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CHARACTERIZATION OF THE FUNGAL COMMUNITIES ASSOCIATED WITH *AQUILARIA CRASSNA* PIERRE EX LECOMTE PLANTATIONS IN FRENCH GUIANA

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Abstract:

Aquilaria is a tree species belonging to the family of the Thymeleaceae. When the tree is wounded, it can produce a blackened wood, also known as agarwood. The blackened wood has a darker colour than healthy wood and gives off a strong fragrance greatly appreciated by perfumers and by certain oriental religious communities. Production of this blackened wood is deemed to depend on environmental factors, including fungi.

The purpose of this work was to set up an experiment in Régina and Cacao, in French Guiana, to characterize the organization of microbial communities, particularly fungi, associated with Aquilaria crassna Pierre ex Lecomte, in order to understand their roles in agarwood formation.

In this study, we used mass sequencing with reversible terminators (Illumina). Of the initial 120 samples from which DNA was extracted, 27 samples of healthy wood, 29 samples of wounded wood and 3 soil samples were kept. These 59 samples were used to generate an average 37,890 sequences per sample. After data processing, we used 921 unique sequences spread across these 59 samples. Some majority OTUs were found for the wood samples. The soil samples showed the same trend, along with greater OTU diversity.

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With a view to producing agarwood of controlled quality in experimental Aquilaria plantations in French Guiana, we determined the majority genera present in the wood of these trees before and after wounding, and some majority genera depending on the different plots in Cacao and Régina. The results indicated that some genera were common to each other and to the literature, but others were fairly characteristic of a specific geographical zone, such as Periconia for Régina and Paraphaeosphaeria for Cacao.

***Key words**: Agarwood, Aquilaria, French Guiana, Illumina, ITS (Internal transcribed spacer)* (*Mass Sequencing, Operational Taxonomic Units (OTU).*

INTRODUCTION

The genus *Aquilaria* contains 21 tree species (Lee *et al.* 2017) and belongs to the family of the Thymelaeaceae. This genus is endemic to Southeast Asia and is of substantial cultural and commercial value thanks to the production of an oleoresin, brought about by wounding of the wood or by pathogens, notably wood-decay fungi. The modified wood is known as “agarwood”.

Agarwood has been used for centuries to make incense, and highly prized and expensive essential oils. The price of a kilogram of essential oil mostly ranges between 5,000 and 10,000 USD, (Barden *et al.* 2000; Blanchette *et al.* 2015). Around 10% of trees produce agarwood in the natural environment (Barden *et al.* 2000) and that percentage increases in plantation trees, though production still remains low. For example, in some Indian plantations it remains below 33% (Kalita *et al.* 2015). This explains how the resource has gradually been exhausted in its natural environment. As a result, most species of the genus *Aquilaria* are classed as CITES II (CITES 2004) and figure on that organization’s red list. Consequently, since 2014, some *A. crassna* plantations have been planted in French Guiana as part of an *ex-situ* conservation project for certain species of the genus *Aquilaria,* and for their exploitation under conditions suited to their biology (Zaremski 2018).

In some studies, it has proved possible to characterize the microorganisms found in *Aquilaria* wood, notably fungi that are naturally present, such as *Aspergillus, Arthrinium, Botryodyplodia, Diplodia, Dokmaia, Fusarium, Penicillium* or *Trichoderma* (Soehartono and Mardiastuti 1997; Mohamed *et al.* 2010; Zaremski *et al.* 2018). Some of those fungi play an active role in agarwood formation by helping to stimulate the defence reactions of the tree, causing it to secrete an oleoresin. The fungus *Lasodiplidia theobromae* (Pat.) Griffon & Maubl. seems to be constantly present in *Aquilaria* trees when they produce agarwood, while the proportions between *Fusarium solani* and *Cunninghamella bainieri* Naumov seem to depend on the individual trees. Agarwood formation is triggered by wounding the trunk and is prolonged by microorganisms present in the wood (Mohamed *et al.* 2014). The wood then produces an oleoresin rich in volatile and non-volatile molecules (Naef 2011), which delay the spread of the fungus and also activate the healing process. When *Aquilaria* wood is healthy, it is whitish and shiny. When it is loaded with oleoresin, it turns from pale beige to black as the oleoresin oxidizes, its density greatly increases and it becomes increasingly fragrant as the process evolves.

In an earlier study, some *Aquilaria* spp. samples from French Guiana, Cambodia, Thailand and Laos were taken from aboveground organs and roots to sequence the fungal ITS2 regions (Zaremski *et al.* 2018). In all, 693,961 sequences were obtained and grouped into 535 Operational Taxonomic Units (OTUs; Ascomycetes 87%, Basidiomycetes 10.5%). The fungal communities differed considerably between the aboveground organs and roots. Some fungi specific to a geographical area were found, along with others common to the different study sites. These results particularly enabled us to set up our experimental design in French Guiana to characterize the organization of microbial communities, particularly fungi, in order to understand their roles in agarwood formation.

We thus characterized the microbial communities associated with *A. crassna* before any wounding of the trunk, and after recent wounds left exposed to the open air. To that end, wood was removed after felling from some healthy trees and from some trees wounded six months earlier. The possible soil-borne origin of these communities was sought by analysing the microbial communities in the plantation soil. We used sequencing with reversible terminators (Illumina) to obtain profiles of the fungal communities associated with *A. crassna*. The high-throughput sequencing technique used (Illumina MiSeq/Double paired-end) enabled mass amplification of fungal genetic markers (ITS) that were large (between 250 and 450 base pairs) and hypervariable, in order to group the sequences, based on their homology with each other, in OTUs, which are considered by some authors as “molecular species”. These techniques give hundreds of thousands of sequences per run, resulting in several thousand usable sequences per modality, which amounts to a particularly interesting sequencing depth rich in taxonomic information.

This study was undertaken in experimental *A. crassna* plantations in Cacao and Régina, in French Guiana.

MATERIAL AND METHODS

Study sites

This work was undertaken in French Guiana, in the village of Cacao and the village of Régina), where the inhabitants are mainly of Lao origin and are widely involved in faming activities.

The *Aquilaria* trees used in this study came from an experimental plantation. They were planted in January 2014.

In Cacao, the trees were planted on six hectares, intercropped with *Citrus* trees, mainly mandarin (around 210 trees /ha), at a spacing of 4 x 4 m between trees. The soil was covered with a nitrogen-fixing Fabaceae cover crop, *Arachis pintoi* Krapov. & W.C. Greg. We divided the field in Cacao into three plots based on their topography. Plot A and plot B were on the same side of the field, on a plateau. Plot C was in a sloping section of the field. The trees in plot A came from a mixture of seeds of varying provenances (Cambodia, Thailand, Laos). The trees in plots B and C were grown from seeds originating from Laos.

