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Research Article

HISTOCHEMICAL STUDIES ON FUNGAL-INDUCED AGARWOOD

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ABSTRACT
This paper deals with a comparison of non-infected (normal) and infected parts of the agarwood (*Aquilaria malaccensis* Lamk. Family: Thymelaeaceae) from a histological and histochemical perspective. Although there is no difference in the structural aspects, the infected agarwood shows the presence of terpenoids, phenolics, phenolic-terpenoid complexes, lipid droplets and alkaloids and the absence of starch in the disintegrating cells of the included phloem, as well as in the adjacent cells of the wood, especially ray and axial parenchyma cells. We could notice two different fungi (in all the samples examined), that colonize the wounded region where the agarwood is formed, ramifying in the remains of the included phloem and adjacent wood cells both intercellulary and intracellulary. Since all the chemicals are deposited in the already dead cells of the wood and in the space formed by the disintegrating included phloem cells and in the cells of the wood adjacent to the hyphae and also these deposits are present in the fungal hyphae it is concluded that agarwood chemicals are the products of fungi that use the cell wall materials, including lignin, of the host cells to produce them. The other evidences to show that the fungi utilize the dead cells of the wood, especially their walls and starch grains, are the absence of nuclei in the cells of regions of agar formation, the demonstration of lignin degradation through the production of the enzyme laccase by the fungus (since no living host cell that can produce laccase is present in that region) and the actual histochemical demonstration of lignin depolymerization in the cells of the wood of infected region. Since no living cells are present, the host wood or included phloem cells are not likely to be involved in these biochemical reactions leading to agar wood production and only the fungi must have been involved.

Keywords: *Agarwood, Aquilaria malaccensis, Fungi, Included Phloem, Laccase, Lignin Depolymerization*

INTRODUCTION
Agarwood (from *Aquilaria malaccensis* Lamk. Family: Thymelaeaceae) is a highly priced forest product that is used in scent, fragrances (especially when burnt and as distillation products), medicines, aromatherapy and religious ceremonies of Buddhist, Hindu, Islamic and Chinese traditions (Liu *et al.*, 2013; Manohara 2013).

It is also a drug whose medicinal value is very varied: stimulant, digestive, sedative, antiallergic, aphrodisiac, carminative, neuroleptic and relieves vomiting, belching, cough, rheumatism, high fever, thyroid and lung cancer, mental disorders, eye and ear defects, skin disease and poisoning (Pant & Rastogi 1979, 1980; Bhandhari *et al.*, 1982; Natarajan *et al.*, 1983; Yang *et al.*, 1989; Okugawa *et al.*, 1993; Kim *et al.*, 1997a, b; Anonymous 2004).

Its aromatic properties make it as one of the costliest materials from time immemorial and these properties are essentially due to a complex mixture of chemical compounds present in it (Naef 2011; Chen *et al.*, 2012; Chu *et al.*, 2013): *P*-methoxycinnamic acids, agarotetrol, agarol, agarospirol, α and β

agarofuran, gmelofuran, dihydroagarofuran, 4-hydroxydihydroagarfuran, oxo–nor–agarofuran, cardinanes, eudesmanes, valencanes, eremophyllanes, guanines, prezizanes, vetispiranes, chromones, thialkaloid liriodenine and many other simple volatile aromatic compounds. There are 39 chromones in several of *Aquilaria* species of which 16 are unique to agarwood. In view of the above, the value of agarwood exported from Singapore alone each year is around 2000 was $ 1.2 billion (Hansen, 2000). The essential oils of this wood sell from $ 100/kg for the low quality to $ 30,000/kg for the best quality. Because of overexploitation of agarwood that essentially grows in Bangladesh, S. China, Indonesia, Malaysia, N.E. India, and parts of East Indies, it has become a vulnerable category of threatened plants as per IUCN Red Listed Taxa List made in 1998.

The normal wood of this species, which is white and soft, is without the aromatic compounds. The agarwood, induced in it by natural or artificial wounding and after getting infected with a host of fungi (around 15 fungi are so far recorded) and bacteria develops all the aroma (Nobuchi and Hamami 2008; Akter *et al.,* 2013).

