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Section 1 **Biology**

Chemical composition of agarwood of *Aquilaria crassna* Pierre ex. Lecomte induced by Basidiomycetes from French Guiana

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ABSTRACT

Aquilaria trees subjected to stress induce the formation of a transformed wood named agarwood. The formation of agarwood is a plant defense mechanism that occur in complex interactions with environmental microorganisms. Agarwood chemical compounds are mainly chromones and oxygenated sesquiterpenes such as eudesmol, agarospirol, jinkoh-eremol and valerianol, which are valued in perfumery. Its derivatives, notably the essential oil are therefore expensive. Agarwood essential oil costs 5,000 to 10,000 US $ per kg. Because of this important value and the scarcity of production from the wild, operators are planting *Aquilaria* and implementing various methods to induce agarwood. Many of these methods are deleterious for trees and even can alter the chemical composition of the essential oil.

In this context, a consortium of farmers from French Guiana and scientists and from Centre de cooperation Internationale en Recherches Agronomiques pour le Développement (CIRAD) are developing the Aquil@Guyane project. This project aims at cultivating *Aquilaria* trees in French Guiana to produce agarwood of controlled chemical composition, respecting the biology and ecology of trees.

In this frame we have tested the inoculation of trees with cubic and fibrous rot fungi to induce agarwood development in tree. Fungal strains used in this experiment have been isolated in the vicinity of Guianese *Aquilaria* plantations. Selected strains have been cultivated on wood test-sticks; infected sticks were then place in holes made with a drill in the trunks of *Aquilaria*. The yield of agarwood and chemical compositions of essential oils extracted from the so obtain blackened wood have been analyzed enabling to compare the different treatments. First, we

evidence that the controlled inoculation of *Aquilaria* tree with selected fungal strains results in the production of agarwood and thus essential oils. The quality, in terms of composition of essential oils obtained among treatments was distributed between reference white wood and agarwood. The fibrous rot fungi were the most effective in terms of agarwood development and quality and yield of essential oils.

Keywords: Agarwood, *Aquilaria*, French Guiana, basidiomycetes, hydrodistillation, Lignivorous fungi, Sesquiterpenes

1. 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, and more particularly volatile oils obtained from the wood are 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. *Aquilaria* is the most used genus to obtain agarwood. This genus currently comprises 21 species (Lee *et al.* 2017). Agarwood is also extracted in the closely related genera *Gyrinops* and *Gonystylus* (Gratzfeld and Tan 2008).

Healthy *Aquilaria* wood is white. In reaction to stress in complex interactions with environmental microorganisms, particularly fungi, the tree produces locally significant quantities of secondary metabolites, the accumulation of which leads to the formation of transformed wood; the so-called agarwood (Naziz *et al.* 2019). Agarwood, is rich in oxidable aromatic molecules that cause a black coloration, and has a high oleoresin and oil content 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 (Fig. 1). The last two groups of 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 black wood can exceed 1 for agarwood belonging to 75 to 80-year-old trees (Sadgopal 1960) and becomes increasingly fragrant over time.

