ONLINE ISSN 2069-7430
ISSN-L 1841-4737
PRO LIGNO
www.proligno.ro
Vol. 19 N° 1 2023
pp. 3-23

CHEMICAL COMPOSITION OF AGARWOOD FROM *AQUILARIA CRASSNA* PIERRE EX. LECOMTE PLANTED IN FRENCH GUIANA, DEPENDING ON THE INDUCTION METHOD

Clara ZAREMSKI
Office Français de la Biodiversité
DEAAF - Parc Rebard, Cayenne, French Guiana
Tel: (+594) 06 94 23 60 92
E-mail: clara.zaremski@ofb.gouv.fr

Nadine AMUSANT
CIRAD - UMR ECOFOG AgroParisTech, CNRS, INRA, Université des Antilles, Université de Guyane
Kourou; BP 701, 97387 Kourou Cedex, French Guiana
E-mail: nadine.amusant@cirad.fr

Marc DUCOUSSO
CIRAD - UMR 113, Laboratoire des Symbioses Tropicales et Méditerranéennes, Cirad/IRD/Inra/Agro-M/UM2
Campus international de Baillarguet - TA A - 82 / J, 34398 Montpellier Cedex 5, France
E-mail: marc.ducousso@cirad.fr

Georges MICHALOUD
Retired from CNRS, UMR 5120 AMAP - CIRAD, Montpellier, France/599 rue de Fontcouverte, 34070 Montpellier, France

Benjamin HEUCLIN
CIRAD, UMR AGAP, F-34398 Montpellier, France. AGAP, Univ Montpellier, CIRAD, INRAE, Montpellier SupAgro, Montpellier, France. TA A-108/1 Avenue Agro- polis, 34398 Montpellier Cedex 5, France
E-mail: benjamin.heuclin@cirad.fr

Claude ANDARY
Retired from Faculty of Pharmacy, University of Montpellier and from CEFE, UMR 5175, CNRS, Montpellier, France/17 rue des Millepertuis, 34830 Clapiers, France
E-mail: claude.andary@gmail.com

Chantal MENUT
IBMM - UMR 5247, Université de Montpellier, CNRS, Bâtiment E, Faculté de Pharmacie, 15 Avenue Charles Flahault, BP 14491, 34093 Montpellier, France
E-mail: chantal.menut@univ-montp2.fr

Alba ZAREMSKI*
CIRAD, UMR AGAP Institut, F-34398 Montpellier, France. AGAP Institut, Univ Montpellier, CIRAD, INRAE, Montpellier SupAgro, Montpellier, France. TA A-108/1 Avenue Agropolis, 34398 Montpellier Cedex 5, France
E-mail: alba.zaremski@cirad.fr

Abstract:

Agarwood, also known as Oud in perfumery, is a wood modified through contact with microorganisms. It emits a sweet and heady fragrance that is rare and precious. It derives its rarity from its very existence, from the trees of the genus Aquilaria. Indeed, agarwood arises from an interaction between the wood of those trees and associated microorganisms, which, after the trunk has been wounded, induce a reaction leading to the production of secondary compounds that give the wood its typical black coloration after oxidation. The compounds involved are mainly chromones and oxygenated sesquiterpenes, such as eudesmol, agarospirol, jinkoh-eremol and valerianol.

Demand for agarwood on the international market has increased considerably over the last ten years or so. Its derivatives, including the essential oil extracted from it, are therefore expensive. Agarwood essential oil fetches US$ 5,000 to US$ 10,000 per kg, and is the most expensive oil on the market. That explains why this product is coveted by the owners of the trees, who attempt to compensate for its rarity by practising various agarwood induction methods.

The methods, such as making holes or hammering nails into the trunks of the trees, or inoculating mixtures of biochemical products, are often harmful to the trees and detrimental to the composition of the

*Corresponding author

essential oil extracted from them. Consequently, a consortium of farmers in French Guiana and scientists from CIRAD (French Agricultural Research Centre for International Development) is implementing the Aquil@Guyane project, designed to grow Aquilaria trees originating from Southeast Asia in French Guiana, with a view to producing agarwood with a controlled chemical composition that respects the biology and the ecology of the trees, while helping to conserve the genus Aquilaria, which is classed as being under threat of extinction (in CITES annex II) in its natural range.

To that end, a biological induction trial was launched. Two induction methods were tested: a way of inducing black wood by environmental microorganisms, achieved by making wounds in the trunk, contaminated or not with soil from the plantation, and a second way of inducing black wood by fungal strains grown in the form of pure strains, selected for their wood-decay trait and because they are common fungi in French Guiana. For the second method, experiments were carried out specifically with brown-rot and white-rot fungi. In order to compare the two methods, we studied the area of inoculation propagation, the volatile compounds making up the essential oils extracted from the blackened wood, and essential oil yields. We were thus able to show that the second black wood induction method was effective in producing agarwood with a controlled chemical composition and a yield that seemed to be better that the usual commercial yield.

It should be noted that white-rot fungi, especially Ganoderma resinaceum, Gloeophyllum trabeum and Pycnoporus sanguineus, proved to be the most effective for induction, for the quality of the chemical composition, and for essential oil yields.

Key words: *Agarwood, Aquilaria, white wood, black wood, wood-decay fungi, volatile compounds, French Guiana, steam distillation, white rot, brown rot, sesquiterpenes.*

INTRODUCTION

Agarwood, also known as Oud, is used in perfumery for the sweet, intense fragrance of the oleoresin extracted from it. For instance, the oleoresin obtained from the wood is used in perfumes with heady oriental notes and is the epitome of luxury in fragrance terms. That luxury comes from the rarity of the product, particularly as it has to be of high quality. In fact, agarwood is a wood transformed by a complex interaction between the genera of trees in the Thymelaeaceae family and associated microorganisms, especially fungi, and more particularly the Ascomycetes. *Aquilaria* is the tree genus most used to obtain agarwood. The genus currently comprises 21 species (Lee *et al.* 2017), but agarwood can sometimes occur in certain species belonging to the genera *Gyrinops* and *Gonystylus* (Gratzfeld and Tan 2008).

Healthy *Aquilaria* wood is white. In complex interactions with microorganisms, particularly fungi, the tree produces a wood that is transformed in terms of its high secondary metabolite contents, produced in reaction to biotic stress (Naziz *et al.* 2019). The transformed wood, or agarwood, is rich in oxidable aromatic molecules that cause a black coloration, and has a high oleoresin and oil content: around 150 molecules (Naef 2011), including steam-distillable volatile molecules and molecules that are not distillable, but are extracted by polar solvents (acetone, methanol, water etc.). These extracts and the steam-distillable oil obtained from agarwood form a complex mixture of different groups of chemical structures, primarily sesquiterpenes, such as agarospirol, β-agarofuran, 10-epi-γ-eudesmol, guaiol, jinkoh-eremol (Deep and Tajuddin 2019; Tan *et al.* 2019), along with chromones such as 2-(2-phenylethyl)-chromones, 5,6,7,8-tetrahydro-2-(2-phenylethyl)-chromones and diepoxy-tetrahydro-2-(2-phenylethyl)-chromones. The last two molecules are good indicators for identifying agarwood (Naef 2011). Under the effect of oxidation, agarwood displays a higher density (the density of healthy wood is around 0.4; the density of the blackened wood can reach1.028 for agarwood belonging to 75 to 80-year-old trees (Sadgopal 1960) and becomes increasingly fragrant over time.

When the tree is wounded, it becomes exposed to environmental microorganisms. According to several authors (Soehartono and Mardiastuti 1997; Mohamed *et al.* 2010; Zaremski *et al.* 2018), the genera of fungi that colonize *Aquilaria* are *Aspergillus, Arthrinium, Botryodyplodia, Diplodia, Dokmaia, Fusarium, Penicillium* and *Trichoderma*. According to Mohamed *et al.* (2014), fungal communities appear to prefer trees that grow in a darker and more humid environment, which suits *Aquilaria,* an undergrowth species.

Around 10% of trees produce agarwood in the natural environment (Barden *et al.* 2000) and that figure does not rise much in plantation trees, for which production is equally low. For example, in certain Indian plantations, it is under 33% (Kalita *et al.* 2015). The fact that the agarwood trade is lucrative, with essential oil varying in price from US$ 5,000 to US$ 10,000 (Barden *et al.* 2000; Blanchette *et al.* 2015), explains why the resource has gradually been exhausted in its natural environment. Consequently, most species of the genus *Aquilaria* are on the CITES red list, classed CITES II (CITES 2004). In addition, certain species of *Aquilaria,* primarily *A. malaccensis* Benth., *A. crassna* Pierre ex. Lecomte and *A. filaria* (Oken) Merr. (Naziz *et al.* 2019) are cultivated, notably in China, Thailand, India, Malaysia and other Southeast Asian countries. However, in order to obtain agarwood, growers are often very aggressive in their inoculation of microorganisms into the trees and, eventually, this approach can adversely affect the quality of the essential

oils extracted from the trees, and in extreme cases leads to premature death of the trees. The aggressive methods most often reported (Blanchette *et al.* 2015; Naziz *et al.* 2019; Tan *et al.* 2019) are wounds caused by cuts, or by holes made by hammering nails into the trunks, the holes being used, or not, to inject mixtures of microorganisms and/or chemical additives to increase the stress of the tree. The resulting agarwood therefore no longer meets the requirements of the market.

Thus, to overcome these problems, we tested two new ways of inducing black wood in *A. crassna* growing in a plantation in French Guiana. Our aim was to develop some natural techniques that would be easily adoptable by farmers, while remaining environment-friendly and adapted to the surroundings.