The wounded and fungal infected wood becomes locally dark brownish or black colored. Because of the high economic value of agarwood many investigators have studied the structure of the normal wood and agarwood (Rao *et al.,* 1992; Nobuchi and Hamami 2008; Mohamed *et al.,* 2013), as well as the phytochemistry of the fragrant chemicals produced (Sathyanathan *et al.,* 2012). However, the actual source of the chemicals, i.e. whether produced by the fungi (and bacteria) that invade the wounded regions, or by the wood cells triggered by microbial infection or by both is a matter of debate for a long time (Ng *et al.,* 1997).

A few studies have claimed that artificial infection of the wood by the fungi induces the production of chemicals characteristic of agarwood (Cui *et al.,* 2013). A few have reported that the living wood parenchyma cells produce the aromatic materials (Mohamed *et al.,* 2013), while others have reported their production by the included phloem tissue strands that are embedded in the wood (Rao *et al.,* 1992); more modern researches that implicate the probable formation of secondary metabolites as the work of endophytes (fungi and bacteria) in most, if not, all plants (Sachin *et al.,* 2013), suggest that in agarwood also the invading fungi (and not really endophytes) might be responsible for the production of the aromatic chemicals since the normal wood has neither aroma nor fungal infection. Hence,

the present histochemical studies were carried out on the normal and agarwood in order to throw light on the above problem.

MATERIALS AND METHODS

Collection and Authentication of Samples

The dried material of agarwood was collected from Amguri (Sivasagar district) and Kakogen (Jorhat district), Assam, India by the second author.

Histology

Transverse sections, taken by using a razor blade, were stained with Toluidine blue O (TBO) in 0.05% in benzoate buffer (benzoic acid 0.25 g in 200ml water pH 4.4) (Krishnamurthy, 1988), washed with water, observed under a microscope (Olympus BX 41, Tokyo, Japan) and the photographic images were captured using a digital Olympus camera fixed with the microscope. The images were processed on Image Pro Express 6.0.

Histochemistry

Specimens were soaked in water and transverse sections were taken using sharp razor blades. The sections (at least 20 for each of the procedures) were stained using specific procedures mentioned by Krishnamurthy (1988) for localizing starch, lignin, tannins, and other phenolics and total lipids. Dragendorff reagent was used for localizing alkaloids (Yoder and Mahlberg, 1976), while terpenoids were localized using vanillin in acetic acid and perchloric acid (Abraham 1988) and antimony chloride (Hardman and Sofowora, 1972) methods.

Photographic images were captured as above. Histochemical localization of the enzyme laccase active during lignin degradation was done using the method of Li (1989).

RESULTS AND DISCUSSION

Results

Anatomy, Histochemistry and Histoenzymology

Non-Infected wood: Wood shows the presence of growth rings, represented by tangentially narrow and radially seriated 2-5 layers of thin walled, lignified fibers (Figure 1a). Wood is diffuse porous and pore is in singles or in radial multiples of 2 to 3. Single pore is almost circular, while in clusters pores are not circular but tangentially flattened. Some pores are smaller than the others. Vessels elements short with crowded side wall pitting. Perforation plate simple, mostly horizontal, occasionally oblique. Axial parenchyma is of the paratracheal scanty type (Figure 1b). Rays in transverse section 1-3 layer thick, more commonly single layered. Rays in tangential longitudinal sections (RLS) are uniseriate or very occasionally biseriate. Some uniseriate rays are biseriate in the body and uniseriate on the wings; mostly homocellular but occasionally heterocellular (Figures 1c, d). Fibers libriform, ray cells contain phenolic deposits around cell walls on the inside and not as distinct bodies. Starch grains very common in xylem parenchyma and included phloem cells, and one to few in each cell. Ray cells near the included phloem also contain starch grains (Figure 1e). TBO stains lignin of fibers and vessel elements bluish green. Phloroglucinol stains lignin to dark red color. Alkaloids could not be detected in non-infected wood. Included phloem present in patches, which are oval, ellipsoid or tangentially flattened. Most of these phloem patches die as one proceeds from periphery to center of the wood. Only some of the peripheral phloem patches may have living cells with nuclei. Similar is the case with axial and ray parenchyma cells although these cells as well as the phloem cells may retain the starch grains.