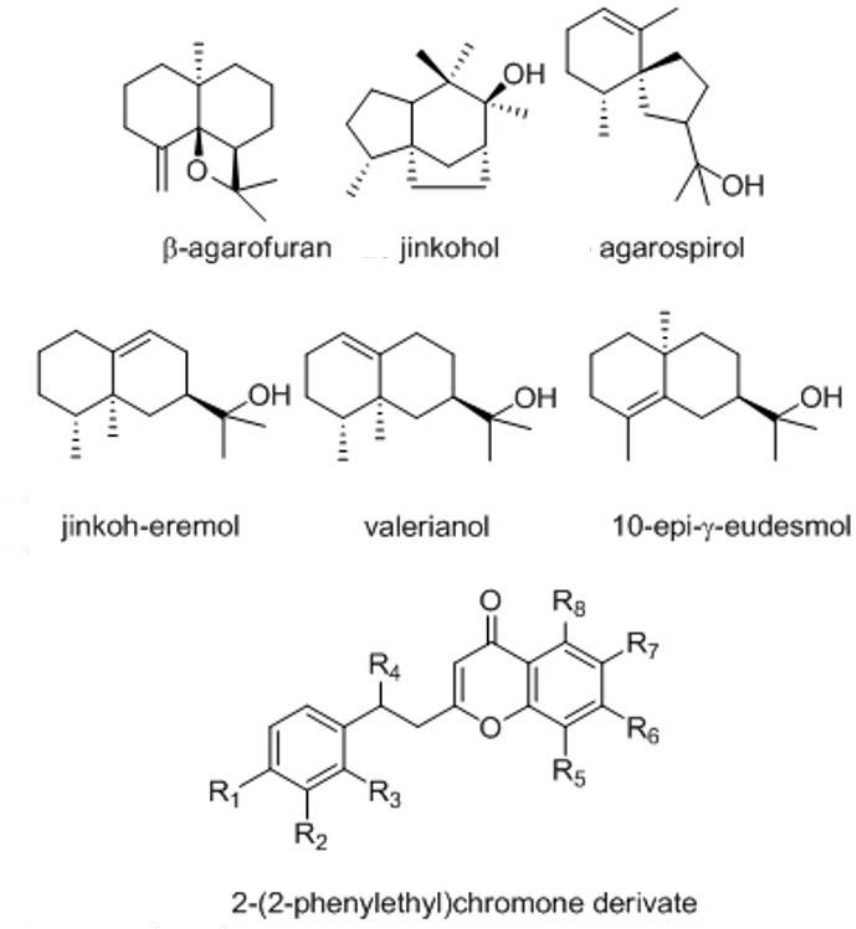

Figure 1: Chemical structures of the main compounds of agarwood.

According to several authors (Soehartono and Mardiastuti 1997, Mohamed *et al.* 2010, Zaremski *et al.* 2018), the genera of fungi that colonize *Aquilaria* and that way may play a role in agarwood development 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.

In the natural environment, about 10% of trees produce agarwood (Barden *et al.* 2000). Plantations are specifically designed to produce agarwood and despite efforts to increase production of agarwood, in some Indian plantations, only a third of the trees produce agarwood (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, classified 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, notably in terms of essential oil composition and presence of residues such as pesticides or heavy metals.

To overcome these problems, we tested a new method to induce agarwood development in *A. crassna* growing in a plantation in French Guiana. The proposed method is based on the inoculation of *Aquilaria* trees with selected cubic and fibrous rot Basidiomycetes fungi from the close environment of the plantation. Test wood sticks are used as support for the inoculum that is introduced in the tree trunk by drill-made holes. Then essential oil yields and compositions have been analyzed in the different treatments compared to some agarwood control samples from Southeast Asia, to white wood and agarwood obtain with much more traditional methods.

2. EXPERIMENTAL METHODS

2.1 Study site

Figure 2: French Guiana is located in South America. Cacao is established near the Comté River (www.assistancescolaire.com; www.cartes-2-france.com)

This work was undertaken in French Guiana, in the village of Cacao (Fig. 2). The inhabitants of Cacao are mainly of Lao origin and are widely involved in farming activities. The village is located 70 km southwest of Cayenne, the capital city of French Guiana, along the Comté River; 4° 34' 17'' North and 52° 28' 11'' West. French Guiana mostly lives 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. The trees of the genus *Aquilaria* chosen for this study came from an experimental plantation managed in partnership by CIRAD and a group of Guianese farmers. The plantation were established in January 2014 using seeds imported from Laos.

2.2 Biological material

The novel methodological objective was to choose local species adapted to the Guianese 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.

In order to test fungal strains ability to stimulate agarwood production in *A. crassna* three steps are necessary for inoculum production. The first step was to identify and select fungi of interest in the close vicinity of the *Aquilaria* plantation. The second step was to isolate fungal strains and to produce pure mycelium on culture medium. The third step was to make the fungi grow in wood test-sticks, in order to use the fungi-colonized test-sticks to inoculate the trees. Then drill-made holes were done to enable the introduction of fungi-colonized test-sticks in *Aquilaria* trunks.

2.2.1 Identifying and selecting Guianese fungi for the study

Thirty-nine fungal fruiting bodies from forest sites near Cacao were characterised based on SSU-rDNA sequences obtained using SR6/SR10R and SR7/SR1R primers. Fresh living fungi were collected on dead wood lying on the ground (25 fungi) and on dead standing trees (14 fungi). 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* thus confirming morphological indentification (Zaremski *et al.* 2019).