The field in Régina, which was smaller (2 ha; around 500 trees/ha), corresponded to a single plot, plot P. The *Aquilaria* trees were not intercropped (monoculture) and the soil was relatively bare.

Experimental design

Aquilaria spp. wood samples were taken from 80 trees in March 2018: 20 trees per plot (A, B, C and P) and, of those 20 trees per plot, 10 were healthy and 10 had been wounded 6 months earlier. Sampling was preferentially done outside the rainy season, to ensure easier access to the plots. Holes measuring 3 mm in diameter and 2 to 3 cm in depth were made aseptically in each tree trunk with a drill, 1.30 m from the ground.

The sawdust from the holes was also aseptically collected with sterile tongs. The drill bit and the tongs were disinfected with alcohol then flamed before removing each sample. The samples were placed in sterile 2-ml Eppendorf tubes placed in bags containing Silicagel, then stored in the laboratory refrigerator in Cayenne. On arrival at the laboratory in Montpellier, the samples were stored in the refrigerator at -20°C pending molecular analyses.

Description of the samples

Supplementary Table II (Annex 1) gives the list of 120 samples from which DNA was extracted and which were sorted for sequencing. In the table, the samples are identified according to the sampling site (Cacao, Régina, Laos, lab (laboratory), plot (A, B, C, P), sample type (wood, fruiting body, mycelium, negative control), the healthy or wounded status of the tree, weight in grams, and the name of the sample depending on the position of the tree in the plot.

The study involved 120 samples subjected to total DNA extraction, PCR amplification of the fungal ITS, sequencing of the PCR products, sequence analysis by BlastN, the constitution of OTUs and their assignment to lines, then an analysis of the entire dataset: 54 samples of wounded wood, 42 samples of healthy wood, 8 samples of blackened wood (agarwood) from Laos, 3 samples of soil from Cacao, 9 samples of fungi serving as the Positive Control, 4 “Negative Control” samples (pure water).

DNA extraction and quantification

Once the 117 wood samples had been aseptically taken, they were used to extract total DNA using the DNeasy® PowerSoil® Kit (Qiagen 2018) protocol, after finely grinding around 130 mg of wood powder in liquid nitrogen using a marble pestle and mortar to break down the plant cell walls. After checking DNA purity and quantifying its concentration by fluorimetric assay (Quantus®, Qubit®) molecular analyses, notably DNA amplification by PCR with targeted primers on the ribosomal internal transcript spacers (ITS) and F-Risa analyses, were carried out to select samples for sequencing.

The three soil samples were also extracted using the DNeasy® PowerSoil® Kit (Qiagen 2018) protocol, apart from grinding in liquid nitrogen.

PCR amplification

Amplification was carried out in a reaction volume of 50 µL containing 5 µL of DNA matrix (50 ng), 10 µL of 5X Green Go Taq buffer, 4 µl of dnTP, 2 µL of each primer (ITS1 and ITS4 at 10 mM), 0.3 µL of Taq polymerase, and water (26.7 µl) to top up to 50 µL.

DNA-free controls were also used to test for any contamination in the reagents and buffers. PCR was carried out on a Perkin Elmer Applied Biosystems apparatus: Gen Amp PCR system 9700 or 2400.

Agarose gel electrophoresis

An 8 µL aliquot of the obtained amplification products was migrated on agarose gel (Seakem® LE Agarose Larza) at 2.5%, in a 1X TAE buffer (Tris-Acetate-EDTA) at 110 volts. Each well thus received 8 µl of PCR products and 3 µl of blue loading dye (6xDNA loading dye). The Quick-Load® 2-log DNA ladder

was used. The positive control used was *Antrodia vaillantii* (DC.) Ryvarden; the negative control was ultra-pure water.

The intensity of the PCR reaction, and the integrity and size of the amplified DNA fragments, were assessed after visualization of the migrated products with ethidium bromide (ETB) for 15 minutes, rinsing in water for 5 min and observation under UV light (590 nm).

After validating their quality on agarose, the PCR products were sent for sequencing at the Hydreka laboratory in Lyon (ww.hydreKa.com).

F-RISA analysis

F-RISA analysis is used to group samples to select them for sequencing. The purpose of analysing the fungal ribosomal intergenic spacer is to characterize the fungal communities of samples. The analysis was carried out in accordance with the "Agilent DNA 7500 and DNA 1200" guide (©Agilent Technologies Inc. 2016). Thus, each sample was represented by data corresponding to migration times of the different amplified DNAs.

Using these data, we were able to class the samples in eight groups and select 59 of them. These eight groups helped in the choice of the sequenced samples, though the metadata (types, status, location and plots) were decisive in the selection process. Supplementary Table 3 shows these 59 samples.

Illumina sequencing

Once these analyses had been interpreted, high-throughput sequencing was carried out. Sequencing with reversible terminators (Illumina) was adopted to obtain exhaustive profiles for the fungal communities associated with *A. crassna*.

The high-throughput sequencing technique (Illumina MiSeq /Double paired-end) was used to mass amplify fungal genetic markers (ITS) that were large in size (between 250 and 450 base pairs) and hypervariable, in order to identify the fungal species present.

All identification methods are dependent upon the existence of the sequence in databases, i.e. the fungus, or some phylogenetically close relatives, must have already been identified.

Sequencing was carried out on a MiSeq apparatus (Illumina) at 2x200.

The sequencing protocol used in this study followed the recommendations of the ILLUMINA® Company. The sequences obtained were thus classed in OTUs (Operational Taxonomic Unit).

The primers used to amplify the ITS fragments to be sequenced were (Op De Beeck *et al.* 2014): ITS3F: GCATCGATGAAGAACGCAGC; ITS4R: TCCTCCGCTTATTGATATGC.

The data were processed with R software, particularly the Dada2 package (package version: 1.10.1) following the recommended processing parameters (Callahan *et al.* 2016) Taxonomic annotation was carried out with the Bayesian RDP classifier (Wang *et al.* 2007) against the UNITE training set (Nilsson *et al.* 2019). The data generated in this way were checked and the diversity indices calculated with the *Phyloseq* package (McMurdie and Holmes 2013).

RESULTS AND DISCUSSION

Overall sequencing results

Sequencing of the 56 wood samples and three soil samples generated an average of 37,890 sequences per sample. It should be noted that three of the samples generated few sequences (samp-39, samp-54, samp-75). After data processing, 76% of the sequences were kept, on average, indicating good quality sequencing.

Thus, 931 unique sequences spread across 60 samples (59 + positive control) were kept after data processing. To check whether the diversity of the samples had been well described, rarefaction curves were produced (Fig. 1 Rarefaction curve for the sub-samples). The semi-parabolic curves, which levelled off depending on the number of sub-samples, indicated that the discovery of new OTUs slowed down. Thus, for the wood samples, it can be said that the majority OTUs were revealed. The soil samples displayed the same trend. It was also found that the soil samples had greater OTU diversity.