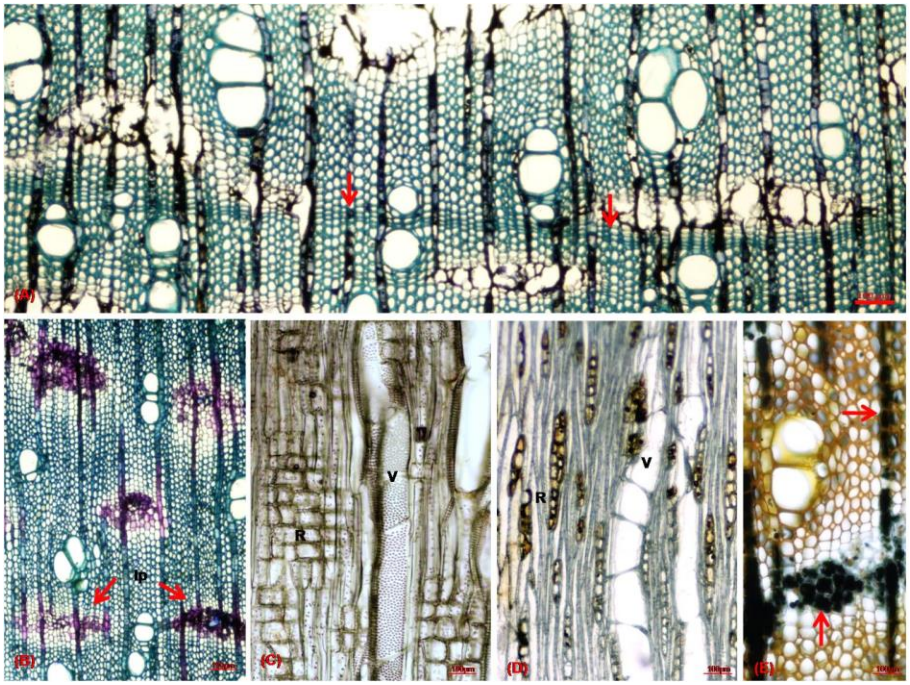

Figure 1: Histology and Histochemical Localization of Constituents of Normal Agarwood
A- T.S. of portion of normal wood stained with TBO showing annual growth ring (indicated in arrows);
B- T.S. of normal wood stained with TBO showing its diffuse porous nature and included phloem (IP);
C- R.L.S. of normal wood unstained showing ray (R) and vessel elements (V);
D- T.L.S. of normal wood showing rays (R) and vessel elements (V);
E- T.S. of normal wood showing presence of starch grains (stained with Lugol's Iodine) in ray and included phloem cells

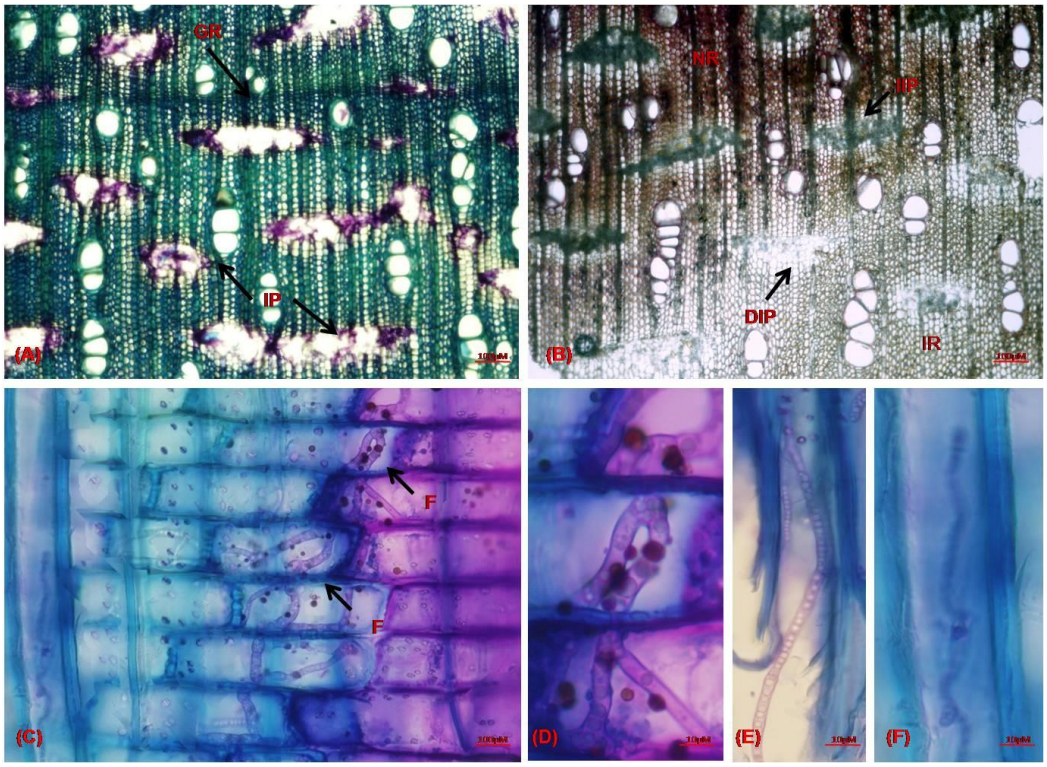

Figure 2: Structure and Fungal Colonization, Products of Fungal Metabolisms of Infected Agarwood