Pure culture mycelial strains have been isolated from fruiting bodies by transferring a piece of the pileus trama into Petri dishes field with nutritive malt-agar medium. Of this collection, we choose two fibrous rot fungi: *Ganoderma resinaceum* Boud. (GR), *Pycnoporus sanguineus* (L.) Murrill (PS) and two cubic rot fungi: Brown-rot fungi: *Poria placenta* (Fr.) Cooke (PP), *Antrodia vaillantii* (DC.) Ryvarden (AV). These fungi have been chosen for their high frequency in the natural environment, making them easily available, and for their good ability to produce mycelium under laboratory conditions.

2.2.2 Inoculum production with the selected fungi

Each strain has been cultivated in 12.5 x 12.5 x 1.5 cm Petri dishes containing 40 mL of Malt-agar culture medium. In each Petri dish, 5 previously sterilised wood test-sticks (0.7 x 0.7 x 10 cm) made of Pine for cubic rot and Beech for fibrous rot have been regularly placed (1 cm space between test-sticks) on the surface of the cultivation medium. Sealed Petri dishes have been incubated for 2 months at to 22 ± 2°C. After this incubation period, the development of each strains in the wood test-sticks has been visually checked; only well-colonized wood test-sticks have been used for *Aquilaria* trunk inoculation in the field.

2.2.3 Inoculation of the trees on the field

Inoculation was carried out on a total of 18 trees over 40 cm diameter as follows (Fig. 3):
- After disinfection with alcohol and flaming, removal of a 4 x 4 cm square of bark using a wood chisel, which was also flamed-disinfected.
- After disinfecting the drill bit with alcohol and flaming, drilling into the middle of the square up to a 10 cm depth.
- Immediate insertion of the wood test-stick in the hole,
- Sealing of the hole by applying a few ml of pine-tar resin to the inner side of the square of bark removed earlier.
- 10 holes have been made per tree, moving upwards (20 cm) but also radially (120°) “in a spiral”.

Inoculation was carried out on three trees per strain. In control trees, Pine (PC) or Beech (BC) wood test-sticks without any fungus were inserted in *Aquilaria* trunk (total of 2 x 3 = 6 trees) as for inoculation with the 4 selected strains.