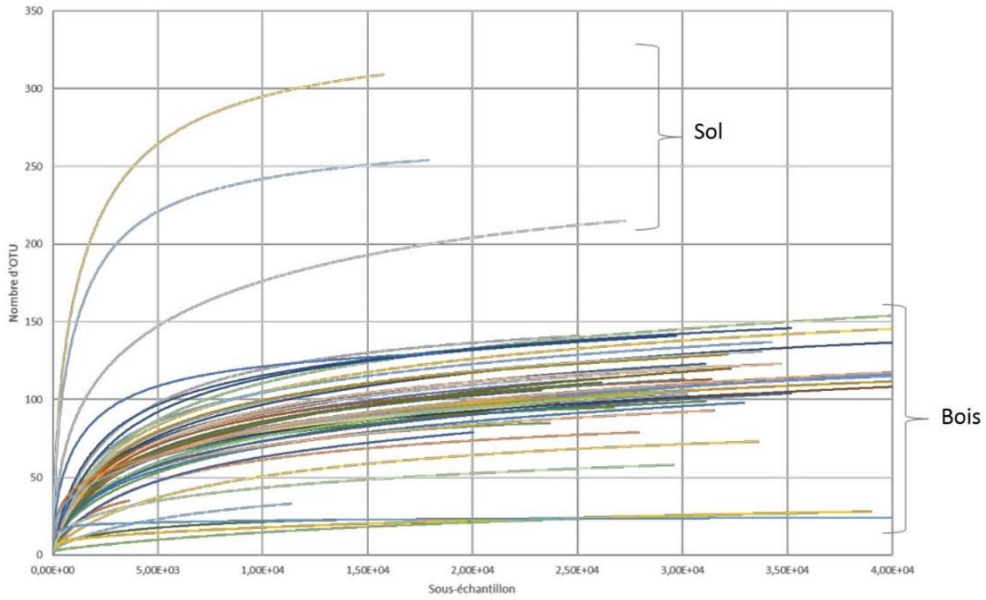

Fig. 1.
Rarefaction curve for the sub-samples (number of sub-samples = 50). The curves levelled off depending on the number of sub-samples: the number of new OTUs slowed down. It can be said that the majority OTUs were revealed. The soil samples displayed greater OTU diversity.

General results after taxonomic assignment

Taxonomic assignment was carried out using the UNITE database (Table 1). Out of 951 sequences, eight were discarded, as they were only present once, in a single sample. This resulted in 913 sequences.

Table 1

Number of OTUs per taxonomic level

	Phylum	Class	Order	Family	Genus	Species
Total number of OTUs	913	913	913	913	913	913
Number of OTUs assigned to level	734	488	422	353	289	181
Number of OTUs not assigned	179	425	491	560	624	732

An abundance analysis of the phyla showed that the majority phyla were firstly the Ascomycetes, followed by the Basidiomycetes (Fig. 2).

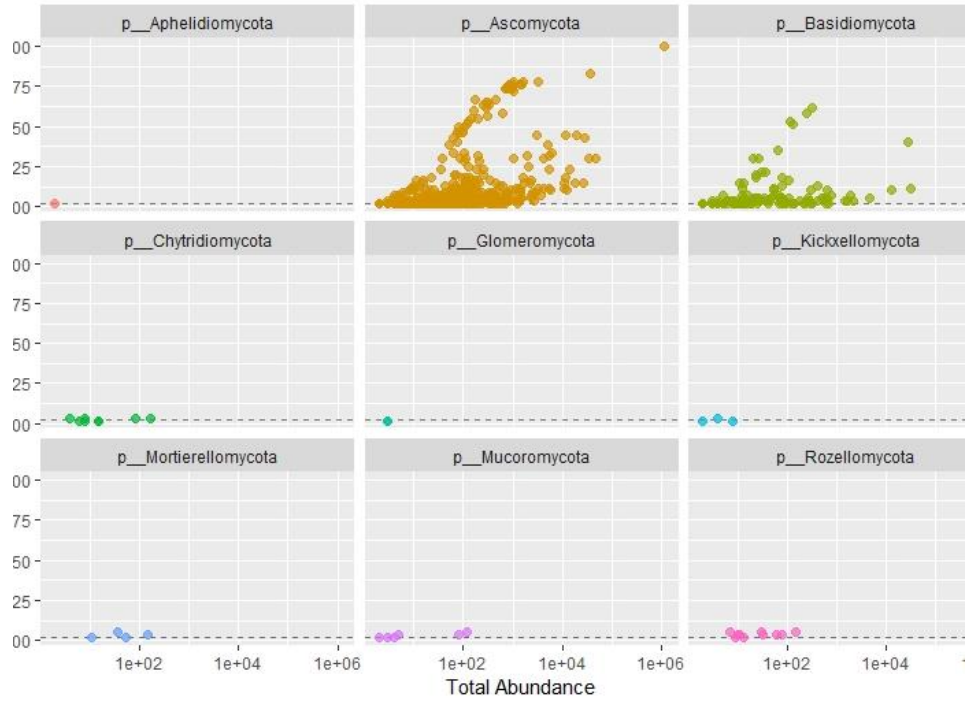

Fig. 2.

Phylum abundance. Ascomycota, Basidiomycota, Mortierellomycota, Mucoromycota and Rozellomycota had abundance values over 5%. Ascomycota and Basidiomycota proved to be the main phyla. The total abundances are shown on the x-axis, i.e. the sums of sequences in all the samples. The percentages of samples or sequences present at least once are shown on the y-axis. Fungal diversity depending on the plots and health status of the wood.