A- T.S. of a portion of wood stained with TBO to show highly disintegrated included phloem (IP) and the lignin stained to a green color due to depolymerisation. Note also the limit of the growth ring (GR);

B- Wood at the transition point between normal (NR) and infected region (IR) stained with Phloroglucinol-HCl (note the normal lignin stained to reddish brown while in the infected region lignin does not take the normal color; note also the intact included phloem (IIP) with starch grains in normal wood and disintegrating included phloem (DIP) without starch grains;

C- R.L.S. of wood stained with TBO to show the colonization of fungi (F) of at least two types (one stained bluish green and the other magenta) in the disintegrated phloem (stained purple violet) and the adjacent ray cells (stained bluish green);

D- A portion of phloem enlarged to show the fungi and the products of their metabolism both inside their hyphae and in the dead phloem cells;

E- A fiber cell enlarged to show the fungal hypha with serial septation of hyphae to produce asexual spores;

F- A similar region enlarged to indicate the penetration of fungal hyphae in the lumen

Infected Wood: All the agarwoods collected by us were noticed only in the dead wood regions of the stem. No living wood could be seen that has either the fungi or the agar chemicals. Infected wood is structurally almost similar to non-infected wood and is restricted to the core region of stem, with a thin zone of functional wood cells at the periphery (sapwood). The included phloem is disorganized and distorted and often only holes are seen in their original places of occurrence (Figure 2a). Many ray and axial parenchyma cells also show disorganization and distortion. The persisting axial and ray cells near phloem patches are fully packed with brownish black contents. The lignified of fibers and vessel elements are stained to a greenish color with TBO and to a dull/no color with Phloroglucinol (Figure 2b). Fungal hyphae ramify the cells of the wounded region. At least two types of fungi are noticed: One has branched mycelium with broad hyphae and stained to a magenta color with TBO and the other with narrow hyphae

stained bluish with TBO; the narrow ones often produce a series of spores (Figures 2c, d). They occupy the disintegrating ray and axial parenchyma and the phloem cells. The fungi with broader hyphae produce various kinds of substances which can be located inside the hyphae as well as just outside them, probably after being released by them. They also colonized the adjacent cells of the wood including fibers and vessel elements and get located in their lumen. Some of hyphae of the fungus with narrow mycelium repeatedly undergo transverse septation to form a series of asexual spores. It is this fungus that extensively spreads in the wood tissue (Figures 2 e, f).