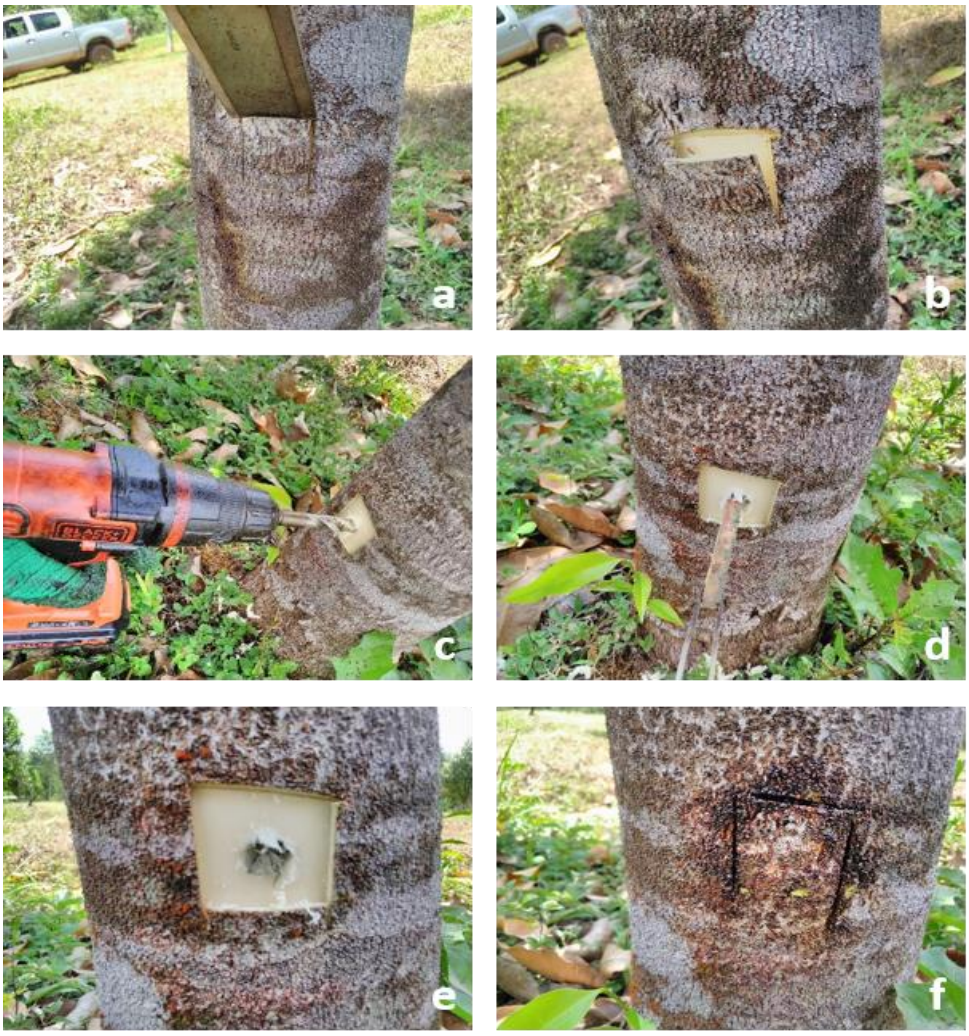

Figure 3: Inoculation process image by image: a: opening of a window in the bark using a wood chisel; b: opening a window in the bark; c: using a drill to make a hole in the trunk; d: inserting fungus-colonized wood test-stick into *Aquilaria* trunk; e: view of the window through the bark after the full insertion of the inoculum; f: after inoculation sealing of the window using pine-star resin.

2.3 Analyzes of the composition of essential oils

Essential oils compositions have been analysed using a GC-MS spectrometer after agarwood steam-distillation after nine months of inoculation. Compositions obtained for each inoculation treatments have been compared with white wood and agarwood from Laos use for the circumstance as control. Extraction process of essential oils applied to the white wood and the agarwood control was exactly the same as the different treatments.

Within the different treatments, agarwood was separated from the white wood, carefully taken and grind into fine chips (1 mm and below). Twenty grams of chips per tree was steam-distilled in 350 mL of milli-Q water, to which an internal standard, tridecane, was added in the Clevenger. 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 (3 h, 6 h, 17 h). Following these initial tests, we launched the set of analyses under the following conditions: 5mL of tridecane (0.04 mg/mL) and a steam-distillation time of 8h.
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.

GC/MS analyses were performed using an Agilent 5977 apparatus MSD series equipped with a silica capillary column HP-5 MS (5%-phenyl-methylpolysiloxane; 15m x 180 µm; film thickness 0.18 µm) interfaced with a quadrupole detector (single quadrupole acquisition Method-MS parameters report), source temperature 230°C, Quadrupole temperature 150°C; the temperature program was 50°C for 5 min, 50-250°C at 10°C/min, then kept at 250°C during 20 min; injector temperature, 250°C; MS transfer line temperature, 250°C; carrier gas, helium at a flow rate of 0.7 ml/min; injection type, split, 20:1 (1 µl of the sample); ionization voltage, 70 eV; electronmultiplier 1000 eV; scan range 33-400 amu; scan rate, 1.56 scan/s. The identification of the constituents was based on comparison of their relative retention indices (calculated from the retention times of a n-alcanes series) with those of published data in the literature and by matching their mass spectra with those obtained from the NIST14, NIST98, FFNSC 2.L. libraries spectra and from literature data.

3. RESULTS AND DISCUSSION

3.1 Essential oil yields from all the samples after steam-distillation

In PS and GR treatments and also in PC treatment, yield over 0.1% have been observed. The highest yield has been obtained in PS treatment. In AV and PP treatments, the yield was calculated as 0.032% which is particularly low, even lower than the Beech control (BC) treatment (Fig. 4).