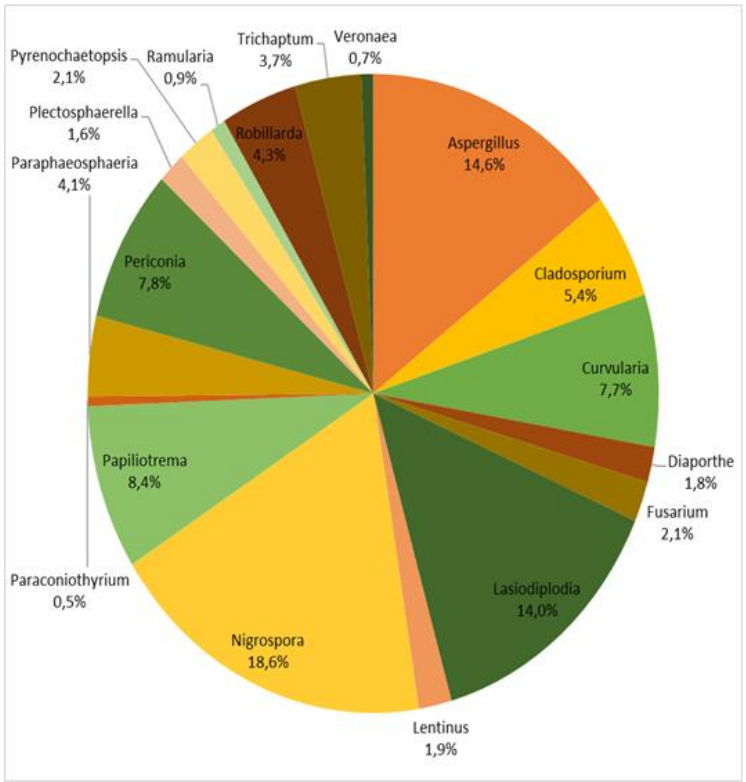

Fig. 3.

Distribution of the main fungal genera across all the samples (wood from French Guiana, wood from Laos, soil from plot C).

The distribution of the main fungal genera obtained for all the samples (wood from the healthy or wounded trees in French Guiana, soil from plot C, agarwood from Laos) is shown, like (Fig. 3). It can be seen that the most frequent genera in all these samples were *Nigrospora* (18.6%), *Aspergillus* (14.6%), *Lasiodiplodia* (14.0%), *Papiliotrema* (8.4%), *Periconia* (7.8%) and *Curvularia* (7.7%).

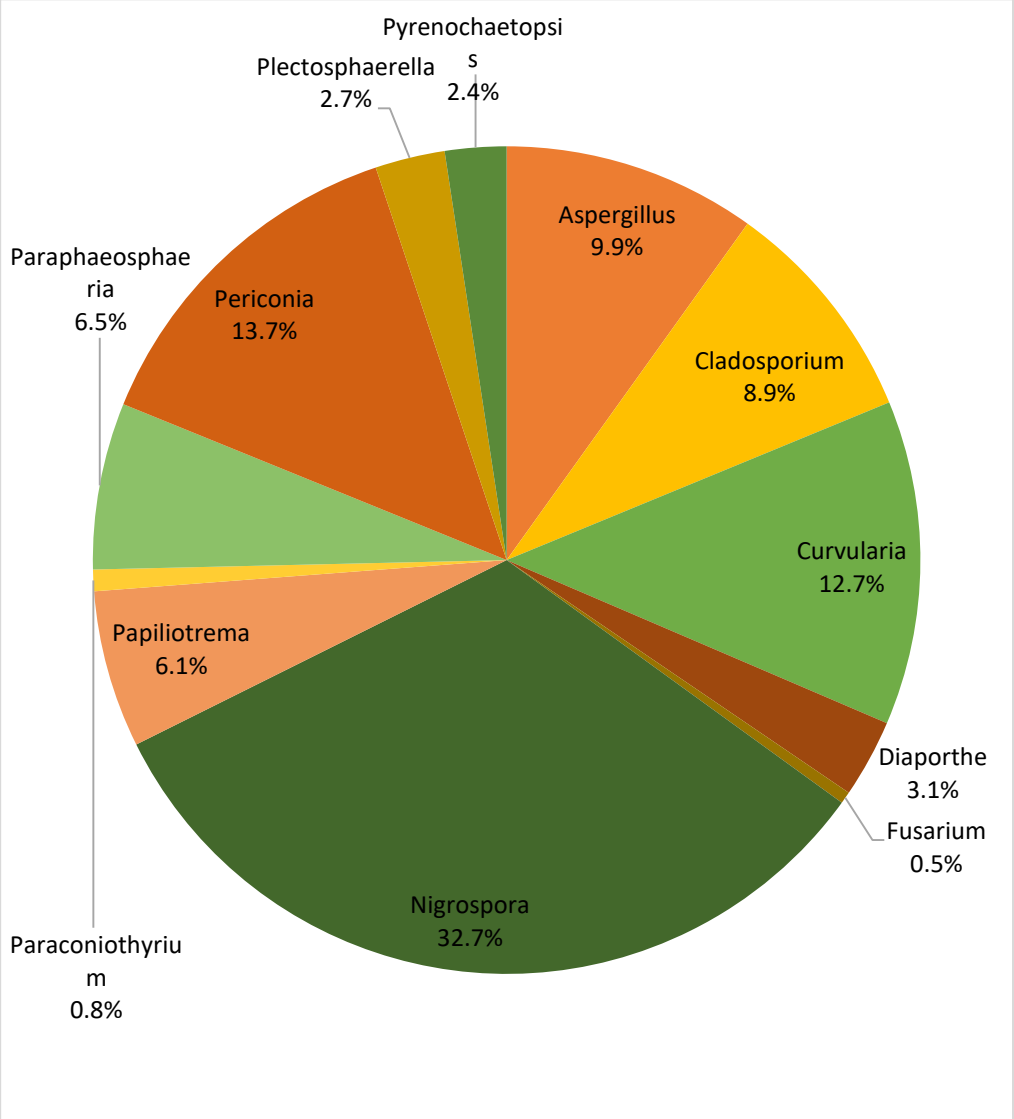

Fig. 4.
Distribution of the main fungal genera on the healthy and wounded wood samples from French Guiana.

The distribution of the main fungal genera obtained from the wood samples (healthy and wounded) in the four Guianan plots is shown, like (Fig. 4). It can be seen that the most frequent genera in all these samples were *Nigrospora* (32.7%), *Periconia* (13.7%), *Curvularia* (12.7%), *Aspergillus* spp. (9.9%), *Cladosporium* (8.9%), *Paraphaeosphaeria* (6.5%) and *Papiliotrema* (6.1%).