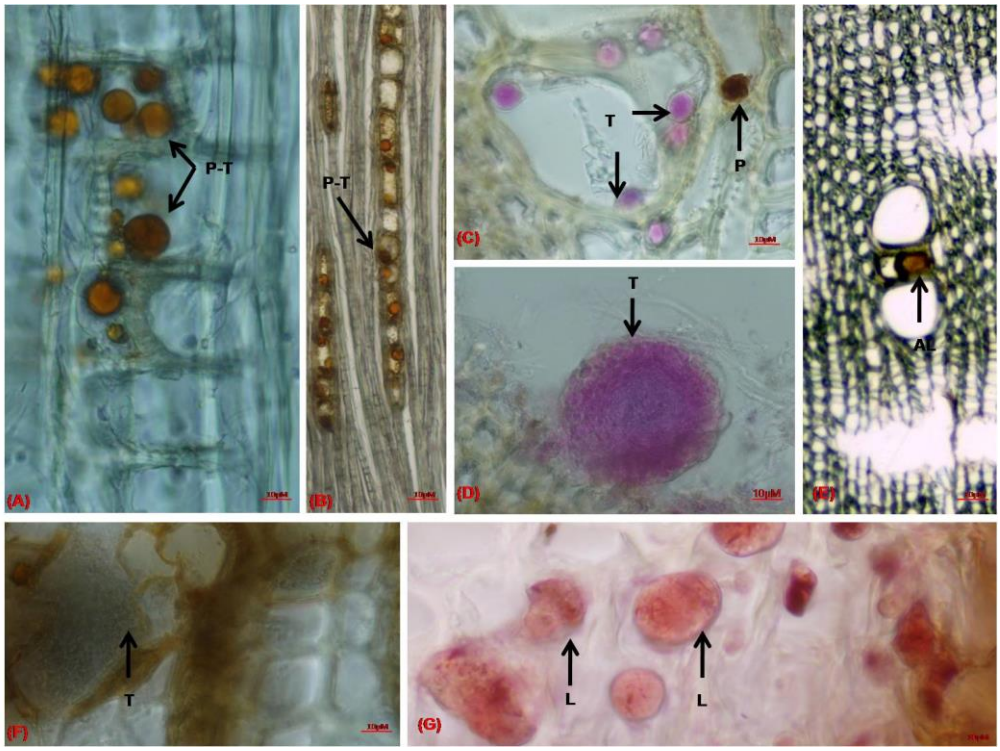

Figure 3: Histochemical Localization of Infected Agarwood
A & B- Portions of R.L.S. and T.L.S. of wood respectively showing ray cells filled with pheonol-terpenoid (P-T) complex deposits stained with vanillin- perchloric acid;
C- T.S. of a portion of wood stained with vanillin-perchloric acid show the pure terpenoid (T) deposits stained to a magenta colour and unstained phenols (p);
D- magnified image of c showing the clear view of terpenoid;
E- T.S. of a portion of wood stained with Dangendroff's reagent to show alkaloid (A) deposits;
F- T.S. of a portion of wood stained with Antimony chloride to show the grey color terpenoid (T) deposits;
G- T.S. of a portion of wood stained with Sudan III showing lipid droplets (L)

Terpenoids could be detected in two forms using vanillin-perchloric acid staining procedure. The first are brownish magenta-red deposits in which terpenoids form complexes with phenolic materials (as these also answer, histochemically, for phenolic substances) (Figures 3a, b), while the second are pure terpenoid bodies showing magenta coloration in the infected phloem region (Figures 3c, d) as well as in the adjacent parenchyma cells of the wood (especially ray cells). Terpenoids were also detected using antimony chloride method and the deposits are stained to a grey color. Terpenoid deposits could also be located in the fungal hyphae as well as just outside of them by both these histochemical procedures.
There are also exclusive deposits of phenolic materials, mostly amorphous, in the phloem cells and ray cells. Lipid droplets could also been located in the fungal-infected region as well as inside their hyphae.

The lipid droplets, phenolics and terpenoids account for the aroma produced in these infected regions of wood.

Starch is slowly degraded by the fungi and could not be detected in the fully infected region (Figures 3e-g); perhaps they are hydrolyzed. Alkaloids are also detected in the infected wood region.

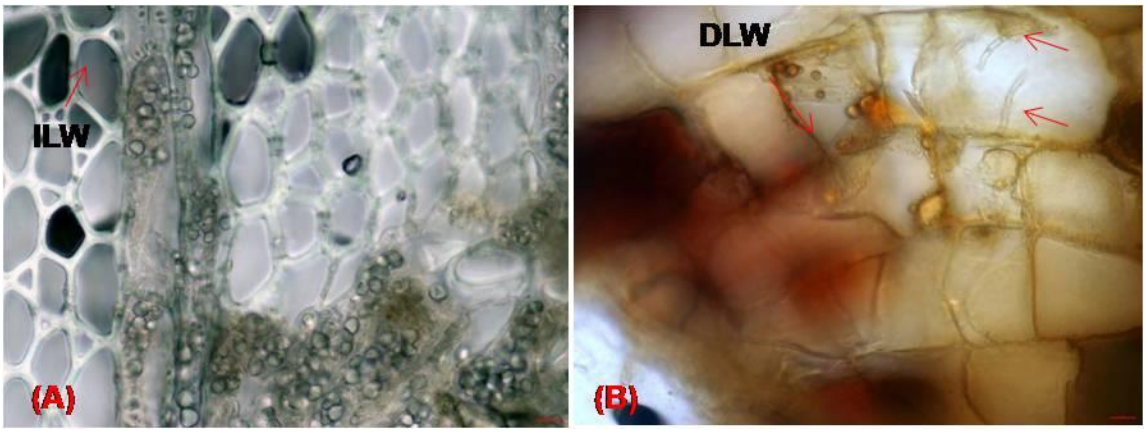

Figure 4: Histoenzymology Studies for Localization of Laccase Enzyme Activity on Lignin Cells Leads to Depolymerization

A- A portion of normal wood enlarged to show the absence of laccase enzyme activity and it shows the intact lignin wall (ILW) note also the absence of fungal hyphae.