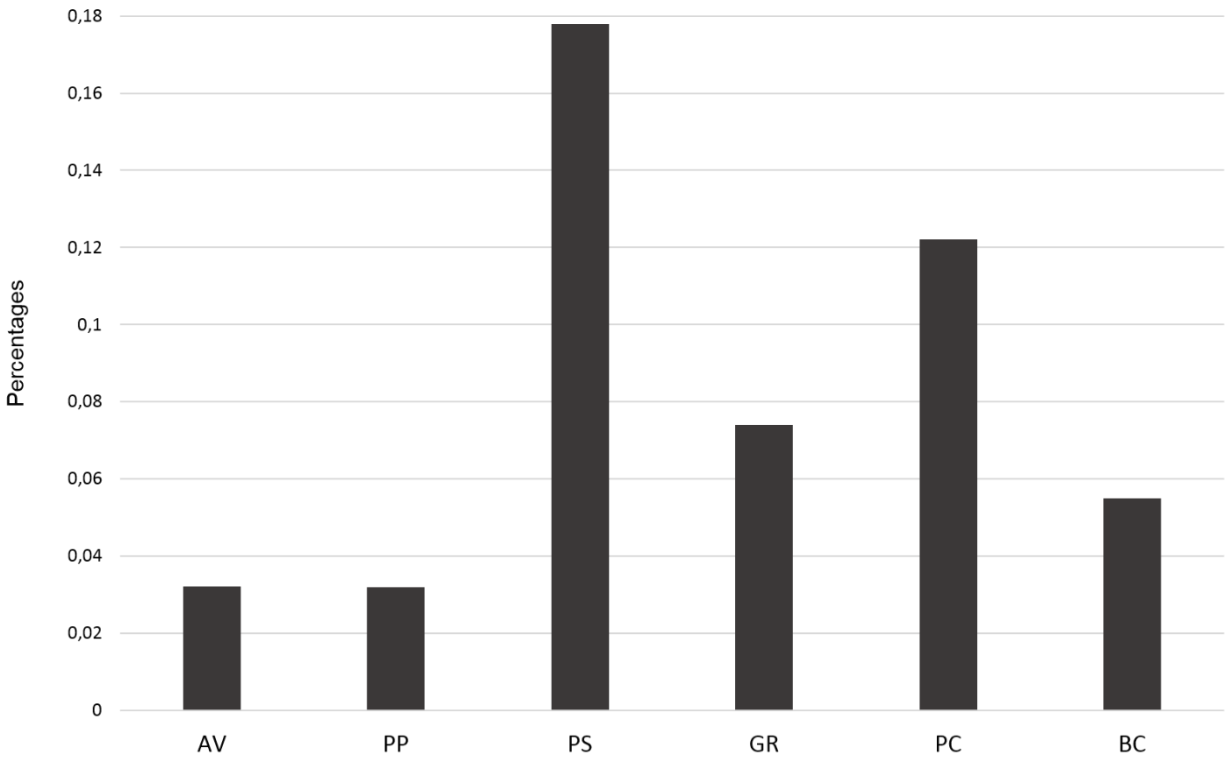

Figure 4: Essential oil yields for the different types of samples inoculated with environmental fungi and with Guianan wood-decay fungi: *Antrodia vaillantii* (AV), *Poria placenta* (PP), *Pycnoporus sanguineus* (PS), *Ganoderma resinaceum* (GR), Pine Control (PC), Beech Control (BC).

3.2 Typical components obtained by steam-distillation

3.2.1 Reference samples

The white wood from Laos mostly revealed the presence of fatty acids, notably palmitic acid, which is also present in agarwood, but in very much lower quantities. For agarwood from Laos, we mostly found oxygenated sesquiterpenes (Fig. 5). Eudesmol, jinkoh-eremol, rotundone and valenca-1(10),8-dien-11-ol, are typical of this agarwood; valerianol and agarospirol are also found in white wood, but in much lesser quantities

