5936</b>
<b>Fungi</b>				
<b>Arthrimum sp.</b>				<b>0.3984</b>
<b>Aspergillus</b>	<b>0.1267</b>	<b>0.0422</b>		<b>1.5936</b>
<i>A. aculeatus</i>		0.0422		1.5936
<i>A. flavipes</i>				0.0797
<i>A. nomius</i>				0.1594
<i>Aspergillus</i> sp.	0.1267			
<b>Candida</b>	<b>1.5632</b>	<b>19.9831</b>	<b>2.2814</b>	<b>8.1275</b>
<i>C. carpophila</i>	0.2535	6.8441		8.1275
<i>C. tropicalis</i>	1.3097	13.1390	2.2814	
<b>Chaetomium sp.</b>				<b>0.1594</b>
<b>Cladophialophora sp.</b>				<b>0.1594</b>
<b>Clonostachys candelabrum</b>				<b>0.3187</b>
<b>Cryptococcus</b>	<b>6.8019</b>		<b>1.6054</b>	<b>2.6295</b>
<i>Cryptococcus aff taibaiensis</i>	6.8019			
<i>Cryptococcus podzolicus</i>			1.6054	2.6295
<b>Cunninghamella bainieri</b>				<b>0.9562</b>
<b>Eupenicillium javanicum</b>				<b>0.0797</b>
<b>Exophiala alcalophila</b>			<b>0.4647</b>	
<b>Fusarium</b>		<b>0.0845</b>		<b>0.3984</b>
<i>F. solani</i>				0.3984
<i>Fusarium</i> sp.		0.0845		
<b>Gibberella fujikuroi</b>				<b>4.0637</b>
<b>Gliocladium cibotii</b>				<b>0.1594</b>
<b>Meira sp.</b>	<b>0.2957</b>			
<b>Neodeightonia subglobosa</b>				<b>12.5100</b>
<b>Nirograna sp.</b>				<b>0.7171</b>
<b>Penicillium</b>		<b>1.9434</b>	<b>3.3376</b>	<b>6.6135</b>
<i>P. citreonigrum</i>		0.3802		0.0797
<i>P. citrinum</i>	0.8450	1.4787		0.7171
<i>P. coffeae</i>				0.0797
<i>P. daleae</i>				0.3984
<i>P. oxalicum</i>		0.0845	0.0845	
<i>P. radicum</i>				0.3187
<i>P. rapidoviride</i>				0.3187
<i>P. simplicissimum</i>			2.7038	4.8606
<i>P. soppii</i>				3.7450
<i>P. verruculosum</i>			0.5492	0.1594
<i>Penicillium</i> sp.				0.7171
<i>Penicillium</i> sp.				0.0797
<b>Pestalotiopsis sp.</b>		<b>0.2112</b>		
<b>Phialemonium curvatum</b>				<b>0.0797</b>
<b>Phialocephala sp.</b>				<b>0.1594</b>
<b>Pseudallescheria sp.</b>				<b>0.0797</b>

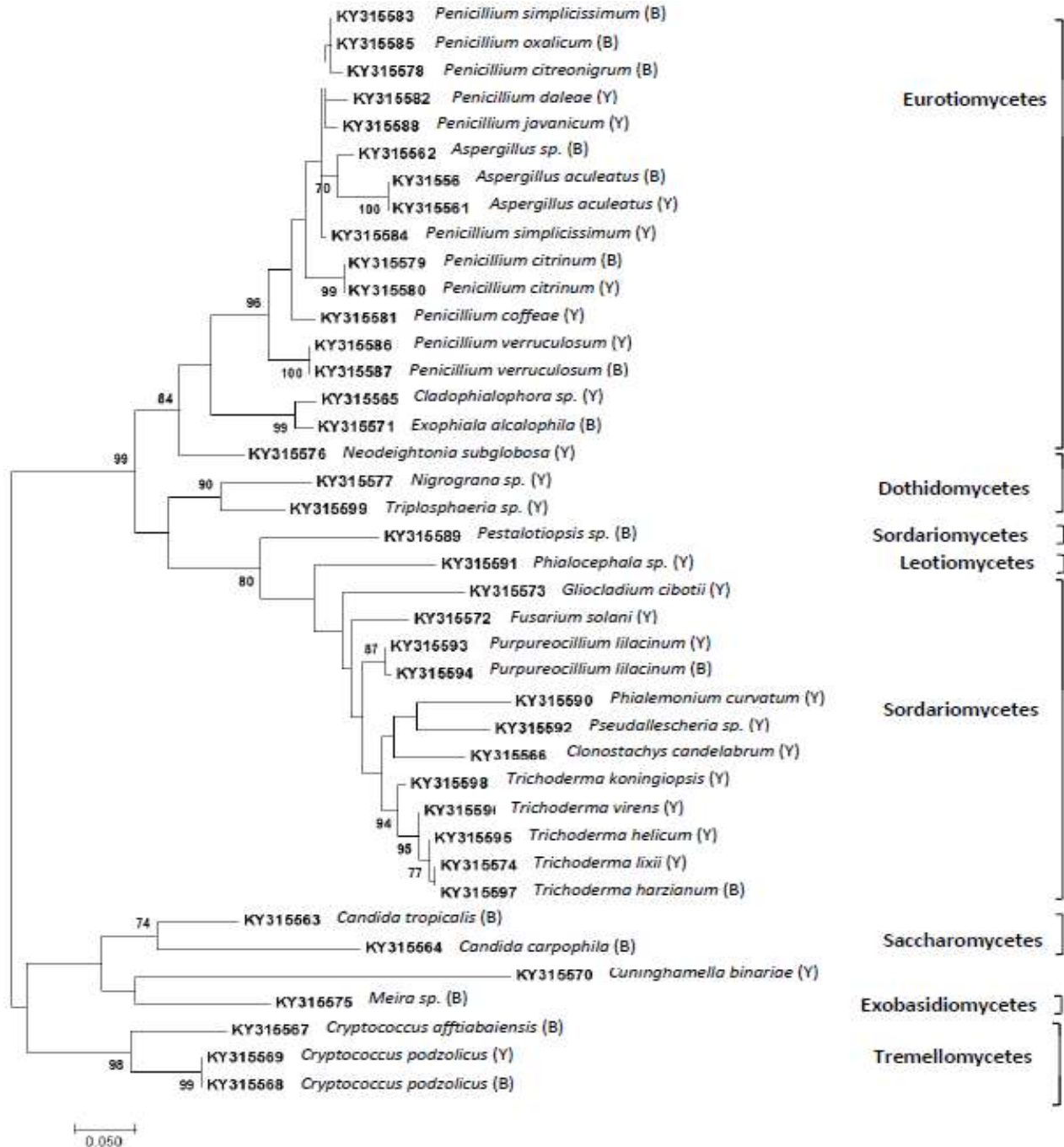
Table 1. Contd.