Two novel techniques were tested, namely inoculation of fungi from French Guiana and/or inoculation of pure strains on a wooden test-piece. The first technique involved inducing contamination by fungi from the environment after making a large cut to expose the wood. Contamination by environmental fungi was achieved with or without applying soil from the plantation. The second way of inducing contamination involved introducing selected fungi, grown in pure strains, via wooden test-pieces. We were thus able to compare the presence of secondary compounds typical of the blackened woods obtained by these different induction methods, and we compared them to some agarwood control samples from Southeast Asia, with a view to analysing their secondary compound composition. We also determined the oleoresin yields from blackened woods obtained by these different induction methods.

MATERIAL AND METHODS

Study site

This work was undertaken in French Guiana, more specifically in the village of Cacao, in the municipality of Roura (Fig. 1). The inhabitants of Cacao are mainly of Lao origin and are widely involved in farming activities. The village is located on the Comté River, in northeastern French Guiana. Its coordinates are 4° 34’ 17’’ North and 52° 28’ 11’’ West. Cacao lies around 70km southwest of Cayenne, the principal town of French Guiana. French Guiana is in South America, near the equator, at 4° latitude North (53° longitude West), on the Guyanas plateau. French Guiana is relatively flat, with the entire region lying between 100 and 200 metres above sea level. The climate is humid equatorial with very low variations in temperature. However, there is a distinction between dry seasons from August to November and from February to March, and wet seasons from April to August and November to February. The biome is humid equatorial.

Fig. 1.

Localisation of the village of Cacao, in French Guiana (4°34’17’’) (***www.cartes-2-france.com).***

The trees of the genus *Aquilaria* chosen for this study came from an experimental plantation managed in partnership by CIRAD and a group of farmers. The trees were planted in January 2014. We divided the field into three plots (Fig. 2). Plot A and plot B were on the same side of the field on a plateau. Plot C was planted on a sloping part of the field. The trees in plot A came from a mixture of seeds of various provenances. The trees in plots B and C were grown from seeds originating from Laos. For this study, we selected trees located only in plots B and C. The *Aquilaria* trees were intercropped with *Citrus* trees, mainly mandarin. The trees were planted at a spacing of 4 x 4 metres. The soil was covered with a nitrogen-fixing Fabaceae cover crop: *Arachis pintoi* Krapov & W.C. Greg.

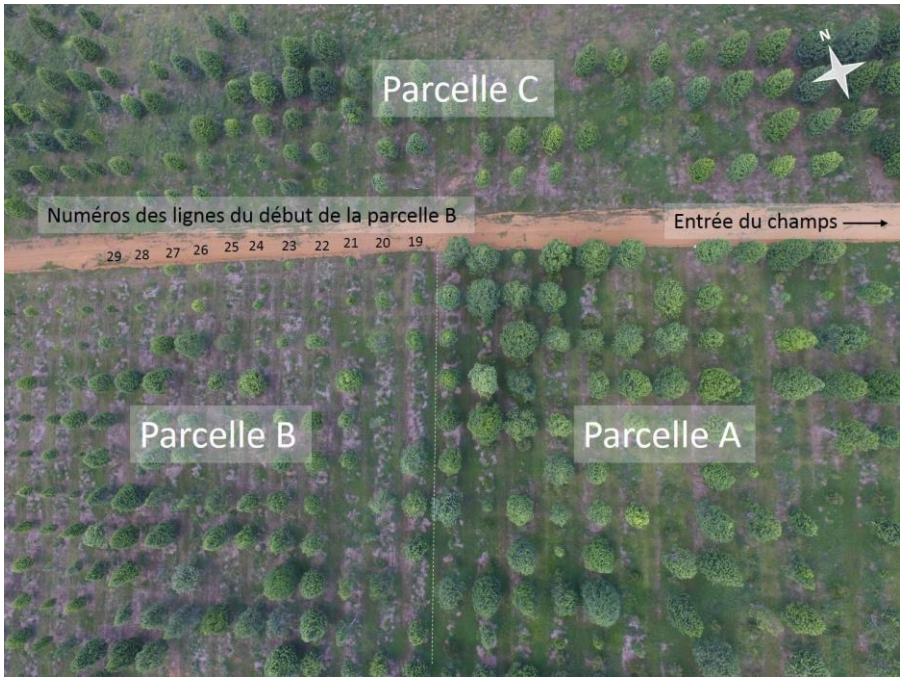

Fig. 2.
Division of experimental plantation field.

Biological material: selection of the fungi used

The novel methodological objective was to choose local species adapted to the Guianan biomes and use no other elements but the biological material, i.e. the fungi and their support medium, sapwood. In addition, the experiments were carried out mindful of repeatability and of skill transferral between the different stakeholders, with a view to cash crop production.

It took three stages to prepare the inoculum for introduction into the trees, in order to test the fungal species' ability to stimulate agarwood production in *A. crassna* plantations trees growing in French Guiana. The first stage was to identify and select fungi of interest for the study from fungi harvested in zones near the study sites. The second stage was to produce pure mycelium from cultures of Badiomycete fungi on culture medium. The third stage was to grow the fungi on wooden test-pieces placed in Petri dishes, in order to inoculate the trees. The test-pieces colonized by the fungus were used to inoculate the trees for the study.

Stage 1: Identifying and choosing Guianan fungi for the study
White-rot and brown-rot fungi, according to Fougerousse (1979).

Among the fungi, those that attack wood do so by breaking down either lignin, cellulose, or both at the same time, and are called wood-decay fungi (destroying wood) or xylophilous fungi (living in wood). These fungi may be specialized by being dependent upon softwood species, hardwood species, or both at the same time. They belong to two main groups, depending on their specificity in decaying wood: a group that causes brown rot, and another group causing white rot. The agents of brown rot destroy cellulose, but cause little damage to lignin, leading to a darker colouring of the wood. The wood-decay process involving white rot is more complex, as all the constituents of the woody membranes can be destroyed. This decay process often begins with lignin decay and continues with the decaying of all the constituents of the wall, in varying order depending on the fungus.

We carried out a taxonomic study based on sequences of the ribosomal (r) DNA small subunit (SSU) using the two primer pairs SR6/SR10R and SR7/SR1R, on 39 fruiting bodies of fungi from forest sites near Cacao and Régina, two villages in eastern French Guiana close to the experimental sites (Zaremski *et al.* 2019). The fungi were collected fresh, in good condition, on dead wood lying on the ground (25 fungi) and on dead standing trees (14 fungi) (Fig. 3). Of the 39 samples, BlastN analyses revealed taxonomic proximity to the genera *Antrodia, Coriolopsis, Fomitopsis, Ganoderma, Poria, Lentinus, Pycnoporus, Auricularia, Gloeophyllum, Trametes, Fomitopsis* and *Rigidoporus*.

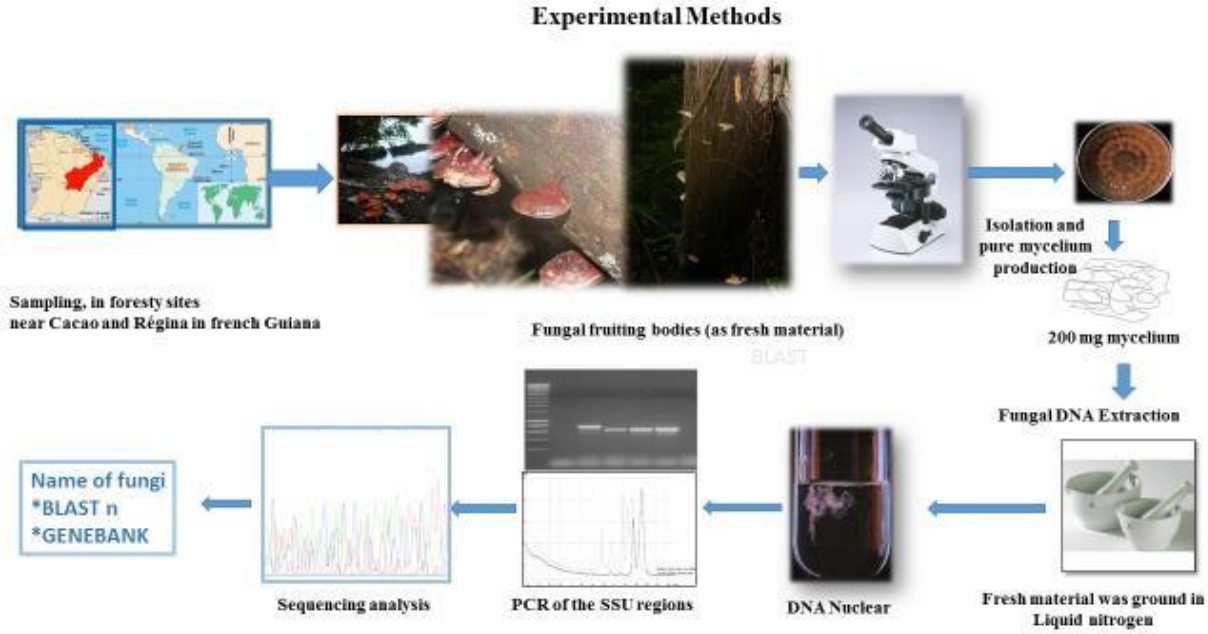

Fig. 3.
Identifying and choosing Guianan fungi for the study.