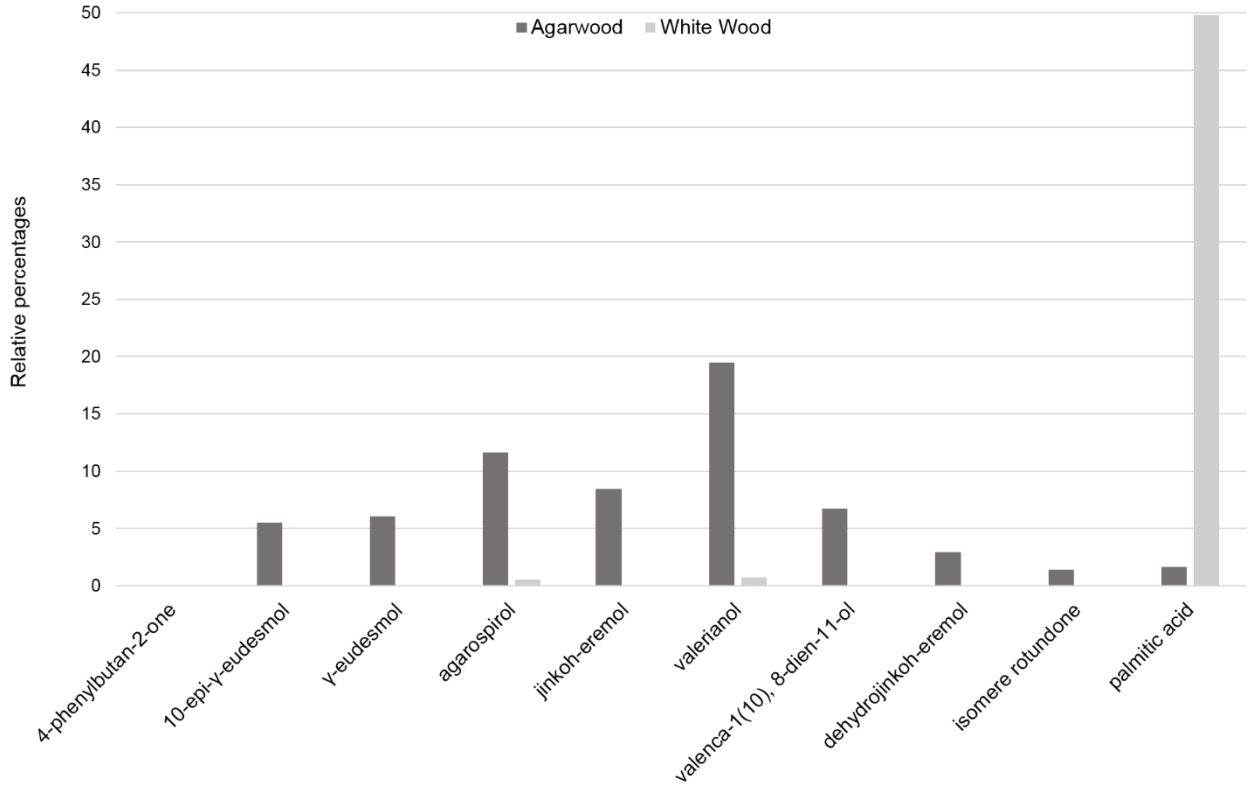

Figure 5: Relative percentages on the apolar column of the main typical volatile compounds in agarwood and white wood from Laos used as reference.

3.2.2 Samples from the different treatments

The proportions of the different molecular structure families indicated that the essential oils from the agarwood obtained from the different treatments mainly contained oxygenated sesquiterpenes and aromatic compounds or derivatives. We found relatively large proportions of 4-phenylbutane-2-one, eudesmols, agarospirol, jinkoh-eremol, valenca-1(10),8-dien-11-ol and valerianol (Fig. 6) that are part of the main compounds of agarwood molecules (Naef 2011). The chemical composition of the essential oils could be distinguished according to the type of rot involved: the relative percentages of compounds in agarwood contaminated by cubic rot fungi, AV and PP, were somewhat similar. That was not the case for the essential oil agarwood obtained with fibrous rot fungi, PS and GR. A comparison of these data with the essential oils obtained from trees treated with control wood test-sticks, pine or beech, allow to interpret the putative role of the fungus in the composition of obtained essential oils. For example, composition observed from PC treatment seemed to be similar to the extracts from trees inoculated with cubic rot fungi while composition from BC treatment allow to obtain lower concentrations than the trees inoculated with fibrous rot fungi. It should be noted that for BC treatment we find palmitic acid. This acid is mainly found in white wood.