<b><i>Pseudozyma</i> sp.</b>	<b>0.4225</b>	
<i>Purpureocillium lilacinum</i>	0.1690	0.3984
<i>Rhodospiridium sphaerocarpum</i>	1.8166	
<i>Rhodotorula mucilaginosa</i>		7.4900
<b><i>Trichoderma/Hypocrea</i></b>	<b>1.6054</b>	<b>4.3825</b>
<i>T. erinaceum</i>		3.2669
<i>T. helicum</i>		0.6375
<i>T. koningiopsis</i>		0.3187
<i>T. lixii</i>	0.2535	0.0797
<i>T. virens</i>	1.3519	0.0797
<b><i>Triplosphaeria</i> sp.</b>		<b>0.0797</b>

Relative abundances are presented as proportions. Braffedon isolates are shown as light gray shaded rows. Yaokro isolates are shown as dark gray shaded rows. Commonly found bacterial and fungal isolates in Yaokro and Braffedon are shown as not shaded rows.



**Figure 2.** Phylogenetic analysis based on the 16S rRNA gene sequences of the bacterial isolates. Branches are grouped by classes. Bootstrap percentages from 1000 replicates > 75 are shown above branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. (B): Braffedon. (Y): Yaokro.



**Figure 3.** Phylogenetic analysis based on the ITS region of the rRNA gene sequences of the fungal isolates. Branches are grouped by classes. The percentage of trees (based on 1000 replicates) in which the associated taxa clustered together is shown above branches > 75. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. B: Braffedon. Y: Yaokro.

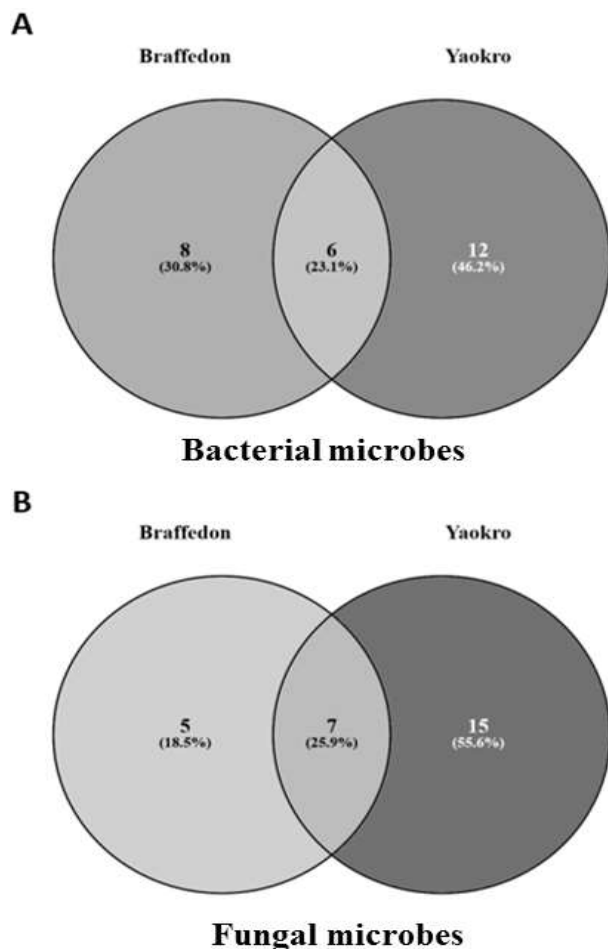
abundant genera in Braffedon were *Candida* (20%) followed by *Pseudomonas* (8.7%) then *Penicillium* (1.9%); and in Yaokro, *Candida* (8.1%) followed by *Rhodotorula* (7.5%) then *Penicillium* (4.8%). No bacteria were commonly isolated from trunk samples in Yaokro and Braffedon. However the opposite occurred for fungal

isolates, since commonly fungal genera identified from trunk samples in both locations included *Aspergillus* and *Candida*. The genus *Fusarium* was limited to trunk samples from Braffedon symptomless palms, while *Klebsiella* and *Candida* were only found in trunk samples from symptomless palms from Yaokro.

**Table 2.** Diversity of microbial communities associated with geolocation of coconut plantations in Yaokro and Braffedon.

Isolate	Shannon H		Simpson D	
	Braffedon	Yaokro	Braffedon	Yaokro
Bacteria	2.7085 <sup>a</sup>	2.4027 <sup>b</sup>	0.091545 <sup>a</sup>	0.147 <sup>b</sup>
Fungi	2.0339 <sup>a</sup>	2.5142 <sup>b</sup>	0.20588 <sup>a</sup>	0.11985 <sup>b</sup>
Bacteria + fungi	3.0975 <sup>a</sup>	3.1489 <sup>a</sup>	0.068594 <sup>a</sup>	0.066162 <sup>a</sup>

<sup>a,b</sup>Different letters indicate significant differences between geolocations at  $p < 0.05$  level with t test.



**Figure 4.** Venn diagram of the bacterial and fungal genera from Braffedon and Yaokro. **A.** Comparison of bacterial microbes either commonly for both geolocations or only in Braffedon or Yaokro. **B.** Comparison of fungal microbes either commonly for both geolocations or only in Braffedon or Yaokro. Venn diagram was done using Venny (<http://bioinfogp.cnb.csic.es/tools/venny/index.html>).