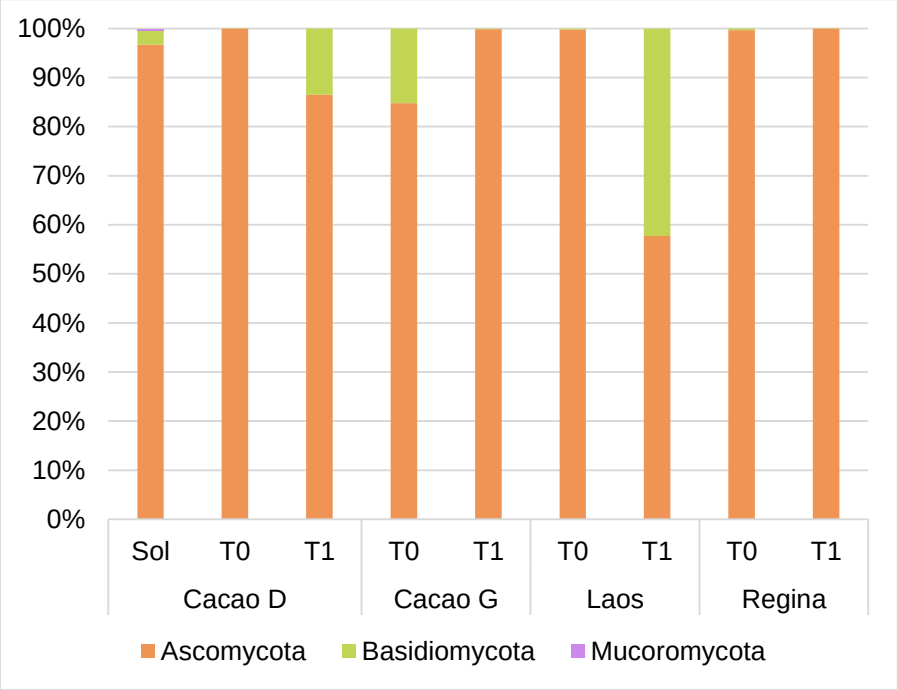

Fig. 5.
Distribution of the fungal phyla depending on the location and status of the sampled trees. The majority phylum is that of the Ascomycetes. That of the Basidiomycetes was particularly present in the agarwood from Laos.

When considering the distribution results for the samples, it can be seen that the main phylum was that of the Ascomycetes, like (Fig. 2, Fig. 5). Basidiomycetes were particularly found in the agarwood from Laos (Laos, T1) and, to a lesser degree, in the soil from Cacao plot D, in the samples of wounded wood from Cacao plot D and in the samples of healthy wood from Cacao plot G, like (Fig. 5).

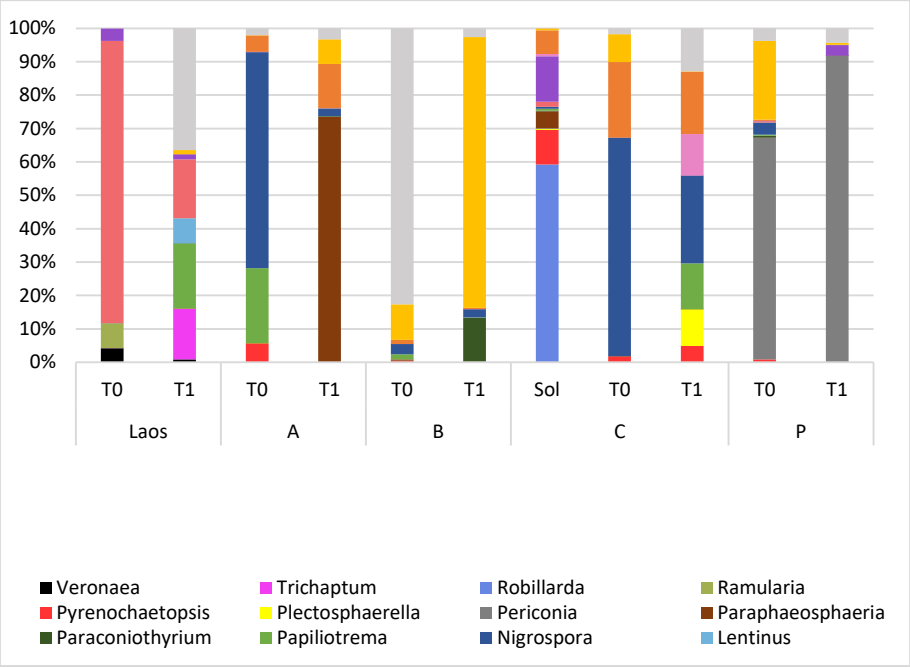

Fig. 6.
Distribution of the main fungal genera depending on the different types of samples and their status. Guianan wood from a healthy tree (T0), Guianan wood from a wounded tree (T1), soil from the four experimental plots in French Guiana (A, B, C, P), white wood from Laos (T0, Laos) and agarwood from Laos (T1, Laos).

The proportions of the main fungal general found in the different types of samples, namely wood taken from healthy or wounded trees in French Guiana, soil samples, samples of white wood and of agarwood from Laos, are shown, like Fig. 6. It can be seen that all the profiles diverged well, that the genus *Aspergillus* seemed to be found in most of the samples, except in the sample of white wood from Laos and in the soil sample. The profile for the white wood from Laos (Laos, T0) displayed a large proportion of the genus *Lasiodiplodia*, a genus only found in the samples from Laos in large proportions, and in very small proportions in the other samples. The fungal genus *Veronaea,* in smaller proportions, was also specific to the samples from Laos. In this modality of white wood from Laos, there was also another genus absent from the other modalities: *Ramularia*. The agarwood from Laos (Laos, T1) was characterized by the presence of the genus *Lentinus* and by several genera in quite similar proportions. The profile for wood from wounded trees in plot C (T1, C) was the same type of profile as Laos T1, which did not really reveal any majority genus. The profile for the samples of soil from plot C displayed one majority genus, *Robillarda*, which was not found for any other profile. It was also characterized by the presence of *Fusarium*, which was also found in the samples from Laos and in the samples of wood from wounded trees in plot P, along with *Pyrenochaetopsis*, which was also found in very small proportions in all the samples except the white wood from Laos. The soil sample also had in common with the other samples from plot C the presence of *Curvularia* in large proportions.