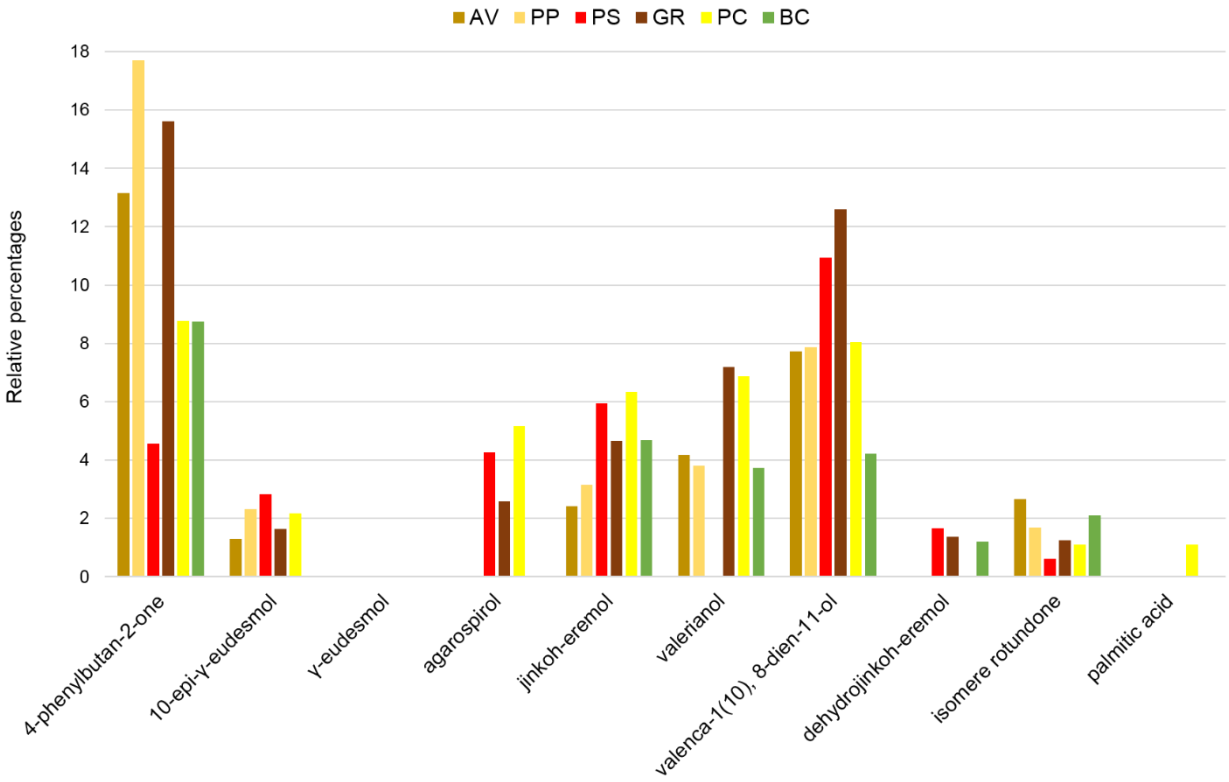

Figure 6: Relative percentages on an apolar column of the main volatile compounds typical of agarwood present in the trees inoculated with Guianese cubic and fibrous rot fungi: *Antrodia vaillantii* (AV), *Poria placenta* (PP), *Pycnoporus sanguineus* (PS), *Ganoderma resinaceum* (GR), Pine Control (PC), Beech Control (BC).

The samples of the GR and PS treatments (fibrous rot) produce an agarwood with volatile compounds close to those of the reference agarwood (Fig. 5). Essential oils extracted from agarwood obtained by inoculating the brown-rot fungi, The samples of the AV and PP (cubic rot) produce an agarwood with volatile compound profiles quite different of the reference agarwood and by the same token also different from those obtained in the GR and PS treatments (fibrous rot).

However, the survival of the fungus inside the trunk, over years and at least the first 9 nine months, necessary time to observe the development of agarwood, has to be checked. At that time, we do not develop a method to assess fungal survival. We just note visual and olfactory indications at harvest. These indications were particularly clear at harvest for fibrous rot fungi with visible mycelium and strong “fungal” smell and unclear with cubic rot. Poorer survival of cubic rot strains along time could be responsible for the difference observed in the quality of essential oils.

3.3 Overall analysis of all the samples

A Principal Components Analysis (PCA) of the data for all the samples (Fig. 7) showed that the reference samples from Laos, agarwood and white wood, were furthest apart on axis 1, which represented the compounds typical of agarwood, such as eudesmol, jinkho-eremol, agarospirol and valerianol; the composition of the essential oil from the agarwood differed from that of the white wood through the presence of those compounds, and the much higher concentration of palmitic acid in white wood.