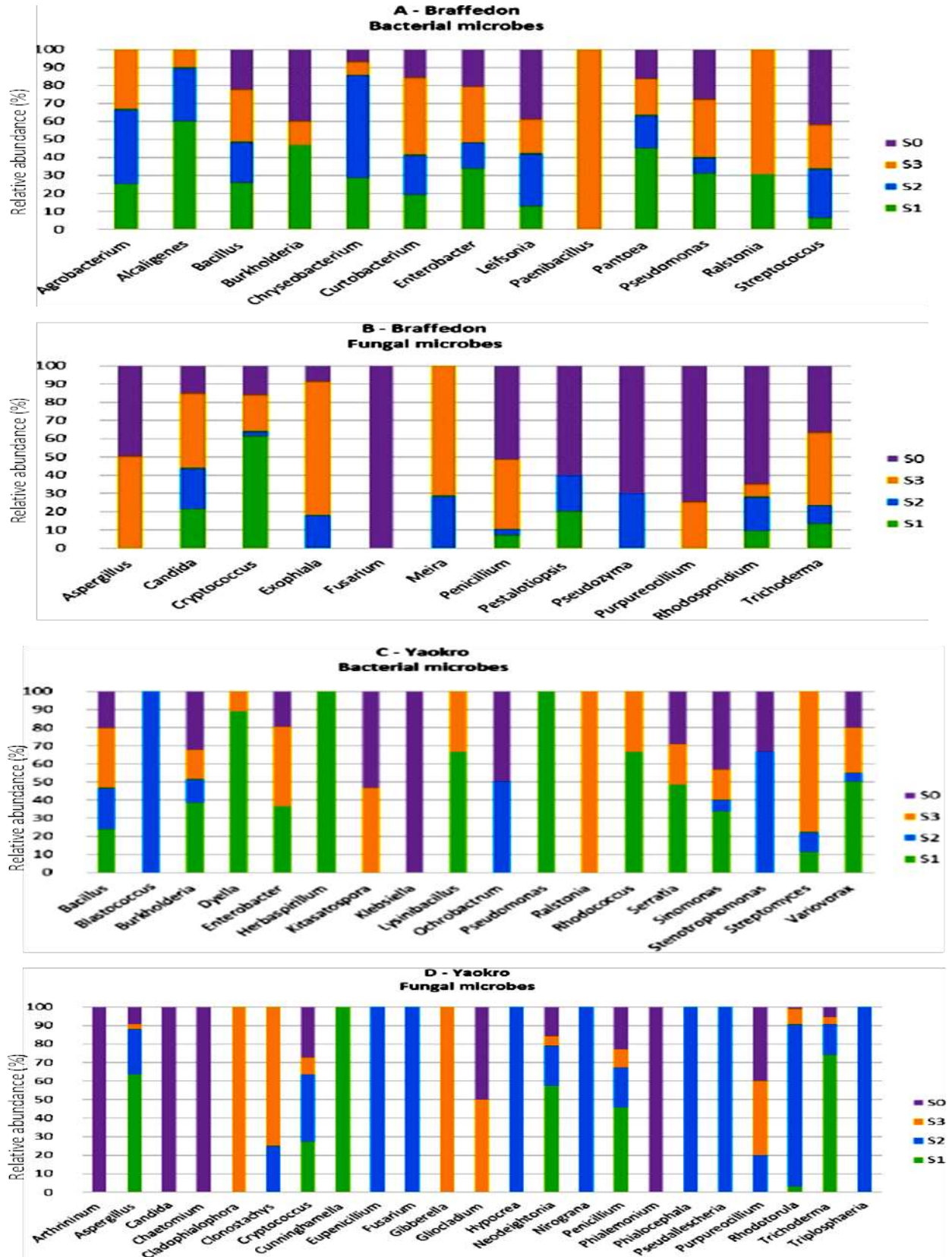
## DISCUSSION

The diversity of bacterial and fungal communities in coconut palms infected and not infected by the CILY

phytoplasma was investigated by coupling a high-throughput culturing method ('dilution-to-extinction') and sequence analysis, in order to increase the range of diversity explored in each sample. The 'dilution-to-extinction' cultivation methodology allows for a better recovery of species since it eliminates interaction between colonies, which supports the findings of bacterial and fungal species that seem to be limited to either Braffedon or Yaokro village.

It is noteworthy that no CILY phytoplasma was detected after PCR testing with the specific primers G813/AwkaSR in any of the six coconut palms collected in Braffedon exhibiting symptoms resembling those of CILY. No phytoplasma DNA was neither detected with the phytoplasma generic primers U5/U3. It is known that phytoplasmas are present at very low concentrations in the phloem of plants they infect (Maejima et al., 2014), so this may have prevented phytoplasma detection in the coconut palms. However, the partial 16S rDNA sequence recovered from P1/P7 amplifications from four out of six symptomatic palms confirmed the presence of *Bacillus megaterium*. Previous studies have confirm the amplification of bacterial DNA particularly *Bacillus* species with primers P1/P7 due to their lack of specificity (Harrison et al., 2002; Yankey et al., 2014). Harrison et al. (2002) reported the detection of *B. megaterium* from Canary Island date palms infected with the lethal decline phytoplasma. Moreover, PCR and sequencing results from our study were supported by the isolation of *B. megaterium* (4.7%, Table 1) from the endophytic bacterial community of coconut palm leaves in the Braffedon village, not found in Yaokro.

The fact that no phytoplasma DNA was detected from the symptomatic palms in Braffedon with the second phytoplasma generic primer pair U5/U3 ruled out the possibility of any detectable phytoplasma in those palms. Instead, *B. megaterium* was detected in the coconut palm phloem and amplified with universal primers P1/P7, hence, the probably higher titre of *B. megaterium* may have prevented any phytoplasma to be detected, if any. CILY-like signs seen in the coconut palms surveyed may be related to other factors, for instance, environmental. Sampling in Braffedon was conducted during April 2016 within the dry season, which fell within a period of a very severe drought during that season in early 2016. This was registered by SODEXAM (*Société d'Exploitation*



**Figure 5.** Relative abundance of bacterial and fungal isolates per disease stage S1, S2, S3 and in symptomless palms (S0). Braffedon bacterial (A) and fungal (B), and Yaokro bacterial (C) and fungal (D) genera are grouped according to the disease stages (S1, S2, S3) from where they were isolated.

et de Développement aéroportuaire, Aéronautique et Météorologique) in Côte d'Ivoire (Diallo, personal communication), so the drought may have had an impact on the agronomic performance of those six coconut palms in Braffedon.