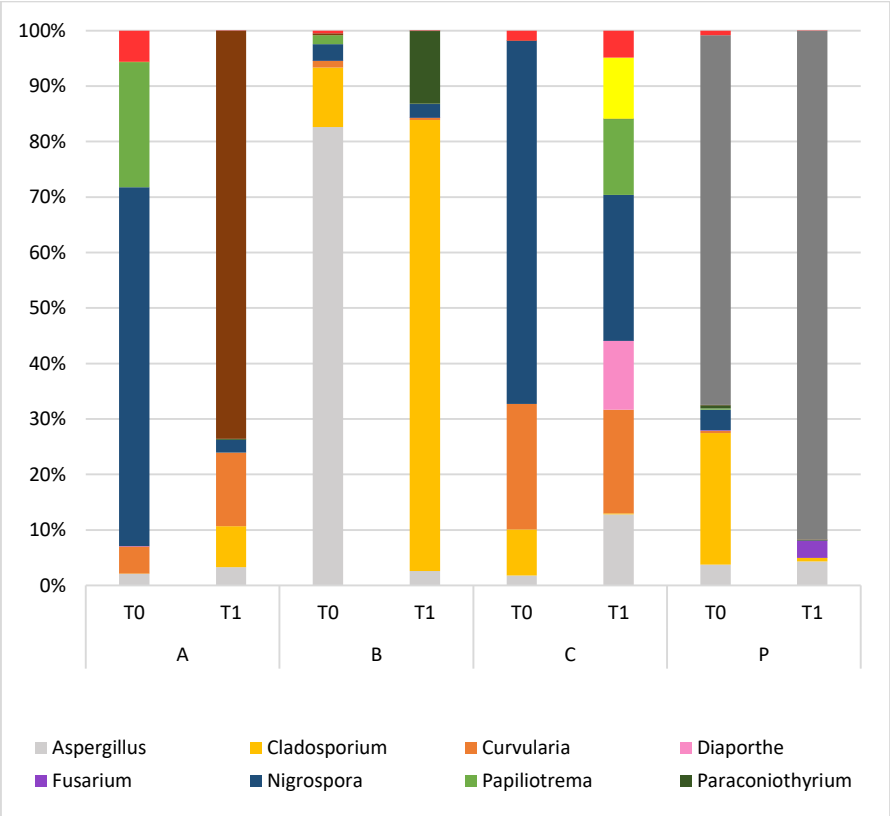

Fig. 7.
Distribution of the main fungal genera for the wood samples from trees in French Guiana depending on their status, healthy (T0) or wounded (T1), for the four experimental plots (A, B, C, P).

The proportions of the different fungal genera present in the wood samples from trees in French Guiana, both healthy (T0) and wounded (T1), for each plot in Cacao (A, B and C) and for the plot in Régina (P) are shown, like (Fig. 8). It can be seen that there was no standard profile in terms of genus, but that each of the modalities seemed to have a majority fungal genus, apart from the wood samples from the wounded trees of plot C, which, in addition, were characterized by several genera specific to them. Plot P displayed some characteristics that were specific to it, notably with the majority presence of the genus *Periconia*, which was only found in those samples and which was the majority genus for the samples from both healthy and wounded trees. On the other hand, in the other plots, the majority genera differed between the samples from the healthy trees and from the wounded trees. The genus *Nigrospora* was found in all the samples, and was the majority genus for the wood samples from healthy trees in plots A and C. The genus *Aspergillus* was found in all the samples, and was the majority genus in the wood samples from the healthy trees of plot B. The genus *Cladosporium* was also found in all the samples, albeit sometimes in very small proportions, but it

was the majority genus in the wood samples from the wounded trees in plot B. The genus *Curvullaria* was found in all the samples (wounded and healthy), but was not the majority genus in any of the samples. On the other hand, the genus *Curvullaria* was found in noteworthy proportions in the samples from plots A and C, whilst it was only in tiny proportions in the samples from plots B and P. In Fig. 8, the genera *Diaporthe* and *Plectosphaerella* were only found for the samples from wounded trees in plot C. Likewise, the genus *Fusarium* was only found for the samples from wounded trees in plot P, in small proportions. *Paraconiothyrium* was only noteworthy in the wood samples from wounded trees in plot B. *Paraphaeosphaeria* was only noteworthy in the samples from wounded trees in plot A. The genera *Papillotrema* and *Pyrenochaetopsis* were found in all the samples, usually in tiny proportions, except for the samples from healthy trees in plot A and the samples from wounded trees in plot C. It can thus be seen that the profiles for plots A and C had certain similarities, while the profiles for plot B were typical of that plot.

Some Chi2 independence tests were carried out on the “Genus & Plot” and “Genus & Status” (healthy or wounded) pairs of variables. The results led us to reject the hypothesis of independence for these two pairs, with a risk of 1%. This shows that some fungal genera were predominant in certain plots and certain plots were specific to certain fungal genera. The same applied for the status (healthy or wounded) of the trees. This confirmed the results presented above in reference to Fig. 7.

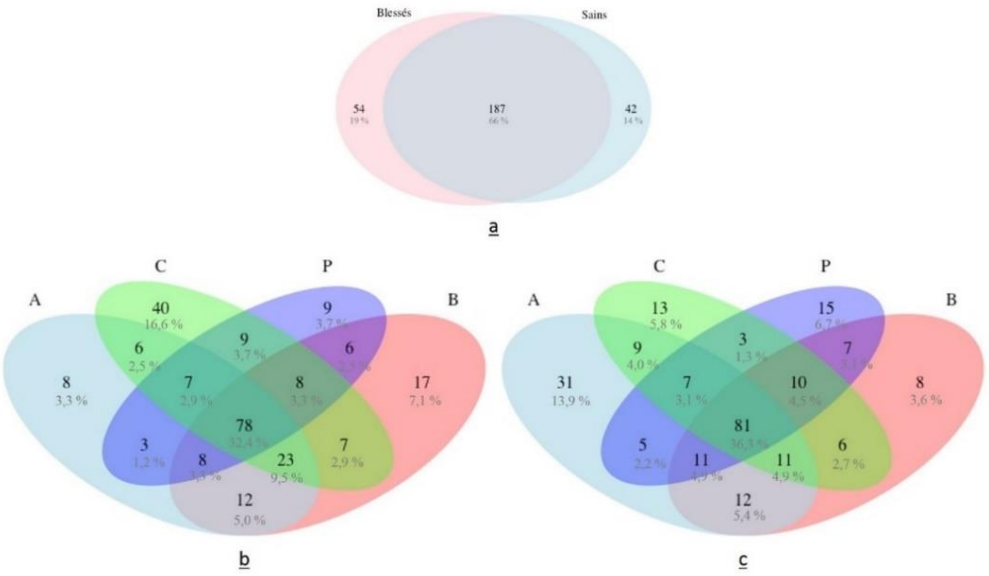

Fig. 8.
Venn diagrams of the total fungal OTU distributions present in the wood samples from French Guiana.
a, All the samples from healthy wood and those from wounded trees
b, The different plots for the wood samples from wounded trees
c, The different plots for the wood samples from healthy trees.

The Venn diagrams show 66% of OTUs common to the fungal communities of the wood samples from healthy trees in French Guiana and those from wounded trees in French Guiana, like (Fig. 8a).

For the wood samples from healthy trees depending on the plots, it can be seen that 36.4% of the OTUs were common to all the plots. It can also be seen that 13.9% of the OTUs were only found in the trees of plot A, as opposed to 6.7% for plot P, 5.8% for plot C and 3.6% for plot B. Thirty percent of the OTUs were therefore found in just one plot, like (Fig. 8c).

For the wood samples from wounded trees depending on the plots, a similar situation was found, with 32.4% of the OTUs common to the four plots, but also with 9.5% of the OTUs common to the three Cacao plots (as opposed to 4.9% for the wood samples from healthy trees). In all, 30.7% of the OTUs were therefore only found in samples from a single plot, of which 3.3% for plot A, 7.1% for plot B, 16.6% for plot C and 3.7% for plot P.