Mycelium was produced in pure cultures from the fruiting bodies identified in this study and was used to produce the inoculum, which we tested for its ability to stimulate oleoresin production on *Aquilaria crassna* plantation trees in French Guiana. Of the fungi grown in pure strains, we chose five white-rot (WR) fungi and two brown-rot (BR) fungi. These fungi were chosen for their high frequency in the natural environment, making them easily available, and for their good ability to produce mycelium under laboratory conditions. All the strains were Guianan strains. The fungi chosen for this study are indicated below:

- For the white-rot fungi: *Coriolopsis polyzona* (Pers.) Ryvarden (CP), *Ganoderma resinaceum* Boud. (GR), *Pycnoporus sanguineus* (L.) Murrill (PS), *Rigidoporus vinctus* (Berk.) Ryvarden (RV), *Gloeophyllum trabeum* (Pers.) Murrill (GT). GT can act as both a brown-rot fungus and a white-rot fungus (Zaremski 2005). While it acts more as a brown-rot fungus in a continental biotope, notably by attacking gymnosperms, it acts as a white-rot fungus in a humid tropical biotope.
- For the brown-rot fungi: *Poria placenta* (Fr.) Cooke (PP), *Antrodia vaillantii* (DC.) Ryvarden (AV).

Stage 2: Producing pure mycelium of the fungi selected for our trials

The growing conditions for the fungi chosen for inoculation were as follows (Fig. 4):

- Dimensions of the sterile plastic Petri dishes: 12.5x12.5x1.5cm;
- Dimensions of the wooden test-pieces: 7x0.7x0.7cm (beech sapwood for white rot or Scots pine sapwood for brown rot. This choice was made depending on the preference of the fungus types for softwood or hardwood trees);
- Number of test-pieces per Petri dish, previously sterilized in an autoclave at 121°C for 20 minutes: 5 test-pieces;
- Spacing of wooden test-pieces in the Petri dishes: from 1cm to 1.5cm;
- Culture medium, for 500ml of distilled water: 20g of Malt + 10g of Agar-agar (Malt 4% & Agar 2%); sterilization by autoclave, 121°C for 20 minutes; after sterilization culture medium left to cool, then poured into the Petri dishes (around 40ml of culture medium per dish);
- Climate chamber: dark, temperature regulated to 22 ± 2°C and a relative humidity (RH) of 70 ± 5%;
- Main items of equipment used: autoclaves, balances, horizontal laminar flow hoods; deionized water.

Fig. 4.
Producing pure mycelium of the fungi selected for our trials.

We cultured pure strains on malt agar in around 12 Petri dishes per fungus, which were the used for our experiment, but also to keep each of these strains in the Laboratory's fungus culture collection.
Culturing was carried out aseptically under the laminar flow hood. The cultures were kept in the dark in a climate chamber for around 20 days. Over those 20 days, we checked that the culture was not contaminated and we monitored its development in the Petri dish. Petri dishes displaying contamination or poor development were discarded.

Stage 3: Fungus development on the wooden-test pieces in the Petri dishes

For fungus growth in the wood, once the culture zone was completely covered by the mycelium of each of the strains, sterile wooden test-pieces (beech sapwood for white rot, or Scots pine sapwood for brown rot) were placed aseptically in the Petri dishes under the laminar flow hood, then kept in the dark in a climate chamber regulated to 22±2°C and a RH of 70±5% for two months. Over those two months, we checked that the culture was not contaminated and we monitored the fungus development in the wood. Petri dishes displaying contamination or poor development were discarded. After two months' incubation, we were able to inoculate the trees in the field.

Plant material: selecting trees for the study

In this study, we selected trees to test seven fungus strains. In addition, we selected some trees taken as negative controls, as they had been inoculated with non-contaminated Scots pine and beech test-pieces. The test was carried out to ascertain the degree of inoculation by defined fungal strains, to obtain an agarwood of controlled chemical composition. Lastly, we also contaminated some trees with a test-piece infested by the fungus *R. vinctus,* the latter being used as a positive control. In fact, Chen *et al.* (2018) showed that it is possible to obtain agarwood with that species of fungus, which they isolated from branches of *A. crassna* producing agarwood. Three trees were used for each of the treatments tested (trial fungi, positive and negative controls), giving a total of 27 (3 x 9) trees inoculated during the experiment. The trees all came from plot B of the experimental field in Cacao and had the same provenance and the same age (5 years old).

Targeted inoculation of fungal strains in trees in the field

The trees were inoculated with test-pieces that were contaminated, or not, on 11 September 2018 by inserting 10 test-pieces per tree. An initial set of trees – one tree per treatment – were felled on 10 June 2019 to analyse the reaction of the trees 9 months after inoculation. Table 1 indicates the trees involved for each set of experimental conditions. In fact, some earlier studies had shown that the longer the time was between wounding and collecting samples, the more there was a chance of finding agarwood formation, with 9 months seeming to be the minimum experimental duration (Rasool and Mohamed 2016).

Table 1

Identification of trees according to the different treatments

Fungus	Trees
Gloeophyllum trabeum	ACG 19.7
Pycnoporus sanguineus	ACG 20.14
Poria placenta	ACG 21.6
Coriolopsis polyzona	ACG 22.13
Antrodia vaillantii	ACG 23.3
Ganoderma resinaceum	ACG 24.8
Rigidoporus vinctus	ACG 34.11
"Pine" negative control	ACG 27.1
"Beech" negative control	ACG 29.8
Inoculation after bark removal and application of plantation soil	ACD 5.3 ACD 6.3 ACG 24.5

Inoculation was carried out as follows:

- After disinfection with alcohol and flaming, removal of a 4 x 4cm square of bark using a chisel, which was also disinfected. (Fig. 5a, Fig. 5b).
- After disinfecting the drill bit with alcohol and flaming, drilling into the middle of the square without bark to a sufficient depth (8cm min), without drilling all the way through the tree. To that end, we chose trees with a diameter over 40cm. (Fig. 5c).
- Insertion of the test-piece (contaminated or not), using a pair of previously disinfected tongs, into the newly made hole, inserting it fully. (Fig. 5d, Fig. 5e).
- Sealing of the hole by applying a few ml of pine-tar resin to the inner side of the square of bark removed earlier. Note: once dry, the bark will no longer remain in place (Fig. 5f).
- This operation was repeated 10 times on the same tree, moving upwards but also radially "in a spiral" (Fig. 5g). The experiment was carried out on three trees per "fungus", along with three trees for the control test-pieces.

Fig. 5.
Inoculation by inserting wooden test-pieces.

Inoculating environmental fungi, with or without soil application to a superficial wound on trees in the field

The aim of this methodological approach was to compare two types of superficial wounds enabling infestation by environmental fungi. By adding some soil from the environment to one of the wounds, we expected an acceleration of the process. In order to compare these two types of wounds, we selected trees with at least three stems so that one could be wounded without added soil, another could be wounded with added soil, and a third could serve as the control.

We selected 25 trees in the Cacao plot that had at least three stems, with two stems being subjected to the test and the third serving as the control. This time, contamination was achieved with environmental fungi, with our without the application of soil, in order to apply stress (environmental microorganisms) and we monitored the response of the trees leading to oleoresin production. To that end, we applied stress to two of the stems on each tree by making a wound (25cm long by 5cm wide and 5 to 8mm deep) with a chisel, and leaving it exposed to environmental organisms (Fig. 6):

- On one of the wounded stems, plantation soil was applied and covered with the previously removed piece of bark, to optimize contamination and prevent the soil from being washed away too soon by rain.
- One the other wounded stem, the wound remained in contact with the open air.
- The third stem served as the control.

The reaction of the wood to the applied stress was monitored by visual observations and a chemical analysis of the wood after exposure for 6 months, 12 months, 18 months, 21 months and 5 years. Stress was applied in February 2018 (rainy season) and in September 2018 (dry season).

Fig. 6.
Inoculation of environmental fungi into: control tree, wounded tree with open wound, wounded tree with covered wound.

Visual observations

First of all, the trunks of the trees that had received test-pieces inoculated with the wood-decay fungi from French Guiana were felled, then cut transversally into slices, around the inoculation zones. We then cut each of the slices radially in the inoculation zones to observe the propagation of the response to stress and select a zone to be cut where the black wood/white wood limit was clearly visible (Fig.5h). Cutting was carried out in the joinery shop at the Kourou Wood Sciences Laboratory.

For each of the slices, we took a photo and recorded our observations (Fig. 5i). Based on the latter and on an analysis of the photos by “Image J” software, we considered the variation in colour, exudation and propagation of blackened wood following the tree’s response to black wood induction by inoculating the different fungal strains. The black wood areas were measured lengthwise and widthwise in the zone where the test-piece had been inserted (Fig. 5i). The comparisons of black wood propagation areas depending on the different fungal strains are presented as box plots produced with the R software Ggplot2 package.

The trees subjected to contamination by environmental fungi were examined for black wood production under similar conditions; slices were cut in the inoculation zones and then examined if black wood was observed.

After 6 and 12 months of stress (through the inoculation of environmental fungi), the stems of the trees were cut into 5cm thick slices in the inoculation zones. The transversal side of each slice was photographed to assess the area of black wood propagation.