No significant differences were observed in the overall diversity levels for fungal and bacterial organisms regardless the origin (leaves, trunk or rhizosphere) between Braffedon and Yaokro (Table 2). However, when bacterial and fungal communities were screened separately per location, Yaokro scored a higher number of both bacterial (13) and fungal (15) species limited to the location when compared to Braffedon. Since the CILY phytoplasma was detected in all symptomatic coconut palms from Yaokro, this suggests that the presence of the CILY phytoplasma in the symptomatic coconut palms may be a factor determining the level of diversity of a microbial community in that given geolocation. Bulgari et al. (2014) confirmed that indeed the endophytic bacterial community composition in grapevine is correlated to phytoplasma infection from studies of the endophytic bacterial community in grapevines healthy and infected with the "flavescence dorée" phytoplasma.

*Burkholderia* (18.7%) and *Neodeightonia* (12.5%) were the most abundant bacteria identified from the rhizosphere in Yaokro. Species of *Burkholderia* included *B. cepacia* and *B. nodosa*, known as plant growth promoters, nitrogen-fixing enhancers, and siderophore producers have been used for soil bioremediation for better water management, and biocontrol of soil-borne plant pathogenic fungi (de los Santos-Villalobos et al., 2012; Parke, 2000) and bacteria (Nion and Toyota, 2008). Although it is not clear its role, the fact that *Burkholderia* was mostly isolated from symptomless and S1-like symptom bearing palms suggests it as a candidate to further assess as a possible biocontrol against the CILY phytoplasma.

The plant-growth promoter *Enterobacter* was isolated from rhizosphere only from Braffedon, while *Penicillium* and *Trichoderma* were identified from Yaokro. In all cases these genera were present in both symptomless and symptomatic palms. *Enterobacter* has been isolated from the rhizosphere of coconut palms in India and proposed as a candidate for further biofertilizer development (Gupta et al., 2014); while *Penicillium* and *Trichoderma* have been combined and used as biocontrol against Ganoderma wilt of coconut (Srinivasulu et al., 2001). Since these genera have been tested in coconut palms, further investigation may help clarifying their possible role as potential bioinoculant or biofertilizer to mitigate CILY.

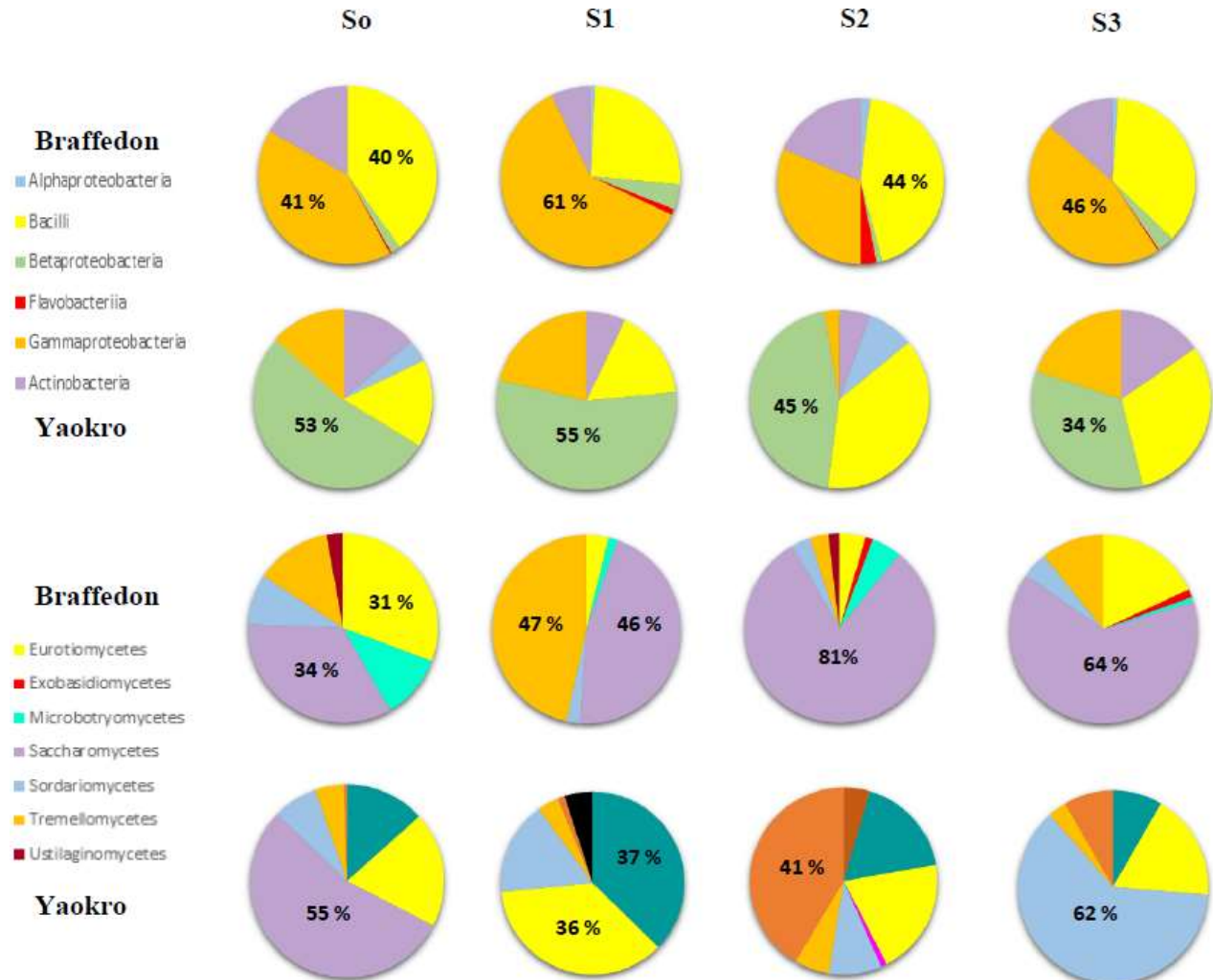
There are no records of *Neodeightonia* as biocontrol. This genus was isolated from symptomless coconut palms, as well as palms with S1-, S2-, and S3-like symptoms; however, it may be more directly associated to the early stages of the CILY phytoplasma colonization since it was mostly isolated from palms exhibiting S1-like

symptoms. *Bacillus* (9.9%) was the most abundant genus isolated from the rhizosphere in Braffedon. In India, *Bacillus* sp. have been isolated from coconut rhizosphere and proved to have antagonistic activity against coconut fungal pathogens (George et al., 2011). *Bacillus* sp. have been also proven as plant growth promoting rhizobacteria to be used as effective bioinoculants to support coconut organic farming (Geetanjali et al., 2015; George et al., 2012). Coconut palms in Braffedon showed signs that although not associated with the CILY phytoplasma, may be related to drought exposure, so more investigation could support a possible role of *Bacillus* sp. in enhancing coconut palms resilience to this environmental constraint.