B- A portion of the infected wood region showing the brown deposits in cells indicating the activity of the enzyme laccase leads to depolymerized lignin wall (DLW) note also the presence of fungal hyphae (arrow) which produce the laccase extracellularly which act on lignified walls and depolymerize them

Histochemical studies involving the localization of laccase, an enzyme involved in lignin degradation, demonstrated that the infected region containing dead cells showed the precipitated products (brown colored) of the enzyme and the thinning of the lignified wall (Figures 4a, b). This observation is substantiated by the poor staining by phloroglucinol.

Lignin depolymerization starts from the periphery of the included phloem patches and proceeds radially in all directions (Figures 5a, b).

The lignified secondary wall is degraded almost totally near the included phloem and stages in degradation could be noticed as one proceeds from the included phloem towards the xylem region (Figures 5 c-e). It is to be mentioned here that the spread of the fungus also starts in the included phloem and up on total degradation of the latter, it spreads to other regions of the wood.

Discussion

A comparison of normal wood with agarwood shows that the latter possesses the same structure. However, it differs from the former in the following respects: (i) fully degenerated included phloem patches with the adjoining axial and ray parenchyma cells also getting disintegrated to various extent; (ii) the presence of two species of fungi that differ in hyphal morphology; they colonize not only the intercellular spaces but also intracellularly in the disintegrating phloem and wood parenchyma cells; the fungal hyphae are also seen in the lumen of fibers and vessel elements as well as the intercellular spaces between them; (iii) lignin in the fungal-infected wood cells and nearby areas stains to a dark greenish color with TBO, in contrast to normal wood lignin, which stains to a more bluish tinge; this is due to depolymerization of lignin into phenolic oligomers and monomers which are stained to a more green color than blue color (Krishnamurthy 1999); this is also substantiated by the loss of reddish color in the walls of the lignified cells when stained with phloroglucinol; these results indicate that the fungi are involved in lignolytic activity since none of the host cells is living in the region and the only living

entities here are the fungi; (iv) one of the enzymes involved in lignin degradation, laccase, could be demonstrated histochemically in the infected but not in the non-infected regions of the wood; laccase in known to be produced extra-cellularly by lignolytic fungi; laccase could not have been produced by host cells in this region, since all cells are already dead; (v) large amount of terpenoid deposits is present in the infected region not only inside the fungal hyphae but also in the lumen of disintegrating cells of the phloem and ray and axial parenchyma as well as in the cavities created by fully disintegrated cells; these terpenoids often form complexes with phenolic materials; (vi) extensive amount of pure phenolic material is also present in the infected region indicating that the production of phenolics is greatly amplified by the lignolytic activity of fungal hyphae; (vii) presence of lipid droplets not only inside fungal hyphae but also outside of them; these perhaps are made of aromatic fatty acids; (viii) presence of alkaloid deposits also, although of a meager amount; (ix) total absence of starch grains, probably due to starch hydrolysis by the fungi.