For the essential oils from the 4 treatments plus 2 controls, they are distributed between white wood and agarwood. Samples from PS and GR treatments are closer to the agarwood than those from the AV and PP treatments that are closer to the white wood. According to this PCA analysis, the samples still differ markedly from agarwood. But, our future studies will check the evolution of the chemical composition over nine months.

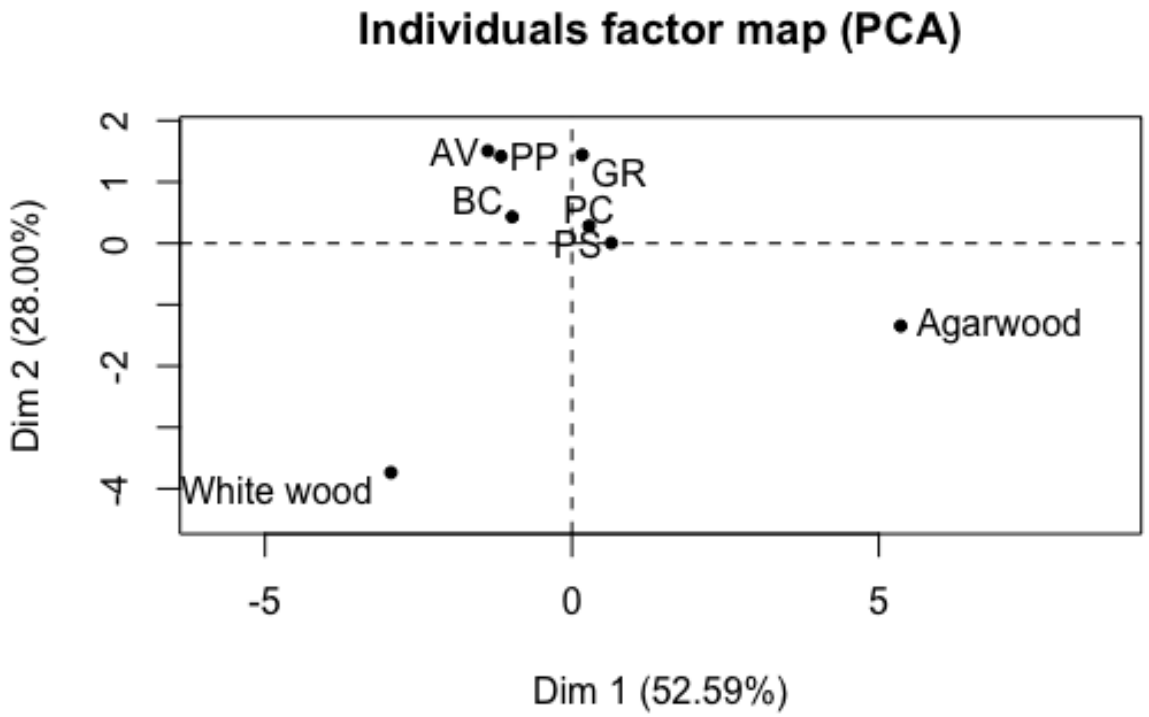

Figure 7: Principal Components Analysis (PCA) of sample distribution according to chemical composition. The PCA was carried out with the R software FactoMineR package. Axis 1 is constructed (89%) based on the variables 10-epi-γ-eudesmol, jinkoh-eremol, agarospirol, valerianol, dehydrojinkoh-eremol, γ-eudesmol. Axis 2 is constructed (78%) based on the variables 4-phenylbutan-2-one, palmitic acid and rotundone. *Antrodia vaillantii* (AV), *Poria placenta* (PP), *Pycnoporus sanguineus* (PS), *Ganoderma resinaceum* (GR), Pine Control (PC), Beech Control (BC)

4. CONCLUSION

This study made it possible to obtain agarwood production in *Aquilaria* trees in French Guiana for the first time. More specifically, it showed that inoculating pure fungal strains by inserting fungus-colonized wood test-sticks into the trunk is a good way of inducing agarwood with a chemical composition which looks more like agarwood than white wood. 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 close vicinity of the plantation. We thus showed that white-rot fungi are the most efficient, and notably *Pycnoporus sanguineus* (PS), which are very common in the tropics and of course in Guiana. Indeed, when exposed to these fungi, the yield and volatile compound composition of the wood resulting from such treatment were the closest to the reference agarwood from Laos used for this study.

5. ACKNOWLEDGEMENTS

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