*Arthrinium*, *Chaetomium* and *Phialemonium* were solely isolated from the rhizosphere of symptomless coconut palms in Yaokro. *Chaetomium* and *Phialemonium* have been long used as biocontrols of several fungal diseases (Hung et al., 2015; Shanthiyaa et al., 2013; Hiratsuka and Chakravarty, 1999), while *Arthrinium* has been recommended as a potential biocontrol of the bayoud disease in palm trees (Calvo et al., 2005). The fact that specific fungal organisms were limited to the rhizosphere of symptomless palms in Yaokro raises many expectations for the possibility of assessing these for their biocontrol potential against the CILY phytoplasma.

Previous studies have shown that the endophytic species composition and frequency vary with different tissues of host plants. In fact, Petrini et al. (1993) suggested that plant organs resemble distinct microhabitats for endophyte colonization. Therefore, endophytic species richness and composition within a tree species will always differ among individual trees, even within homogenous investigation sites (Collado et al., 2007; Petrini et al., 1993). The results of the present study are in consonance with these observations; hence, every palm at a given location is at different risk of CILY, compared to its neighbouring palms.

The occurrence of bacterial and fungal endophytes identified in either Braffedon or Yaokro varies upon the microhabitat and location. For instance, *B. megaterium* was restricted to leaves in Braffedon, while *B. pumilus* and *B. subtilis* were only isolated from trunk samples from Yaokro; *Enterobacter* was found in trunks from Yaokro, and in leaves in Braffedon, while *Curtobacterium*, *Pantoea* and *Streptococcus* were limited to leaf samples from Braffedon. For fungal species, *Aspergillus* and *Candida* were found in trunks and leaves in Braffedon, and in trunk in Yaokro; *Pestalotiopsis*, *Pseudozyma* and *Fusarium* in trunks from Braffedon, and *Meira* sp. and *Cryptococcus* sp. from leaves in Braffedon. *Pantoea agglomerans*, *Curtobacterium*, *Bacillus*, *Burkholderia*, *Pestalotiopsis* have been recorded as biocontrol agents against a broad spectrum of plant pathogens, or part of endophytic communities in a number of crops (Bulgari et al., 2011; Grisan et al., 2011). The above mentioned observations support the fact that apart from the nature of



**Figure 6.** Relative abundance of isolated bacterial and fungal classes from Braffedon and Yaokro.. S0, Symptomless palms. Coconut palms with CILY-like symptoms; S1, Disease stage 1; S2, Disease stage 2; S3, Disease stage 3.

the host tissue, the geographic location may influence the host composition of the microbial community, including the endophytic population (Suryanarayanan and Vijaykrishna, 2001; Petrini et al., 1993), aspects that would worth be further investigated.

*Klebsiella pneumoniae* and *Candida* were solely found in trunk samples corresponding to symptomless coconut palms in Yaokro. There are records of *K. pneumoniae* as part of endophytic communities such as that from soybean (Kuklinsky-Sobral et al., 2005), but not yet from coconut palms. Interestingly, Tagliavia et al. (2014) recently identified *K. pneumoniae* as the major bacterial microbe within the gut microbiota of *Rhyncophorus ferrugineus*, the red palm weevil, one of the major pests of palms, including coconut. *Candida* is a known antagonistic agent used as biological control of postharvest diseases, available as commercial products such as Aspire and Yield-Plus (Sui et al., 2015). The fact that *K. pneumoniae* and *Candida* are only present in

symptomless palms in Yaokro opens up expectations of their possible potential as biocontrol against the CILY phytoplasma, which should be further studied. On the other hand, *Fusarium* was limited to trunk samples from symptomless palms in Braffedon, which may make it a good candidate as bioinoculant to boost plant resilience against drought.

As previously reported, *Proteobacteria* is the most abundant phylum identified in plants (Bulgari et al., 2012). From our study, the dominant bacterial classes identified in Yaokro from all the disease stages, including the symptomless palms were *Betaproteobacteria* followed by *Bacilli*, while in Braffedon the dominant bacterial classes were *Gammaproteobacteria* followed by *Bacilli* (Figure 6). These results coincide with those from Bulgari et al. (2012), where *Betaproteobacteria* was the dominant bacterial class isolated from apple tree roots, healthy and infected with 'Ca. P. mali'.

The dominant fungal classes in Yaokro corresponded

to *Saccharomyces* for the symptomless palms and *Dothiomycetes*, *Urediniomycetes* and *Sordariomycetes*; whereas in Braffedon, the fungal dominant class was *Saccharomyces*. Results show that there is more diversity for the fungal endophytic communities in CILY phytoplasma-infected coconut palms, which suggests that the presence of the CILY phytoplasma may influence the composition of the fungal endophytic community. Furthermore, the 'dilution-to-extinction' cultivation method proved to be a reliable high-throughput method for isolation of both endophytes and rhizosphere-limited species from either Braffedon or Yaokro villages.

Since the endophytic control is one of the desirable sustainable approaches for the possible control of phytoplasma diseases, bacterial and fungal endophytes identified in the present study from coconut palms from either Braffedon or Yaokro should be further investigated to assess their biocontrol potential against the CILY phytoplasma. Most of the bacterial and fungal endophytes isolated from coconut palms from Braffedon and Yaokro have been already reported as potential biocontrols for a number of bacterial, fungal and phytoplasma diseases around the world. These are so far the first results that refer to the rhizosphere, as well as the bacterial and fungal endophytic populations isolated through a high-throughput cultivation method, 'dilution-to-extinction' from coconut palms symptomless and with CILY-like symptoms, as well as, infected and non-infected with the CILY phytoplasma.