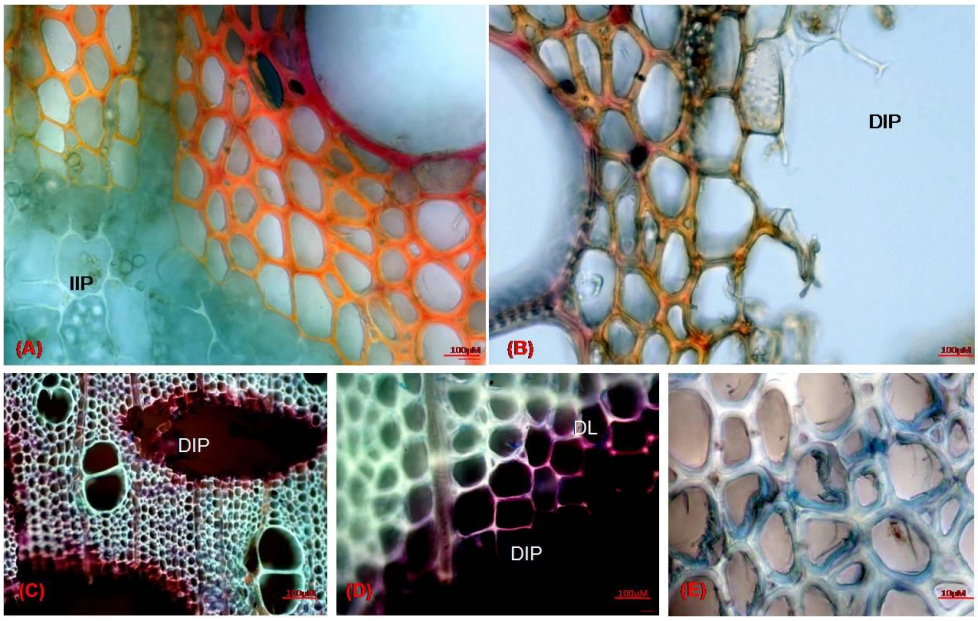

Figure 5: Histochemical Evidences Show the Difference in Non Infected and Infected Wood During the Depolymerization of Lignin in the Cell Wall
A- A portion of non infected wood stained with Phloroglucinol showing reddish colored lignified walls and the included phloem with starch grains stained to a bluish tinge;
B- A portion of infected wood stained with Phloroglucinol shows the depolymerization of lignin through the absence of reddish color and the absence of starch grains in the included phloem cells. Fluorescence images of the transverse section of the infected wood region stained with tryphan blue;
C- Shows full degradations of included phloem patch (DIP) replaced almost by an empty cavity;
D- Periphery of a degraded phloem patch bordered by xylem fibers. Note the almost fully degraded lignified walls (DL) in fibers that have become very thin;
E- A region of wood a little away from the degraded phloem patch to show the degradation of lignified walls in fibers

All the above changes are exclusive to the infected wood, where the only new-comers are the fungi. Many of the chemical components characteristic of agarwood have been histochemically located in the disorganizing tissues as well as in the fungal hyphae. Since the region of wood that gets infected is devoid of living cells even in the axial and ray parenchyma and included phloem, it is impossible for these cells to produce the chemical changes that characterize the agarwood. Hence, it is likely that the fungi utilize

these cells, especially their cell walls and stored starch, for producing all the chemicals of agarwood. This is substantiated by the fact that atleast some agarwood chemicals could also be histochemically demonstrated in the fungal hyphae.

It may be concluded that the aroma of agarwood and the chemicals responsible for this aroma are the products of fungal metabolism in which the host cell wall materials are used as raw materials. The aroma is due to terpenoids, the terpenoid-phenol complexes, the abundant phenolic deposits, lipid droplets, and perhaps the lone alkaloid reported in agarwood in the literature.

The variety of phenols already reported in agarwood might have been derived not only from the phenolic substances already present in the axial and ray parenchyma cells but also from depolymerization of lignin of wood fibers and vessel elements.

The metabolic by-products of starch hydrolysis carried but by the fungi may be involved in the synthesis of aromatic compounds in the infected region. The present histochemical study indicates that the whole aromatic property of agar wood is exclusively due to fungal metabolism in which the host acts merely as a source of raw materials. However, more studies need to be done through pure culture of involved fungi and see whether they produce all the aromatics independently or only in presence of the host wood materials in the culture medium.

Summary

Agarwood is an expensive wood of commerce that is formed in the species of *Aquilaria* as a result of wounding and subsequent infection of fungi. (1) This paper throws light on whether the agarwood chemicals are formed by the host plant, the infecting fungi or by both. (2) A detailed comparison of normal and agar woods through anatomical, histochemical and histoenzymological procedures was made. (3) There is no basic structural difference between the two woods but agarwood showed terpenoids, phenolics, phenolic-terpenoid complexes, lipid droplets and alkaloids and the absence of starch in the cells and in the disintegrated spaces between cells. Two fungi that differ in hyphal morphology colonize and ramify (intercellulary and intracellulary) in the agarwood region. These fungi also showed some of the agarwood chemicals not only inside them but immediately adjacent to their hyphae. (4) Agarwood chemicals are products of fungi that exploit the host cell wall, including lignin, and of starch grains. The host cells are obviously not involved in the synthesis of agar chemicals since they are dead and enucleate where agar formation takes place. Since the only living component in this region are fungi there are likely to be involved in agar wood production and not the host cells which are already dead.

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