Experimental conditions for steam-distillation of the different samples

After visual observation of the slices, the black wood was removed and ground into fine chips (1mm), using a coffee grinder. Around 20 grams of chips per tree was steam-distilled in 350mL of milli-Q water, to which an internal standard, tridecane, was added in the Clevenger. The mass of wood was not always respected in terms of the amount of black wood, which was not always quantitative. A prior series of trials was carried out on some so-called reference samples (uncontaminated), to define the analysis conditions (internal standard concentration: 4 g/L and 0.04g/L) and the steam-distillation time (3h, 6h, 17h). Following these initial tests, we launched the set of analyses under the following conditions: 5mL of tridecane (0.04mg/mL) and a steam-distillation time of 8h.

This analysis was carried out on:

- Reference wood.
- Wood contaminated by microorganisms from the soil.
- Wood contaminated by microorganisms after wounding, without soil.

The mass of wood depended on the quantity of material available, but the same mass:volume ratio was kept for all the analyses.

The volatile constituents obtained by steam-distillation were analysed by gas phase chromatography coupled to a mass spectrometer (GC-MS). For that reason, chromomes, which are nonetheless very important components of agarwood, are not represented here, due to their low volatility.The internal standard was used to calculate yields.

Experimental conditions for the GC-MS analyses

GC-MS is a qualitative analytical technique for organic compounds. It is used to gather information on the structures of compounds that are present in a sample, even in small quantities.

The chromatograph used here was an Agilent 7820 A model and the mass spectrometer was an Agilent MSD 5977 model.

The GC operating conditions were as follows:

- Oven temperature: initial temperature of 50°C held for 5 minutes using programme number 1, namely 10°C/min up to 250°C.
- Injection temperature 250°C, split ratio 20:1.
- Apolar column: Agilent 19091S-577, HP-5ms, 20m x 180µm x 0.18µm.

The MS operating conditions were as follows:

- MS source: 230°C.
- MS quad: 150°C.
- Acquisition mode scan.
- Mass spectrum [33-350].

RESULTS

Observation of blackened wood propagation areas

Trees inoculated with strains of Guianan wood-decay fungi

After 9 months' exposure to the wooden test-pieces inoculated with Guianan white-rot and brown-rot fungi, and the controls, blackening of the wood was seen around the inoculated test-piece (Fig. 7).

Fig. 7.
Observation of blackened wood propagation areas
Trees inoculated with strains of Guianan wood-decay fungi: After 9 months' exposure to the wooden test-pieces inoculated with Guianan white-rot and brown-rot fungi, and the controls, blackening of the wood was seen around the inoculated test-piece. Generally, black wood propagation was found to be greater towards the bark than towards the pith.
Control sample: Pinus (TP), Control sample Beech (TH), Rigidoporus vinctus (RV), Poria placenta (PP), Antrodia vaillantii (AV), Ganoderma resinaceum (GR), Gloeophyllum trabeum (GT), Pycnoporus sanguineus (PS), Coriolopsis polyzona (CP).

Generally, black wood propagation was found to be greater towards the bark than towards the pith. It was also found in all the treatments that there was a black wood propagation front in contact with the white wood that was darker than the blackened wood next to the test-piece.

For the trials using the "Beech Control" (BC) test-pieces, i.e. not inoculated with the fungus, a paler coloration than that in the trials conducted with the fungi developed in contact with the test-piece after nine months. On the other hand, for the trials using the sterilized "Pine Control" (PC) test-pieces not inoculated

with the fungus, we found a wood with a relatively dark coloration, similar to that of the black wood arising from the interaction of *Aquilaria* trees with the white-rot fungi tested in this study. Lastly, the positive control, a tree tested with the white-tot RV strain, produced a blackened wood whose coloration was darker than in the other trials conducted, and we found that the reaction zone displayed greater exudation when the wood was cut.

The response of the wood to colonization by the brown-rot fungi, PP and AV, gave rise to a paler coloration than with the white-rot fungi, GR, PS and CP. It should be noted that wood with a paler coloration was seen after colonization by the GT fungus, seemingly reflecting a less intense response. This last fungus is known specifically to decay lignin, cellulose, or both at the same time (Zaremski 2005). In view of the coloration observed, these results indicated that GT preferentially decayed lignin, and was thus considered to be a white-rot fungus.

The black wood propagation areas depending on the different fungal strains are shown as box plots (in Fig. 8), where four groups are distinguished depending on their blackened wood propagation areas:

- Small areas (< 22.5cm²): PC;
- Small to moderately extended areas ([22.5cm²; 26cm²]): AV, GT, PP, PS, BC;
- Moderately extended areas ([26cm²; 30cm²]): GR, RV;
- Extended areas (> 30cm²): CP.

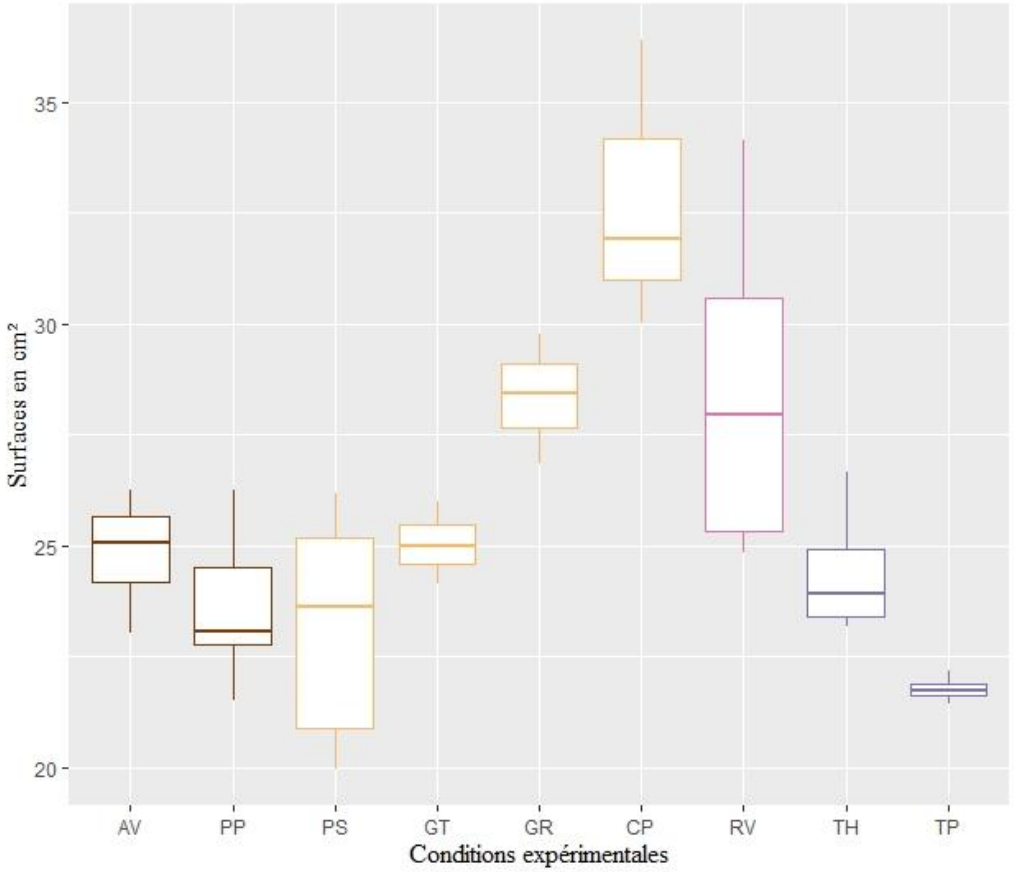

Fig. 8.

Graphic representation of the blackened wood propagation area measurements in the Aquilaria trees planted in French Guiana. The brown-rot fungi are shown in brown and the white-rot fungi in yellow. The positive control is shown in pink and the negative controls in purple. As a reminder: Antrodia vaillantii (AV), Poria placenta (PP), Pycnoporus sanguineus (PS), Gloeophyllum trabeum (GT), Ganoderma resinaceum (GR), Coriolopsis polyzona (CP), Rigidoporus vinctus (RV), Beech Control (TH), Pine Control (TP).

Thus, according to Fig. 8, the wood contaminated by the CP strain displayed the greatest propagation of black wood in the *Aquilaria* trunk 9 months after inoculation in the field, compared to the other treatments tested (white-rot and brown-rot strains, Pine and Beech Controls, without fungus, and the RV Positive Control). Then came the white-rot strains, RV (positive control) and GR. The propagation areas were less extensive for the other strains of white-rot fungi and brown-rot fungi, and for the negative control (“Beech Control”). The “Pine Control” negative control induced the least qualitative response.

Trees colonized by environmental microbial communities, with or without application of plantation soil

After the trees had been stressed for six months or a year by superficial wounding, with or without soil application, radial observations on a slice showed that the reaction of the tree to stress extended over a depth of 2cm (Fig. 9a). The reaction of the tree took the form of black exudates that had solidified (Fig. 9b, Fig. 9c, Fig. 9d). This reaction appeared from the secondary phloem. A mixture of coloured fibres was seen, along with sorts of solidifications caused by the exudates, which were denser to the touch. As expected, the observations carried out on the controls revealed no trace of exudates.

It is interesting to note that, after just six months of induction, the reactions of the trees were apparent and sometimes in the form of very dense solidified exudates (Fig. 9b). However, this dense reaction only occurred down to a very shallow depth, hence over a very small part of the stressed stems. An analysis of the trees stressed for 12 months showed a tree reaction ranging from weak to very dense (Fig. 9c, Fig. 9d), but on a larger number of analysed slices. The exudate resulting from the stress reaction had had more time to spread.