DISCUSSION

General sequencing showed that most of the phyla represented in the samples of this study were Ascomycetes, followed by the Basidiomycetes. Ascomycetes have already been reported in studies on the diversity of microorganisms existing in *Aquilaria* and involved in agarwood induction (Chhipa and Kaushik 2017; Premalatha and Kalra 2013). However, there are fewer studies reporting Basidiomycetes (Zaremski *et al.* 2018). These authors found differences in fungal communities between the aboveground and belowground organs of the tree and reported that, out of the sequences grouped in 535 OTUs, 87% were assigned to the Ascomycetes and 10.5% to the Basidiomycetes.

In our study, we found that Basidiomycetes were particularly found in the agarwood from Laos, and in smaller proportions in some samples from the wood of trees in French Guiana, as well as in the soil samples, like (Fig. 5).

An analysis of the OTUs revealed that the majority genera found in the samples as a whole were *Nigrospora*, *Aspergillus*, *Lasiodiplodia*, *Papiliotrema*, *Periconia* and *Curvularia.* The fungal genera *Cladosporium* and *Paraphaeosphaeria* were only found in wood samples from healthy or wounded trees in French Guiana. Although the genera *Nigrospora*, *Aspergillus*, *Lasiodiplodia, Cladosporium* and *Curvularia* appeared to be present in *Aquilaria* trees producing agarwood in India (Chhipa and Kaushik 2017), China (Gong et Guo 2008), Malaysia (Mohamed *et al.* 2014) and Indonesia (Turjaman *et al.* 2016), some other genera seemed to be specific to our study, including *Papiliotrema*, *Periconia* and *Paraphaeosphaeria.*

What is also noteworthy is that some of these genera are pathogens, such as *Lasiodiplodia,* or *Nigrospora* and others are endophytic, such as *Nigrospora* and *Papiliotrema.* Thus, some studies were carried out in Asia to inoculate *Aquilaria* trees with pathogenic fungi (Chen *et al.* 2017) and others with endophytic fungi (Monggoot *et al.* 2017, Turjaman *et al.* 2016). The genus *Lasiodiplodia,* which is a potential plant pathogen, is often used in a mixture with *Fusarium*, which is also a pathogen, to induce agarwood formation in *Aquilaria* wood (Chen *et al.* 2017).

In our study, the genus *Lasiodiplodia* was typical of the samples from Laos, in which the genus *Fusarium* was also found; this genus is also found in large proportions in soil (Leplat 2012).

Fig. 7 and the Venn diagrams brought out differences between plots and between healthy and wounded wood samples, like (Fig. 8). Indeed, although 66% of the OTUs were found to be common to the wood samples from healthy and wounded trees, and that 36.3% and 32.4% of the OTUs were common to healthy trees and wounded trees respectively, for all the plots, the proportions of certain fungal genera, depending on the modalities, displayed differences with each other.

Plot P, the only plot at Régina, was characterized by the presence of *Periconia*, a genus not mentioned in the literature, and especially present in wounded trees associated with *Fusarium*, whereas in healthy trees *Periconia* was also the majority genus, but was associated with *Cladosporium* and *Nigrospora*.

There were also differences between the Cacao plots, especially plot B, for which the wood samples from healthy trees mostly revealed the genus *Aspergillus* and, to a lesser degree, *Cladosporium*, unlike the wood samples from wounded trees, which mostly revealed *Cladosporium* and, to a lesser degree, *Paraconiothyrium*, a genus specific to this modality and which was not found in the soil, or in the samples from Laos, either.

Cacao plots A and C displayed closer profiles, notably with the presence of *Nigospora* as a majority genus for the wood samples from healthy trees, Curvularia, *Pyrenochaetopsis* and *Aspergillus*. Nevertheless, these samples differed through a large proportion of *Papillotrema* for plot A and a large proportion of *Cladosporium* for plot C. In addition, the Venn diagrams, like (Fig. 8c) indicated that these plots (A and C) only had 4% of common OTUs for the wood samples from healthy trees.

In the same plots, Cacao A and C, the genus abundance profiles were different for each of them. Indeed, for plot A, the majority genus was *Paraphaeosphaeria*, which was only found in very small proportions in the other wood samples, but which was also found in the soil.

On the other hand, the wood samples from wounded trees in plot C did not display a profile indicating a majority genus, but indicated several genera present in noteworthy proportions: - *Apergillus* and *Nigrospora*, present in all the wood samples; *Pyrenochaetopsis,* present in the healthy wood samples from the different plots and in the soil; - *Curvularia*, a genus common to the healthy and wounded wood samples from plots A and C and present in the soil; - *Papillotrema*, also found in samples from the healthy trees of plots A and B; - *Diaporthe* and *Plectosphaerella*, typical of the wounded wood modality of plot C.

Thus, the wood sample analysis revealed large differences between Régina and Cacao, with the genus *Periconia* dominating in Régina. It should be remembered that *Aquilaria* tree cropping practices are not the same in the two sectors: difference in know-how between farmers, plantation intercropped with *Citrus* trees in Cacao, existence of an *Arachis pintoi* cover crop in Cacao. It should be noted that this genus is not reported in the literature either.

The soils in Régina are sandy and fluviomarine (Boye *et al.* 1979) and Régina is particularly susceptible to rainfall (Météo-France 2016a, b).

On the other hand, Cacao is on a granitic and alluvial soil (Boye *et al.* 1979).

Plots A and B were on a plateau, while plot C was on a slope. In addition, the trees in plot A came from a mixture of seeds of various provenances in Southeast Asia, while those of plots B and C were grown from seeds from Laos. Consequently, it was difficult in Cacao to reveal a majority fungal strain; such fungi are mostly found in studies on *Aquilaria* trees in Southeast Asia (Chhipa and Kaushik 2017; Gong and Guo 2008; Mohamed *et al.* 2014; Turjaman *et al.* 2016).

Our study, which completed the earlier study by Zaremski *et al.* (2018), showed that many of the strains we found in the samples from French Guiana were also strains found in the samples from Laos, or in the literature. However, some of the strains were an exception, such as *Periconia,* typical of Régina*,* and *Paraphaeosphaeria*, somewhat typical of Cacao. This could be defined as a factor of differentiation between essential oils extracted from trees in French Guiana and those extracted from trees in other countries, even creating a difference between the villages of Cacao and Régina. Because, although wounding lies behind the formation of agarwood, it is the associated microorganisms that prolong its formation and characterize it (Mohamed *et al.* 2014).