The study offers a baseline for future research on culturable endophytic or rhizosphere microbial communities that may be used as future biocontrols, or bioinoculants to enhance plant resilience against CILY or environmental constraints like drought in Côte d'Ivoire. At this stage it may be too early to recommend which bacterial or fungal species could be effectively used to tackle CILY in Côte d'Ivoire without having further evidences on the assessment of their role as potential biocontrols. Further testing should be done to explore the richness of the endophytic microbial population profiles, and to further clarify the possible environmental and physiological factors that may govern their occurrence in both Braffedon and Yaokro. Once more data and research evidence become available, the present research outcomes can be used as groundwork to help designing new strategies for the effective management of CILY in Grand-Lahou.

## CONFLICTS OF INTERESTS

The authors have not declared any conflict of interests.

## ACKNOWLEDGEMENTS

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## REFERENCES

- Aakra Å, Utåker JB, Nes IF, Bakken LR (1999). An evaluated improvement of the extinction dilution method for isolation of ammonia-oxidizing bacteria. *J. Microbiol. Methods* 39:23-31.
- Arocha Rosete Y, Diallo H, Konan Konan J, Yankey N, Saleh M, Pilet F, Contaldo N, Paltrinieri S, Bertaccini A Scott J (2017). Detection and differentiation of the coconut lethal yellowing phytoplasma in coconut-growing villages of Grand-Lahou, Côte d'Ivoire. *Ann. Appl. Biol.* 170(3):333-47.
- Bangels E, Peusens G, Beliën T (2010). Integrated management of phytoplasma diseases in pome fruit: an overview of efficacy results of IPM insecticides against pear Psylla (*Cacopsylla pyri*). *Commun. Agric. Appl. Biol. Sci.* 75:255-263.
- Batugal P, Rao VR, Oliver J (Eds.) (2005). Coconut Genetic Resources. International Plant Genetic Resources Institute – Regional Office for Asia, the Pacific and Oceania (IPGRI-APO), Serdang, Selangor DE, Malaysia.
- Baudoin L, Lebrun P, Berger A, Myrie W, Been B, Dollet M (2008). The Panama Tall and the Maypan hybrid coconut in Jamaica: did genetic contamination caused a loss of resistance to lethal yellowing. *Euphytica* 161:353-360.
- Brower JE, Zar JH, von Ende C (1998). Field and laboratory methods for general ecology. Boston, Mass, WCB McGraw-Hill, 273p.
- Bulgari D, Bozkurt AI, Casati P, Çağlayan K, Quaglino F, Bianco PA (2012). Endophytic bacterial community living in roots of healthy and 'Candidatus Phytoplasma mali'-infected apple (*Malus domestica*, Borkh.) trees. *Antonie Van Leeuwenhoek* 102:677-687.
- Bulgari D, Casati P, Brusetti L, Quaglino F, Brasca M, Daffonchio D, Bianco PA (2009). Endophytic bacterial diversity in grapevine (*Vitis vinifera* L.) leaves described by 16S rRNA gene sequence analysis and length heterogeneity-PCR. *J. Microbiol.* 47:393-401.
- Bulgari D, Casati P, Crepaldi P, Daffonchio D, Quaglino F, Brusetti L, Bianco PA (2011). Restructuring of endophytic bacterial communities in grapevine yellows-diseased and recovered *Vitis vinifera* L. plants. *Appl. Environ. Microbiol.* 77:5018-5022.
- Bulgari D, Casati P, Quaglino F, Bianco P (2014). Endophytic bacterial community of grapevine leaves influenced by sampling date and phytoplasma infection process. *BMC Microbiol.* 14(1):198.
- Calvo MA, Arosemena L, Agut M, Adelantado C, Aissaoui A (2005). Biological control of Bayaud in the palm trees by strains of *Arthrinium*. *J. Biol. Sci.* 5:236-238.
- Collado J, Platas G, Paulus B, Bills GF (2007). High-throughput culturing of fungi from plant litter by a dilution-to-extinction technique. *FEMS Microbiol. Ecol.* 60(3):521-533.
- Compant S, Brader G, Muzammil S, Sessitsch A, Lebrühi A, Mathieu F (2013). Use of beneficial bacteria and their secondary metabolites to control grapevine pathogen diseases. *BioControl* 58:435-455.
- D'Amelio R, Massa N, Gamalero E, D'agostino G, Sampò S, Berta G, Faoro F, Iriti M, Bosco D, Marzachi C (2007). Preliminary results on the evaluation of the effects of elicitors of plant resistance on chrysanthemum yellows phytoplasma infection. *Bull. Insectol.* 60: 317-318.
- Danyo G (2011). Review of scientific research into the Cape Saint Paul Wilt Disease (CSPWD) of coconut in Ghana. *Afr. J. Agric. Res.* 6:4567-4578.
- de los Santos-Villalobos S, Barrera-Galicia GC, Miranda-Salcedo MA, Cabriales JJP (2012). *Burkholderia cepacia* XXVI siderophore with biocontrol capacity against *Colletotrichum gloeosporioides*. *World J. Microbiol. Biotechnol.* 28:2615-2623.
- Deng S, Hiruki C (1991). Amplification of 16S rRNA genes from culturable and nonculturable Mollicutes. *J. Microbiol. Methods* 14:53-61.
- Dimitriu PA, Lee D, Grayston SJ (2010). An evaluation of the functional