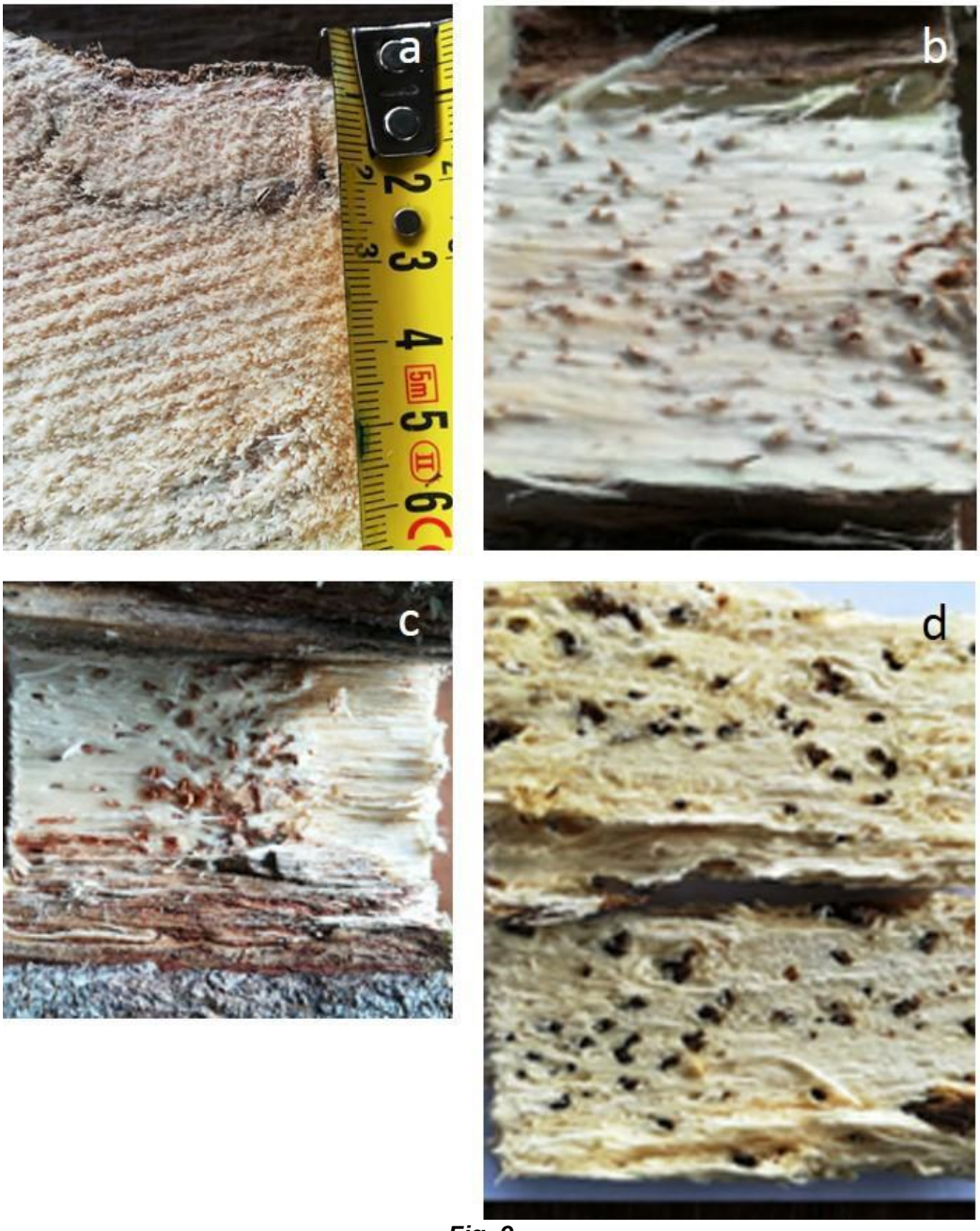

Fig. 9.
Trees colonized by environmental microbial communities, with or without application of plantation soil.

Typical components of the experimental samples obtained by steam-distillation

1. Reference samples

The reference samples were essential oils obtained by steam-distillation of agarwood and white wood from *A. crassna* originating from Laos, extracted in our laboratory, and an agarwood essential oil obtained commercially. These samples enabled us to demonstrate the production of agarwood following the experiments conducted in French Guiana.

As expected, the white wood from Laos mostly revealed the presence of fatty acids, notably palmitic acid (49.84%). For the black wood from Laos, in the reference samples we found a majority presence of oxygenated sesquiterpenes (71.82%) and, to a lesser extent, hydrocarbonated sesquiterpenes (4.91%).

When comparing the composition of the essential oils from the commercial agarwood and from the black wood and white wood from Laos, we found compounds such as valerianol, agarospirol, eudesmol, jinkoh-eremol and valenca, which are typical of agarwood. Nevertheless, although the profiles between the commercial agarwood and the black wood from Laos remained similar, different proportions were found for some of the markers, such as valerianol and agarosprirol (higher in the black wood from Laos than in the commercial agarwood). Lastly, when comparing the composition of the white wood to that of the agarwood, we found a very small quantity of some specific compounds of agarwood. Details of all the compounds characterized in these samples are shown as supplementary elements in Table 2.

Table 2

Reference samples

Details of all the compounds characterized in these samples are shown as supplementary elements

				% relative on apolar column		
Compounds	***Ri literature***	***Ri apolar calculated***	***Ri polar calculated***	***Laos black***	***Laos white***	***Agarwood "commercial"***
hexanoic acid	967	993	1846	-	12,76	-
hexahydro-benzoic acid	1124	1120	2094	-	0,96	-
octanoic acid	1167	1171	2062	-	0,94	-
4-phenylbutan-2-one	**1241**	**1248**	**1864**	**traces**	**-**	**1,18**
hexyl caproate	1385	1386	~1610*	-	0,47	-
α-humulene	1452	1467	1675	1,05	-	traces
MW = 220	-	1487	1732	2,76	-	9,95
α-selinene	1498	1507	~1675*	traces	-	traces
ɣ-cadinene	1513	1526	1763	traces	-	-
δ-cadinene	1522	1534	1760	2,29	-	-
α-calacorene	1544	1556	1924	1,57	-	-
***α*-agarofuran**	**1548**	**1563**	**1861***	**traces**	**-**	**0,90**
(E)-nerolidol	1561	1568	2036	1,17	-	-
MW = 218	-	1576	1899	1,20	-	3,24
10-epi-ɣ-eudesmol	**1622**	**1637**	**2111**	**5,51**	**-**	**5,04**
ɣ-eudesmol	**1630**	**1646**	**2172**	**6,06**	**-**	**2,68**
agarospirol	**1646**	**1651**	**2180**	**11,62**	**0,52**	**4,43**
hinesol	1640	1654	2191	2,74	-	traces
jinkoh-eremol	**1643**	**1660**	**2205**	**8,44**	**-**	**6,35**
valerianol	**1656**	**1668**	**2218**	**19,46**	**0,70**	**9,54**
MW = 218	-	1675	-	2,00	-	-
valenca-1(10), 8-dien-11-ol	**?**	**1687**	**2322**	**6,72**	**-**	**6,36**

dehydrojinkoh-eremol	**1673**	**1692**	**2337**	**2,93**	**-**	**2,08**
acorenone B MW = 218	1700	1707		2,18	-	1,14
MW= 220	-	1714	-	2,92	-	7,61
MW = 218	-	1719	-	2,77	-	- 7,01
MW = 222	-	1757	2297	2,79	traces	-
tetradecanoic acid MW =	-	1760	-	1,20	-	-
228	1758	1761	~2690*	-	1,	- 0,94
MW = 218	-	1766	2302	- 2	39	-
pentadecanol **isomere**	-	1768	2374	, 2	0,97	**0,69**
rotundone iso- mere	1773	1779	-	2	-	- 6,40
dehydrofukinone	**1712**	**1783**	**2361**	-	0,	-
MW = 218	1775	1814	2430	**1,37**	70	-
MW = 242	-	1826	2470	2,80	**-**	-
MW = 242	-	1834	-	1,87	-	-
hexadecanol methyl palmi-	-	1847	-	-	-	- 0,80
tate palmitoleic acid palmitic	1874	1881	~2380*	-	2,	0,88
acid	1912	1927	~2210*	-	60	-
isomere	1939	1945	~2940*	-	1,16	-
dihydrocolumellarine oleic	1960	1972	~2931*	- 1	0,65	
acid	1918	1978	-		0,75	
	2135	2146	~3157*		3,82	
	2132	2168	-		49,84	
Total determined (%)				99,2	92,01	77,22

[X] = ref MS
* = RI polaire litterature (RI = retention indice)

2. Samples of black wood from trees colonized by environmental microbial communities, with or without application of plantation soil

An analysis of the essential oils from samples contaminated with soil microorganisms (WS), or not (NS), revealed the presence of oxygenated sesquiterpenes and aromatic compounds or derivatives.

In quality terms, an analysis of the black wood compounds from all the WS and NS samples showed that the compounds typical of agarwood, such as eudesmol, agarospirol, jinkoh-eremol, valerianol and valenca, were only present in very low quantities and proved absent in some individuals, especially the NS specimens. On the other hand, palmitic acid, which is more typical of white wood (Wang *et al.* 2018), was present in three of the samples (ACG 24-5 ST, ACD 5-3 AT, ACG 24-5 AT) in relatively large quantities. Details for all the compounds characterized in these samples can be found in Table 3. It should be noted that tree ACG 24.5 displayed some specific characteristics, whether or not it was contaminated with soil microorganisms. Indeed, the essential oil from the wood contaminated by soil microorganisms displayed a higher proportion (19.59%) of oxygenated sesquiterpenes than the essential oils from the other trees (from 0% to 6.12%). The essential oil extracted from the same tree, but having undergone the other contamination method, had a higher proportion of fatty acids (74.33%) and the lowest proportion of aromatic compounds or derivatives (2.54%).