CONCLUSION

Several studies have been undertaken in Asia to characterize microorganisms, notably fungi, associated with the genus *Aquilaria*, for agarwood production. Some genera are particularly reported, such as *Lasiodiplodia* and *Fusarium.* As part of the Aquil@Guyane project with its experimental *Aquilaria* tree plantations in French Guiana, and with a view to producing agarwood of controlled quality, we determined the majority fungal genera present in the wood of trees before and after wounding, compared to the literature, to wood samples from Laos and to soil samples. The results indicated that certain genera are common to each other and to the literature, but that others are fairly typical of a geographical zone, such as *Periconia* for Régina and *Paraphaeosphaeria* for Cacao. Moreover, in Cacao, the wounded trees in plots A and B interacted with two main strains, namely *Paraphaeosphaeria* and *Cladosporium* respectively, while the trees in plot C interacted with several genera, none of which was a majority genus. In Régina, Periconia was the majority genus in the wood samples from both healthy trees and wounded trees.

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REFERENCES

©Agilent Technologies, Inc. (2016). Agilent DNA 7500 and DNA 12000. Kit Guide. Agilent Technologies, pp. 24.

Barden A, Anak NA, Mulliken T, Song M (2000) Heart of the matter: agarwood uses and trade and CITES implementation for *Aquilaria malaccensis*. Traffic network report*,* TRAFFIC International, Cambridge, 60 p. Url: http://www.iucn.org/dbtw-wpd/edocs/Traf-072.pdf

Blanchette RA, Jurgens JA, Heuveling van Beek H (2015) Growing Aquilaria and production of agarwood in hill agro-ecosystems. In: Integrated Land Use Management in the Eastern Himalayas. Eds K. Eckman and L. Ralte. Delhi, India, pp. 66-82.

Callahan BJ, Sankaran K, Fukuyama JA, McMurdie PJ, Holmes SP (2016) Bioconductor Workflow for Microbiome Data Analysis: from raw reads to community analyses. F1000Res 5, pp. 1492.

Chen X, Sui C, Liu Y, Yang Y, Liu P, Zhang Z, Wei J (2017) Agarwood Formation Induced by Fermentation Liquid of *Lasiodiplodia theobromae*, the Dominating Fungus in Wounded Wood of *Aquilaria sinensis*. Current Microbiology 74(4):460-468.

Chhipa H, Kaushik N (2017) Fungal and Bacterial Diversity Isolated from *Aquilaria malaccensis* Tree and Soil, Induces Agarospirol Formation within 3 Months after Artificial Infection. Frontiers in Microbiology, 8.

Convention on International Trade in Endangered Species (CITES) (2004) “Convention on international trade in endangered species of wild fauna and flora. Consideration of proposals for amendment of appendices-I and - II *Aquilaria* spp. and *Gyrinops* spp.”. *In*: Proceedings of the Thirteen Meeting of the Conference of the Parties, Bangkok, Thailand, October 2 to 14, 2004.

Gong LJ, Guo SX (2009) Endophytic fungi from *Dracaena cambodiana* and *Aquilaria sinensis* and their antimicrobial activity. Afr J Biotechnol 8:731-736.

Kalita J, Bhattacharyya PR, Boruah HP, Unni BG, Lekhak H, Nath SC (2015) Association of *Zeuzera conferta* Walker on agarwood formation in *Aquilaria malaccensis* Lamk. Asian J. Plant Sci. Res. 5:4-9.

Leplat J (2012) Développement saprotrophe de *Fusarium graminearum* : rôle respectif de différents habitats naturels du champignon dans le processus d'infection du blé en Bourgogne ; recherche d'indicateurs prédictifs du risque de fusariose. Thèse Université de Bourgogne, Unité Mixte de Recherche 1347 Agroécologie, pp. 306.

McMurdie PJ, Holmes S (2013) phyloseq: An R Package for Reproducible Interactive Analysis and Graphics of Microbiome Census Data. PLoS ONE,8: e61217

Mohamed R, Jong PL, Zali MS (2010) Fungal diversity in wounded stems of *Aquilaria malaccensis*. Fungal Diversity 43:67-74.

Mohamed R, Jong PL, Nuru Irdayu I (2014) Succession patterns of fungi associated to wound-induced agarwood in wild *Aquilaria malaccensis* revealed from quantitative PCR assay. World Journal of Microbiology and Biotechnology 30(9):2427-2436.

Monggoot S, Popluechai S, Gentekaki E, Pripdeevech P (2017) Fungal endophytes: an alternative source for production of volatile compounds from agarwood oil of *Aquilaria subintegra.* Microbial Ecolog 74(1):54-61.

Naef R (2011) The volatile and semi-volatile constituents of agarwood, the infected heartwood of *Aquilaria* species: a review. Flavour and Fragrance Journal 26(2):73-87.

Nilsson RH, Larsson KH, Taylor AFS, Bengtsson-Palme J, Jeppesen TS, Schigel D, Kennedy P, Picard K, Glöckner FO, Tedersoo L, Saar I, Kõljalg U, Abarenkov K (2019) The UNITE database for molecular identification of fungi: handling dark taxa and parallel taxonomic classifications. Nucleic Acids Res. 47: D259–D264

Op De Beeck M, Lievens B, Busschaert P, Declerck S, Vangronsveld J, Colpaert JV (2014) Comparison and validation of some ITS primer pairs useful for fungal metabarcoding studies. PLoS ONE 9: e97629

Qiagen (2018) DNeasy® PowerSoil® Pro Kit Handbook. Qiagen, pp. 24.

Premalatha K, Kalra A (2013) Molecular phylogenetic identification of endophytic fungi isolated from resinous and healthy wood of *Aquilaria malaccensis*, a red listed and highly exploited medicinal tree. Fungal Ecology 6(3):205-211.

Soehartono T, Mardiastuti A (1997) The current trade in Gaharu in West Kalimantan. Biodiversitas Indonesia 1: 1-10

Turjaman M, Hidayat A, Santoso E (2016) Development of agarwood induction technology using endophytic fungi. *In*: Mohamed R (ed), Agarwood: Science Behind the Fragrance Tropical Forestry, Springer, Singapore, pp. 57–71.

Wang Q, Garrity GM, Tiedje JM, Cole JR (2007) Naïve Bayesian Classifier for Rapid Assignment of rRNA Sequences into the New Bacterial Taxonomy. Appl Environ Microbiol. 73:5261-5267.

Zaremski A (2018) Rapport d'exécution Intermédiaire du 1er janvier 2017 au 31 décembre 2018. Le projet Aquil@Guyane: "Bases scientifiques et techniques pour la création d'une filière d'huiles essentielles d'*Aquilaria* (Agarwood) " haut de gamme et de produits dérivés, en Guyane. Agritrop CIRAD, Kourou, pp. 24.

Zaremski C, Malandain C, Sibourg O, Andary C, Michaloud G, Zaremski A (2018) NGS identification of fungi potentially implicated in the production of agarwood from *Aquilaria* spp. Trees. PRO LIGNO 14(3):9-18.

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