- significance of peat microorganisms using a reciprocal transplant approach. *Soil Biol. Biochem.* 42:65-71.
- FAOSTAT-Food and Agriculture Organization of the United Nations (2015). Economic and Social Department: The Statistical Division. FAOSTAT [WWW Document]. URL <http://www.fao.org/faostat/en/#home> (accessed 10.14.16).
- Franklin RB, Mills AL (2006). Structural and functional responses of a sewage microbial community to dilution-induced reductions in diversity. *Microbiol. Ecol.* 52:280-288.
- Garcia-Chapa MA, Batlle A, Laviña A, Camprubí A, Estaún V, Calvet C (2004). Tolerance increase to Pear decline phytoplasma in Mmychorrizal OHF-333 pear rootstock. *Acta Hort.* 657:437-441.
- Geetanjali K, Sakthivel T, Subramani K, Manigundan Velmurugan A, Gautam RK (2015). Characterization of Coconut Rhizobacteria for Plant Growth Promoting Traits. *J. Andaman Sci. Assoc.* 20:136-140.
- George P, Gupta A, Gopal M, Chandra-Mohanar R, Thomas L, George V (2011). Antagonistic activity of coconut rhizospheric and endophytic *Bacillus* spp. against *Ganoderma applanatum* and *Thielaviopsis paradoxa*. *J. Plantation Crops* 39:278-284.
- George P, Gupta A, Gopal M, Chandra-Mohanar R, Thomas L, George V (2012). Screening and *in vitro* evaluation of phosphate solubilizing bacteria from rhizosphere and roots of coconut palms (*Cocos nucifera* L.) growing in different states of India. *J. Plantation Crops* 40:61-64.
- Golinska P, Wypij M, Agarkar G, Rathod D, Dahm H, Rai M (2015). Endophytic actinobacteria of medicinal plants: diversity and bioactivity. *Antonie Van Leeuwenhoek* 108:267-289.
- Gopal M, Gupta A, Nair RV (2005). Variations in hosting beneficial plant-associated microorganisms by root (wilt)-diseased and field-tolerant coconut palms of West Coast Tall variety. *Curr. Sci.* 89:1922-1927.
- Griffiths BS, Ritz K, Wheatley R, Kuan HL, Boag B, Christensen S, Ekelund F, Sorensen SJ, Muller S, Bloem J (2001). An examination of the biodiversity-ecosystem function relationship in arable soil microbial communities. *Soil Biol. Biochem.* 33:1713-1722.
- Grisan S, Martini M, Musetti R, Osler R (2011). Development of a molecular approach to describe the diversity of fungal endophytes in either phytoplasma-infected, recovered or healthy grapevines. *Bull. Insectol.* 64: S207-S208.
- Gupta A, Gopal M, Thomas GV, Manikandan V, Gajewski J, Thomas G, Seshagiri S, Schuster SC, Rajesh P, Gupta R (2014). Whole genome sequencing and analysis of plant growth promoting bacteria isolated from the rhizosphere of plantation crops coconut, cocoa and arecanut. *PLoS One* 9:e104259.
- Hammer O, Harpe DAT, Ryan PD (2001). PAST: Paleontological Statistics Software: Package for Education and Data Analysis. *Palaeontologia Electronica*, <http://palaeo-electronica.org>.
- Harrison NA, Davis RE, Helmick EE (2013). DNA Extraction from Arborescent Monocots and How to Deal with Other Challenging Hosts. In: Dickinson, M. Hodgetts, J. (Eds.), *Phytoplasma: Methods and Protocols*. Humana Press, Totowa, NJ, Pp. 147-158.
- Harrison NA, Davis RE, Oropeza C, Helmick EE, Narváez M, Eden-Green S, Dollet M, Dickinson M (2014). "*Candidatus* Phytoplasma palmicola", associated with a lethal yellowing-type disease of coconut (*Cocos nucifera* L.) in Mozambique. *Int. J. Syst. Evol. Microbiol.* 64:1890-1899.
- Harrison NA, Womack M, Carpio ML (2002). Detection and characterization of a lethal yellowing (16SrIV) group phytoplasma in Canary Island date palms affected by lethal decline in Texas. *Plant Dis.* 86:676-681.
- Hiratsuka Y, Chakravarty P (1999). Role of *Phialemonium curvatum* as a potential biological control agent against a blue stain fungus on aspen. *Eur. J. Forest Pathol.* 29: 305-310.
- Hung PM, Wattanachai P, Kasem S, Poeaim S (2015). Efficacy of Chaetomium Species as Biological Control Agents against *Phytophthora nicotianae* Root Rot in Citrus. *Mycobiology* 43:288-296.
- Hutcheson K (1970). A test for comparing diversities based on the shannon formula. *J. Theor. Biol.* 29:151-154.
- Konan Konan JL, Allou K, Diallo HA, Yao DS, Koua B, Kouassi N, Benabid R, Michelutti R, Scott J, Arocha-Rosete Y (2013a). First report on the molecular identification of the phytoplasma associated with a lethal yellowing-type disease of coconut palms in Côte d'Ivoire. *New Dis. Rep.* 28(3):2044-0588.
- Konan Konan JL, Lekadou T, N'goran B, Kouassi N, Gbalou Y (2013b). Project FIRCA (Fonds Interprofessionnel pour la Recherche et le Conseil Agricoles)/Cocotier CNRA – COC No. 588. Etude de la maladie du cocotier identifiée dans le Département de Grand- Lahou, 50p.
- Kra KD, Atta Diallo TH, Toualy MN, Kwadjo KE, Nazaret KEY, Regis YYF, Kouamé KAEP, Séka K, Ouattara BGM, Ibo JG, Konan Konan JL, Arocha Rosete Y (2017). Field schools and plant clinics: effective agricultural extension approaches to fight the coconut lethal yellowing disease and improve livelihoods of smallholder farmers in Grand-Lahou, Côte d'Ivoire. *Int. J. Agric. Ext. Rural Dev.* 5:545-555.
- Kuklinsky-Sobral J, Araujo WL, Mendes R, Pizzirani-Kleiner AA, Azevedo J. (2005). Isolation and characterization of endophytic bacteria from soybean (*Glycine max*) grown in soil treated with glyphosate herbicide. *Plant Soil* 273:91-99.
- Kumar S, Stecher G, Tamura K (2016). MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. *Mol. Biol. Evol.* msw054.
- Kumar ST, Aparna NS (2014). *Cordyceps* species as a biocontrol agent against coconut root grub, *Leucopholis coneophora* Burm. *J. Environ. Res. Dev.* 8:614-618.
- Lane DJ (1991). 16S/23S rRNA sequencing. In: S. E. & M. Goodfellow (Eds.), *Nucleic acid techniques in bacterial systematics*, New York: John Wiley and Sons. Pp.125-175.
- Lherminier J, Benhamou N, Larrue J, Millet ML, Boudon-Padieu E, Nicole M, Blein JP (2003). Cytological characterization of elicitor induced protection in tobacco plants infected by *hytophthora parasitica* or phytoplasma. *Phytopathology* 93:1308-1319.
- Lorenz KH, Schneider B, Ahrens U, Seemüller E (1995). Detection of the apple proliferation and pear decline phytoplasmas by PCR amplification of ribosomal and non-ribosomal DNA. *Phytopathology* 85:771-776.
- Lugtenberg B, Kamilova F (2009). Plant-Growth-Promoting Rhizobacteria. *Ann. Rev. Microbiol.* 63:541-556.
- Maejima K, Oshima K, Namba S (2014). Exploring the phytoplasmas, plant pathogenic bacteria. *J. Gen. Plant Pathol.* 80:210-221.
- Malembic-Maher S, Le Gall F, Danet JL, Dorlhac de Borne F, Bové JM, Garnier-Semancik M (2005). Transformation of tobacco plants for single-chain antibody expression via apoplastic and symplasmic routes, and analysis of their susceptibility to stolbur phytoplasma infection. *Plant Sci.* 168:349-358.
- Martini M, Musetti R, Grisan S, Polizzotto R, Borselli S, Pavan F, Osler R (2009). DNA-dependent detection of the grapevine fungal endophytes *Aureobasidium pullulans* and *Epicoccum nigrum*. *Plant Dis.* 93:993-998.
- Musetti R, Grisan S, Polizzotto R, Martini M, Paduano C, Osler R (2011). Interactions between "*Candidatus* Phytoplasma mali" and the apple endophyte *Epicoccum nigrum* in *Catharanthus roseus* plants. *J. Appl. Microbiol.* 110:746-756.
- Nion Y, Toyota K (2008). Suppression of bacterial wilt and Fusarium wilt by a *Burkholderia nodosa* strain isolated from Kalimantan soil, Indonesia. *Microbes Environ.* 23:134-141.
- Parke JL (2000). *Burkholderia cepacia*: friend or foe?. *The Plant Health Instructor*.
- Perica MC (2008). Auxin-treatment induces recovery of phytoplasma-infected periwinkle. *J. Appl. Microbiol.* 105:1826-1834.
- Petrini O, Sieber TN, Toti L, Viret O (1993). Ecology, metabolite production, and substrate utilization in endophytic fungi. *Nat. Toxins* 1:185-196.
- Rajendran L, Akila R, Karthikeyan G, Raguchander T, Samiyappan R (2015). Related Enzyme Induction In Coconut By Endophytic Bacteria (EPC 5). *Acta Phytopathol. Entomol. Hungarica* 50:29-43.
- Rajendran L, Karthikeyan G, Raguchander T, Samiyappan R (2008). Cloning and Sequencing of Novel Endophytic *Bacillus subtilis* from Coconut for the Management of Basal Stem Rot Disease. *Asian J. Plant Pathol.* 2:1-14.
- Rodriguez RJ, White JR, Arnold AE, Redman RS (2009). Fungal endophytes: diversity and functional roles. *New Phytol.* 182:314-330.
- Romanazzi G, Musetti R., Marzachi C, Casati P (2009). Induction of