ONLINE ISSN 2069-7430
ISSN-L 1841-4737
PRO LIGNO
www.proligno.ro
Vol. 19 N° 1 2023
pp. 3-23

Table 3

Samples of black wood from trees colonized by environmental microbial communities, with or without application of plantation soil: Details for all the compounds characterized in these samples

			Relative percentages present						
Compounds	***Ri literature***	***Ri calculated***	***ACD 5-3 ST[a]***	***ACD 5-3 AT[a]***	***ACD 6-3 ST[a]***	***ACD 6-3 AT[a]***	***ACG 24-5 ST[a]***	***ACG 24-5 AT[a]***	***ACG 24-5-T***
nonane	900	900	1,43	-	0,96	0,91	-	-	2,22
benzaldehyde	952	964	3,58	1,73	2,00	1,61	traces	2,44	-
octanal	998	1004	2,59	-	10,5	2,31	-	-	2,78
benzenacetaldehyde	1036	1047	21,79	2,24	5,34	2,09	0,57	3,30	14,29
dodecane	1200	1200	3,17	1,39	2,2	1,49	-	-	4,97
4-phenylbutan-2-one	1241	1249	2,27	4,23	8,58	17,76	1,52	12,37	-
2-methoxy-4-vinylphenol	1313	1319	2,28	2,24	2,03	0,69	-	-	-
MW = 220	1487*	1488	-	1,18	-	-	0,81	2,31	-
4-(4-butanone		1505	1,91	2,24	4,59	2,99	0,45	4,68	-
benzophenone	1610	1640	2,74	traces	2,50	0,91	-	-	-
10-epi-γ-eudesmol	**1622**	**1637**	**-**		**-**	**-**	**traces**	**1,06**	
γ-eudesmol	**1630**	**1646**	**-**		**-**	**-**	**traces**	**traces**	**-**
agarospirol	**1646**	**1651**	**-**		**traces**	**traces**	**traces**	**1,34**	**-**
jinkoh-eremol	**1643**	**1659**	**-**	**1,42**	**2,11**	**1,16**	**traces**	**3,30**	**-**
valerianol	**1656**	**1667**	**-**	**1,54**	**traces**	**traces**	**traces**	**3,09**	**-**
neo-intermedeol	1660	1672	-		1,71	1,44	1,95	2,70	-
valenca-1(10), 8-ol	1687*	1687	traces	2,04	-		traces	2,78	-
dehydrojinkoh-	1673	1693	-	-	-	-	traces	traces	-
MW = 218	1714*	1714	1,88	3,30	1,75	-	1,39	7,56	-
acorenone B	1700	1740	-	-	-	-	traces	1,76	-
MW = 218	1757*	1758	-		-	-	0,55	1,00	-
MW = 218	1768*	1768	1,36	1,52	-	1,80	2,31	2,65	-
isomère rotundone	**1712**	**1783**	**-**		**2,30**	**1,38**	**1,36**	**1,81**	**-**
isomere dehydrofukinone	1775	1826	-		-	-	-	1,85	-
pentadecanoic acid	1860	1863	-	-	-	-	1,19	-	-
hexadecanol	1874	1881	-	4,66	-	-	-	-	-
methyl palmitate	1912	1926	-	-	-	-	0,33	-	-
palmitoleic acid	1939	1951	-	-	-	-	1,55	-	-
palmitic acid	1960	1982	-	30,46	-	-	49,21	3,71	-
linoleic acid ?	2110		-	-	-	-	22,38	-	-
Total determined (%)			45	60,19	46,57	36,54	85,57	59,71	24,26

*IR from our database (see Table 1)
[a]: experimental conditions of the samples in the experimental part (cf.VII)

3. Black wood samples from trees inoculated with Guianan wood-decay fungi

The list of compounds identified in the black wood obtained after controlled inoculation with strains of Guianan wood-decay fungi and their relative percentages are presented in Table 4. The proportions of the different molecular structure families indicated that the essential oils from the wood mainly contained oxygenated sesquiterpenes and aromatic compounds or derivatives.

We found relatively large proportions of phenylbutane, eudesmol, agarospirol, jinkoh-eremol, valenca and valerianol. The chemical composition of the essential oils could be distinguished according to the type of rot involved: the relative percentages of compounds in black wood contaminated by brown-rot fungi, AV and PP, were somewhat similar. That was not the case for the essential oils in black wood contaminated with white-rot fungi. A comparison of these data with the essential oils obtained from trees treated with uncontaminated beech or pine test-pieces showed the influence of the fungus effect depending on the type of wood species used. For instance, the composition of the extract from the PC negative control seemed to be similar to the extracts from trees inoculated with white-rot fungi. Some fatty acids were found in the extracts from the PC samples. The RV positive control, frequently used to inoculate *Aquilaria* trees, displayed a slightly different profile, notably being the only one in which γ-eudesmol was found.

Table 4

Black wood samples from trees inoculated with Guianan wood-decay fungi
The list of compounds identified in the black wood obtained after controlled inoculation with strains of Guianan wood-decay fungi and their relative percentages.

			Relative percentages present								
Compounds	Ri literature	Ri calculated	GT 1907	PS 2014	PP 2106	CP 2213	AV 2303	TP 2701	GR 2408	RV 3411	TH
benzaldehyde	952	964	traces[a]	traces[a]	traces[a]	traces[a]	traces[a]	traces[a]	0,98[a]	0,91[a]	traces
4-phenylbutan-2-one	1241	1249	6,6	4,56	17,71	4,6	13,15	8,77	15,62	16,73	8.75
MW = 220	1487*	1487	3,32	6,43	3,74	4,08	2,35	5,84	3,54	4,08	4.46
butanone	1473	1505	1,58	0,78	3,31	traces	2,52	1,11	2,58	1,48	traces
α-selinene	1498	1508	-	-	-	traces	traces	traces	-	-	traces
β-dihydroagarofuran	1503	1516	traces	traces	traces	traces	traces	traces	traces	traces	traces
MW = 218	1576*	1576	-	1,10	-	0,74	-	0,8	-	-	**0,80**
10-epi-γ-eudesmol	**1622**	**1637**	**1,83**	**2,84**	**2,31**	**2,9**	**1,3**	**2,17**	**1,63**	**1,74**	**traces**
γ-eudesmol	**1630**	**1646**	**-**	**traces**	**traces**	**traces**	**traces**	**traces**	**traces**	**traces**	**traces**
agarospirol	**1646**	**1650**	**1,4**	**4,26**	**traces**	**1,69**	**traces**	**5,17**	**2,59**	**0,76**	**traces**
hinesol	1640	1654	-	-	-	-	-	-	0,81	-	**traces**
jinkoh-eremol	**1643**	**1659**	**5,08**	**5,94**	**3,15**	**4,13**	**2,42**	**6,34**	**4,65**	**2,19**	**4,68**
valerianol	**1656**	**1668**	**4,61**	**traces**	**3,80**	**4,3**	**4,17**	**6,88**	**7,18**	**6,36**	**3,73**
neo-intermedeol	1660	1670	1,52	traces	1,31	1,25	1,26	1,47	traces	traces	1,42
valenca-1(10), 8-dien-11-ol	**1687***	**1687**	**12,5**	**10,93**	**7,88**	**6,96**	**7,73**	**8,04**	**12,6**	**7,64**	4,22
dehydrojinkoh-eremol	1673	1693	1,69	1,65	traces	traces	traces	traces	1,37	traces	1,20
MW = 218	1714*	1714	13,16	11,13	9,56	6,05	10,47	7,92	14,42	6,61	5,38
Acorenone B	1700	1741	2,3	-	-	1,8	4,13	2,09	1,82	1,76	3,74
MW = 218	1757*	1757	1,47	1,64	traces	1,96	-	1,38	0,65	1	5,85
MW = 218	1768*	1768	1,51	traces	4,84	4,78	1,78	-	-	1,55	1,63
isomère rotundone	**1712**	**1783**	**1,44**	**0,62**	**1,69**	**1,96**	**2,65**	**1,09**	**1,24**	**0,8**	**2,09**
o-costol	1773	1787	0,88	traces	traces	traces	traces	traces	0,65	traces	1,52

isomère dehydrofukinone	1775	1827	1,6	1,61	-	1,88	-	1,35	-	1,17	1,24
palmitic acid	1960	1962	-	traces	-	-	-	1,1	-	-	-
3,6-dimethyl-phenanthrene	2037	2071	-	-	-	-	-	-	-	-	-
1,5-diphenyl-1-penten-3-one	?	2185	-	-	-	-	-	-	-	0,68	-
Retene	2214	2240	-	0,99	4,74	2,34	1,22	traces	0,69	traces	4.56
methyl dehydroabietate	2288	2361	-	1,41	6,16	2,04	6,22	traces	0,96	0,84	5.90
Total determined			62,49	55,89	70,20	53,46	61,37	61,52	73,98	57,71	61,17

*IR from our database (see Table 1)
[a]: experimental conditions of the samples in the experimental part (cf.VII).

4. Overall analysis of all the samples

A Principal Components Analysis (PCA) of the data for all the samples (Fig. 10) showed that the reference samples of extracts from the wood from Laos, black Laos and white Laos, were the furthest apart on axis 1 (horizontal), which represented the compounds typical of agarwood, such as eudesmol, eremol, agarospirol and valerianol; the composition of the essential oil from the black wood differed from that of the white wood through the presence of those compounds, and of palmitic acid, typical of white wood. The essential oil of the commercial agarwood was closer to the essential oil from the Laos black wood. For the essential oils from the samples contaminated by the different fungal strains, the samples closest to the commercial agarwood were those that had been inoculated with the Guianan wood-decay fungal strains, more particularly with the white-rot fungi GR, GT, PS and PC. The trees contaminated with CP and RV, the positive control, and with the brown-rot fungi, AV and PP, stood out slightly from the first group along axis 1, being closer to the white wood that to the commercial agarwood according to that axis. It should be noted that the sample from tree ACG 24.5, contaminated with soil fungi, was also found in this group. The samples from tree ACD 6.3, contaminated, or not, with soil microflora, were grouped together according to axes 1 and 2. According to axis 2 (vertical), notably defined by palmitic acid, which is typical of white wood, the above-mentioned groups were closer to the commercial agarwood than the samples from the Laos black and white woods. However, according to axis 1 and axis 2, the samples with soil and without soil from tree ACD 5.3, and the sample without soil from tree ACG 24.5, were close to the Laos white wood sample.

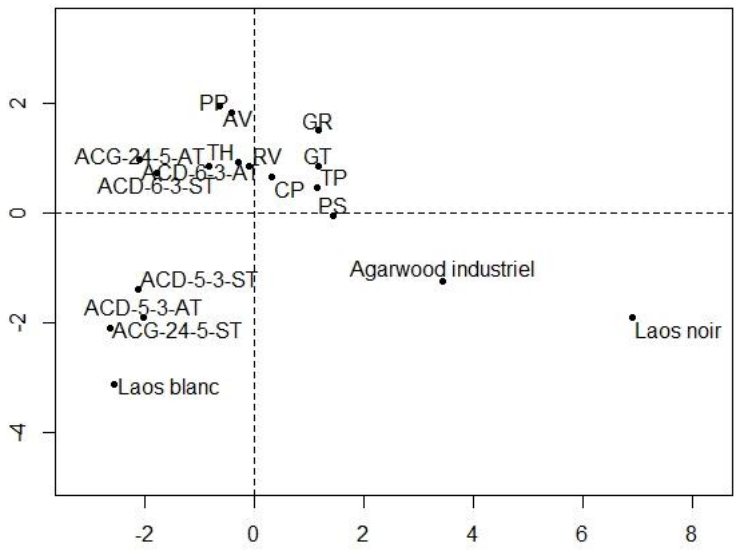

Fig. 10.
Principal Components Analysis (PCA) of sample distribution according to chemical composition, carried out with the R software FactoMineR package
Axis 1 (horizontal) is constructed (89%) based on the variables 10-epi-γ-eudesmol, jinkoh-eremol, agarospirol, valerianol, dehydrojinkoh-eremol, γ-eudesmol.
Axis 2 (vertical) is constructed (78%) based on the variables 4-phenylbutan-2-one, palmitic acid and rotundone.

5. Essential oil yields from all the samples after steam-distillation

The essential oil mass percentage yields obtained after steam-distillation of blackened wood were all under 0.20% (Fig. 11). It needs to be remembered that yields from white wood are 0.1% (1kg of essential oil is extracted for 1,000kg of white wood) and 1% from black wood (1kg of essential oil is extracted from 100kg of black wood), i.e. a factor of 10 between the two types of wood. It can also be noted that three of the trees contaminated with the white-rot fungi, PS, GT, CP, and the negative control PC, had a yield over 0.1%. The wood contaminated with the RV positive control came close, with a yield of 0.098%. The trees contaminated with the brown-rot fungi, AV and PP, gave a lower yield, at 0.032%. The yields of the trees contaminated with environmental fungi were lower, ranging from 0.00024% for tree ACD 5.3 to 0.02% for tree ACD 25.5, (Fig. 5.).

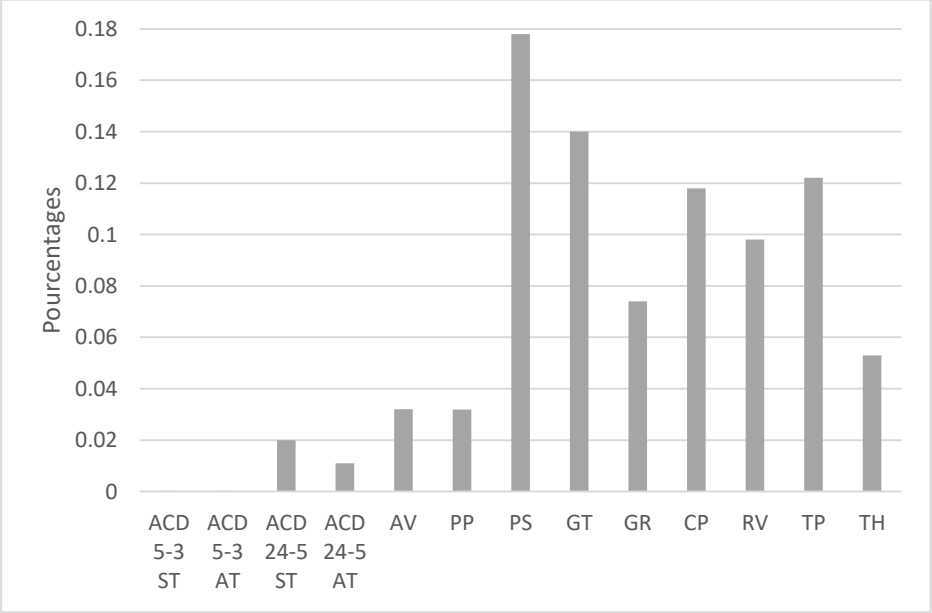

Fig. 11.
Essential oil yields for the different types of samples inoculated with environmental fungi and with Guianan wood-decay fungi. With Soil (WS); No Soil (NS); Antrodia vaillantii (AV), Poria placenta (PP), Pycnoporus sanguineus (PS), Gloeophyllum trabeum (GT), Ganoderma resinaceum (GR), Coriolopsis polyzona (CP), Rigidoporus vinctus (RV), Pine Control (TP).

DISCUSSION

This study set out to identify conditions conducive to agarwood production based on its chemical composition compared to that of reference essential oils from white wood (uncontaminated) and black wood (agarwood). To that end, we conducted two sets of trials. In the first set of trials, stress was achieved by making wounds along the trunk, followed by contamination with fungi from the soil and the environment. In the second set of trials, the trees were specifically contaminated with Guianan wood-decay fungi (white-rot and brown-rot fungi). We also added some controls (beech and pine), so that we could see the influence of the wooden support medium used as the contamination vector, along with a so-called positive control, *Rigidoporus vinctus*, which is commonly used for agarwood production in Asia (Chen *et al.* 2018).

It is true that the literature describes several practices for inducing agarwood production (Naziz *et al.* 2019; Blanchette *et al.* 2015; Tan *et al.* 2019), but the novel nature of this study came from the use of environmental fungi, notably from soil taken from the foot of the tree, applied directly after wounding the tree. In fact, although wounding *Aquilaria* trees is a frequently used technique (Tan *et al.* 2019), applying soil to the wound had yet to be tested. Likewise, mixtures containing microorganisms are commonly inserted into *Aquilaria* tree trunks (Blanchette *et al.* 2015; Tan *et al.* 2019), but in our case we inserted pure strains of selected fungi using wooden test-pieces, which were also used to plug the hole into which they were inserted. This called for the development of a contamination method based on inoculating locally selected fungi.

We used these fungi, belonging to the family of the Basidiomycetes and causing white rot or brown rot, to compare how *Aquilaria* trees responded to such stress. The fungi were selected following an earlier study characterizing the xylophilous and wood-decay fungi common in French Guiana (Zaremski *et al.* 2019). They were selected for their wood-decay nature, their relative abundance in French Guiana and because they included both white-rot fungi, notably decaying lignin, and brown-rot fungi, decaying cellulose.

As expected, our initial observations revealed a reaction of the wood, which was reflected in a more or less developed coloration around the stress zone. However, some differences were found depending on the biotic agents involved. We found that black wood production in response to stress was much more developed when the tree was inoculated with white-rot and brown-rot fungi than with environmental fungi, with or without soil application. The white-rot fungus, *Coriolopsis polyzona,* gave the largest agarwood propagation area, followed by *Ganoderma resinaceum* and *Rigidoporus vinctus*, two white-rot fungi, the latter being the positive control. The brown-rot fungi, the other white-rot fungi and the beech negative control gave smaller agarwood propagation areas. The propagation area associated with the pine negative control was the smallest.

We explained these differences by the difference in the type of wound. Indeed, for the trees contaminated with soil microorganisms, the wound was made more or less on the surface to only a shallow depth (2 cm), while on the trees specifically contaminated by brown-rot or white-rot fungi, using wooden test-pieces, the wound differed in that ten holes with a diameter of 10mm and a depth of 8cm were made along the trunk. These initial observations lead us to recommend a wound down to a sufficient depth to obtain greater agarwood propagation. The differences between the wood-decay fungi came from the type of rot and the fungal strains. Indeed, the white-rot fungi gave larger agarwood propagation areas than the brown-rot fungi. The white-rot fungal strain *Coriolopsis polyzona* seemed to produce the largest agarwood propagation volume in this study.