- resistance in the control of phytoplasma diseases. *Petria* 19:113-129.
- Saracco P, Marzachi C, Bosco D (2008). Activity of some insecticides in preventing transmission of chrysanthemum yellows phytoplasma 'Candidatus Phytoplasma asteris' by the leafhopper *Macrostelus quadripunctulatus* Kirschbaum. *Crop Prot.* 27:130-136.
- Schneider B, Seemüller E, Smart CD, Kirkpatrick BC (1995). Phylogenetic classification of plant pathogenic mycoplasma-like organisms or phytoplasmas. *Molecular Diagnostic Procedures of Mycoplasmaology* 1, P.79.
- Schulz B, Boyle C (2006). What are Endophytes?, In: Schulz, B.J.E. Boyle, C.J.C. Sieber, T.N. (Eds.), *Microbial Root Endophytes*. Springer Berlin Heidelberg, Berlin, Heidelberg, Pp. 1-13.
- Shanthiyaa V, Saravanakumar D, Rajendran L, Karthikeyan G, Prabaka K, Raguchander T (2013). Use of *Chaetomium globosum* for biocontrol of potato late blight disease. *Crop Prot.* 52:33-38.
- Srinivasulu B, Aruna K, Rao DVR (2001). Biocontrol of Ganoderma wilt of coconut palm. in: *South Indian Horticulture*. South Indian Hortic. Assoc.49:240-242.
- Sui Y, Wisniewski M, Droby S, Liu J (2015). Responses of Yeast Biocontrol Agents to Environmental Stress. *Appl. Environ. Microbiol.* 81:2968-2975.
- Suryanarayanan TS, Vijaykrishna D (2001). Fungal endophytes of aerial roots of *Ficus benghalensis*. *Fungal Divers.* 8:155-161.
- Tagliavia M, Messina E, Manachini B, Cappello S, Quatrini P (2014). The gut microbiota of larvae of *Rhynchophorus ferrugineus* Oliver (Coleoptera: Curculionidae). *BMC Microbiol.*14(1):136.
- Tamura K, Nei M (1993). Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. *Mol. Biol. Evol.* 10:512-526.
- Tymon AM, Jones P, Harrison NA (1998). Phylogenetic relationships of coconut phytoplasmas and the development of specific oligonucleotide PCR primers. *Ann. Appl. Biol.* 132:437-452.
- Unterseher M, Schnittler M (2009). Dilution-to-extinction cultivation of leaf-inhabiting endophytic fungi in beech (*Fagus sylvatica* L.)—different cultivation techniques influence fungal biodiversity assessment. *Mycol. Res.* 113:645-654.
- Wertz S, Degrange V, Prosser JI, Poly F, Commeaux C, Freitag T, Guillaumaud N, Roux X (2006). Maintenance of soil functioning following erosion of microbial diversity. *Environ. Microbiol.* 8:2162-2169.
- Wertz S, Degrange V, Prosser JI, Poly F, Commeaux C, Guillaumaud N, Le Roux X (2007). Decline of soil microbial diversity does not influence the resistance and resilience of key soil microbial functional groups following a model disturbance. *Environ. Microbiol.* 9:2211-2219.
- White TJ, Bruns T, Lee S, Taylor JW (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: *PCR Protocols: a Guide to Methods and Applications* (M. Innis, D. Gelfand, J. Sninsky and T. White, eds.). Academic Press Inc., New York. 18(1):315-322.
- Wilson D (1995). Endophyte: The Evolution of a Term, and Clarification of Its Use and Definition. *Oikos* 73:274-276.
- Yankey N, Swarbrick PJ, Nipah JO, Quaicoe RN, Dickinson MJ (2014). Detection of the Cape St. Paul Wilt phytoplasma in coconut palms in Ghana through the combination of visual symptoms assessment and molecular diagnosis using a SecA gene based assay. *J. Plant Pathol.* 96:281-285.