By studying the chemical composition of the samples after contamination, we were able to see the efficiency of the contamination method for agarwood production, by searching for specific markers identified in reference essential oils from Laos and from commercial agarwood.

We were able to confirm that the essential oil from the black wood and commercial agarwood contained oxygenated sesquiterpenes, notably eudesmol, agarospirol, jinkoh-eremol and valerianol, which are typical compounds of agarwood (Naef 2011).

We found that the essential oils from trees contaminated by microorganisms present in soil taken from the foot of the tree, or by environmental fungi without soil application, displayed similar profiles, somewhat characterized by aromatic compounds or derivatives and some other molecules not typical of agarwood. In addition, two of the samples contaminated with soil (ACD 5.3) and without soil (ACG 24.5) had a high palmitic acid content, typical of white wood (Wang *et al.* 2018). However, sample ACG 24.5 contaminated with soil revealed the presence of oxygenated sesquiterpenes, notably jinkoh-eremol and valerianol, and displayed a profile closer to that of the black wood from Laos and the commercial agarwood, revealing agarwood production.

However, the composition results for the essential oil from the black wood obtained by inoculating with selected fungal strains were quite another matter. Indeed, both a high oxygenated sesquiterpene content and the absence of palmitic acid revealed volatile compound profiles close to those obtained for the black wood from Laos and the commercial agarwood, whatever the fungal strains tested, and likewise for the essential oils extracted from the positive control, RV, and the Pine Control, TP.

The samples contaminated with the white-rot fungi, GR, GT and PS, as well as the Pine Control, TP, seemed to produce an agarwood with volatile compounds close to those of the commercial agarwood. This result shows that the existence of certain volatile compounds was not due to the presence of a particular strain of fungus, but was rather due to the wound on the trunk, which would seem to induce colonization by environmental microorganisms. However, the survival of the fungus inside the trunk, over a given period, remains to be demonstrated to prove that the choice of fungal strain is decisive in the characteristics of the agarwood obtained. It is worth noting that the essential oil extracted from the blackened wood of the positive control, inoculated with RV, which we had selected as such because it is a ubiquitous fungus existing in French Guiana and because its use to induce agarwood production was highlighted by Chen *et al.* (2018), had a chemical composition further from that of the commercial agarwood than the essential oil obtained from the blackened wood resulting from inoculation with all the white-rot fungi used in our study. On the other hand, the essential oils extracted from the agarwoods obtained by inoculating the brown-rot fungi, AV and PP, displayed profiles further from those of the commercial agarwood and the Laos black wood than those obtained by inoculation with white-rot fungi.

Thus, the blackened wood obtained by inoculating with selected fungal strains using wooden test-pieces had an assembly of volatile compounds closer to that of the commercial agarwood than the blackened wood obtained by inoculation with environmental fungi, and even closer than the Laos black wood. This was particularly clear for the GR, GT and PS strains.

Lastly, the yield study showed us generally that yields after steam-distillation of black wood from trees inoculated with white-rot fungi, the positive control and the negative control are worthwhile (see Fig. 11): from 0.074% to 0.178%. The yields after steam-distillation of black wood from the trees inoculated with brown-rot fungi were lower than those inoculated with white-rot fungi, at 0.032%.

As expected, the yields after steam distillation of black wood from the trees inoculated with environmental fungi were much lower, ranging from 0.00024% to 0.02%. More precisely, the highest yield was obtained for the blackened wood obtained after inoculation with PS. It was thus interesting to note that the positive control had a lower yield than three of the trees inoculated with white-rot fungi, though the resulting wood was the darkest. In addition, the negative control, which seemed worthwhile in terms of its volatile compound assembly, was less so than PS and GT in terms of yield. This shows that the presence of a fungus contributing to black wood propagation seems to be important from a quantitative viewpoint.

Finally, the comparison between black wood propagation in terms of area, and yields, showed that the area observed does not seem correlatable to the yield. For example, PS, which had the largest yield, did not give a large black wood propagation volume, and the opposite was found for CP.

Given these results, it can therefore be concluded that stress induction by inoculating pure fungal strains using wooden test-pieces is the most appropriate method. This method is novel, as the fungi are inoculated as pure strains. From a quantitative viewpoint, the white-rot fungal strains, and especially *Ganoderma resinaceum* (GR), *Gloeophyllum trabeum* (GT) and *Pycnoporus sanguineus* (PS), were the most effective in producing agarwood with the composition required by the market. These strains do not seem to have been used often in other studies, yet they seem suited to agarwood production in French Guiana.

CONCLUSION

This study made it possible to obtain agarwood production in *Aquilaria* trees in French Guiana and, more specifically, it showed that inoculating pure fungal strains by inserting contaminated wooden test-pieces into the trunk is the best way of inducing agarwood with a controlled chemical composition in *Aquilaria* trees. It is a novel method compared to other methods reported in the literature, it totally excludes chemical additives and uses fungal strains that are frequently found in the environment around Guianan plantations. We thus showed that white-rot fungi are the most efficient, particularly GR, GT and PS, which are very common in French Guiana. Indeed, when exposed to these fungi, the yield and volatile compound composition of the wood resulting from such contamination were the closest to those obtained with commercial agarwood harvested in Laos. We recommend using these fungi for agarwood production with a controlled chemical composition in French Guiana.

ACKNOWLEDGEMENTS

This study was undertaken as part of the Aquil@Guyane Project: Scientific and technical basis for the creation of a supply chain for top-of-the-range *Aquilaria* (Agarwood) essential oils and by-products in French Guiana, jointly funded by Europe, the *Collectivité Territoriale de Guyane* and CIRAD: European Regional Development Fund (ERDF) for French Guiana (agreement No. FEDER/2017/N° 31; and FEDER/2020/N°472).

We particularly thank the farmers, Messrs. Ya Hu, Didier Tcha and Pierre Tcha, for their time in setting up and monitoring the experimental plots, and their faultless availability in helping us to take samples in the field.

Our warm thanks to our colleagues Dr. Jacques Beauchêne, Arnaud Jahn-Oyak, Deborah Fernand, Julien Passelande and Mr Soepe Koese for their participation in the different stages of this study.

REFERENCES

Barden A, Anak NA, Mulliken T, Song M (2000) Heart of the matter: agarwood use and trade and CITES implementation for *Aquilaria malaccensis*. Traffic net- work report, TRAFFIC International, Cambridge, pp. 60. 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.

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.

Chen X, Liu Y, Yang Y, Feng J, Liu P, Sui C, Wei J (2018) Trunk surface agarwood- inducing technique with *Rigidoporus vinctus*: An efficient novel method for agarwood production. PLOS ONE 13(6): e0198111, doi: 10.1371/jour- nal.pone.0198111

Deep K, Tajuddin N (2019) King of scents - Agarwood. Perfumer & flavorist 44(3):42-56.

Fougerousse M (1979) Préservation des menuiseries contre la pourriture. Bois et Forêts des Tropiques 183:49-66.

Gratzfeld J, Tan B (2008) Agarwood - saving a precious and threatened resource. Botanic Garden Conservation International 5(1):27-29, Special issue: Conserving forest biodiversity.

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

Lee SY, Mohamed R, Faridah Hanum I, Lamasudin DU (2017) Utilization of the internal transcribed spacer (ITS) DNA sequence to trace the geographical sources of *Aquilaria malaccensis* Lam. populations. Plant Genetic Resources: Characterization and Utilization 16(02):103–111, doi: 10.1017/S1479262117000016

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

Mohamed R, Jong PL, Nurul 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, doi: 10.1007/s11274-014-1668-2

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, doi: 10.1002/ffj.2034

Naziz PS, Das R, Sen S (2019) The scent of stress: evidence from the unique fragrance of agarwood. Frontiers in Plant Science 10:840, doi: 10.3389/fpls.2019.00840

Pripdeevech P, Khummueng W, Park SK (2011) Identification of odor-active components of agarwood essential oils from Thailand by Solid Phase Microextraction-GC/MS and GC-O. The Journal of Essential Oil Research 23(4):46-53. https://doi.org/10.1080/10412905.2011.9700468

Rasool S, Mohamed R (2016) Understanding agarwood formation and its challenges. In: Agarwood: Science behind the fragrance, Eds Mohamed R., Springer, Singapore, pp. 39-56. https://doi.org/10.1007/978-981-10-0833-7_3

Sadgopal (1960) Explanatory studies in the development of essential oils and their constituents in aromatic plants. Part 1: Oil of Agarwood. SPC 33:41-46.

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

Tan CS, Isa NM, Ismail I, Zainal Z (2019) Agarwood induction: current developments and future perspectives. Frontiers in Plant Science 10:122, doi: 10.3389/fpls.2019.00122

Wang MR, Li W, Luo S, Zhao X, Ma CH, Liu SX (2018) GC-MS Study of the chemical components of different *Aquilaria sinensis* (Lour.) *Gilgorgans* and agarwood from Different Asian countries. Molecules 23(9):21-68. https://doi.org/10.3390/molecules23092168

Zaremski A (2005) Les Aphyllophorales impliquées dans la dégradation du bois: taxonomical characterisation, phylogeny and early detection of infection. Marseille: Université de Provence, Marseille, pp. 148.

Zaremski C, Ducousso-Détrez A, Amusant N, Ducousso M, Zaremski A (2019) Taxonomic study of French Guiana fungi to identify and isolate pure cultured fungi for oleoresin production in Aquilaria: use of sequences from the small ribosomal DNA (r) subunit (SSU) and the two primer pairs SR6/SR10R and SR7/SR1R. PRO Ligno 15(3):3-15.

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