

# A Novel Agarwood Resin Inducement Method Using Mycotoxins of Selected Fungal Species

**Upul Subasinghe** (✉ [upuls@sjp.ac.lk](mailto:upuls@sjp.ac.lk))

University of Sri Jayewardenepura <https://orcid.org/0000-0002-4989-1428>

**R.A.P. Malithi**

Centre for Forestry and Environment, University of Sri Jayewardenepura

**S.W. Withanage**

Centre for Forestry and Environment, University of Sri Jayewardenepura

**T.H.P.S. Fernando**

Rubber Research Institute

**D.S. Hettiarachchi**

Edith Cowan University School of Science

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## Abstract

Agarwood is a dark, fragrant, valuable resinous wood produced in *Aquilaria* and *Gyrinops* tree species in the family *Thymelaeaceae* to protect internal tissues from microbial infections. *Aspergillus niger* and *Fusarium solani* are well known to induce agarwood resin formation. This study demonstrated for the first time that agarwood resin formation can be induced by the mycotoxins of *A. niger* and *F. solani*. Different volumes of mycotoxins extracted from the ASP-U strain (USJCC-0059) of *A. niger* and the FUS-U strain (USJCC-0060) of *F. solani* were inoculated into *A. crassna* trees at 1 m intervals. The impacts of the inoculations were observed through resin content and constituent analysis at 7 months after inoculation. Resin production due to the mycotoxins of ASP-U and FUS-U was restricted to  $\pm 20$  cm and  $\pm 60$  cm, respectively, from the inoculation point. Color of the resinous agarwood varied from yellowish-brown to black. The differences in resin content formation due to the different inoculant volumes of ASP-U and FUS-U were statistically significant, and the average resin contents varied from 0.89%-4.44% and 1.24%-9.20%, respectively. GC-MS analysis detected 27 constituents responsible for the characteristic aroma of agarwood resin. Among them, phenyl butanone, agarofuran, agarospirol,  $\beta$ -caryophyllene, alloaromadendrene oxide and (-)guaiene-1(10),11-diene-15-ol were found in all extracts. These are common in commercially available agarwood induced by fungal species. Hence, we demonstrated that the mycotoxins of specific fungal strains can be used for agarwood production. Therefore, chemically synthesized mycotoxins can be used at commercial-scale, eliminating the difficulties of maintaining live fungal cultures for agarwood production.

## Introduction

Agarwood is the term for the fragrant, resinous heartwood derived from infected tree species of the *Thymelaeaceae* family. The *Aquilaria* and *Gyrinops* genera are commonly used to establish forest plantations for agarwood production, while the other two genera that form agarwood, *Aetoxylon* and *Gonystylus*, are not used at the commercial scale. *Aquilaria* species are abundant from northern India to China, Vietnam and Indonesia. *Gyrinops* species are distributed mainly in eastern Indonesia and Sri Lanka (Subasinghe et al., 2012).

Agarwood has a long history of use as incense and perfume and in traditional medicine (Blanchette, 2003; Subasinghe and Hettiarachchi, 2013) in the Asian and Middle Eastern regions. Agarwood resin is commonly used in Chinese medicine as an analgesic, to treat stomach problems and as an expectorant. It has also been used as a key perfumery agent, known as agar or oud, in the Middle East and India. However, agarwood has now gained popularity among Western perfumery and other cosmetic industries (Tajuddin et al., 2016).

Volatile compounds such as terpenoids, volatile organic constituents and nonvolatile compounds, mainly 2(2-phenylethyl) chromones and fatty acids, are commonly found in agarwood resins. However, the characteristics of agarwood resins vary depending on the region of origin, botanical species, tree age and method of induction of agarwood resin production (Agris, 2005), and these factors determine the market price (Wetwitayaklung et al., 2009).

Agarwood resins are produced as defense mechanisms by the above mentioned genera to mimic internal tissue damage and to activate the wound healing process. Fungi growing in wounded trees have been found to be the main cause of agarwood resin formation (Bhuiyan et al., 2008; Hitihamu et al., 2014). Studies conducted to determine the effectiveness of inoculation with certain fungi for forming agarwood in *Aquilaria* species (Mohamed et al., 2014; Faizal et al., 2017; Subasinghe et al., 2019) found several fungal species, viz., *Fusarium falciforme*, *F. oxysporum*, *F. ambrosium*, *F. solani* (Nobuchi, 2016), *Pythium*, *Botryodiplodia*, *Penicillium*, *Rhizoctonia*, *Acremonium*, *Cystosphaera*, *Thielaviopsis*, *Libertella*, *Trichoderma* and *Scytalidium* (Subasinghe et al., 2019), growing in infected agarwood. However, the natural process of agarwood formation due to these fungal species occurs rarely and slowly. Therefore, high-quality, artificially formed agarwood is popular on the market because the demand for agarwood cannot be met by naturally formed agarwood. For this reason, the inoculation of agarwood-producing trees with suitable fungal species is a common practice at the commercial scale, and this process takes approximately 3-6 years to form agarwood (Bhuiyan, 2008).

Mycotoxins formed as secondary metabolites in certain fungi are capable of causing diseases and death in humans and other animals (Brzonkalik et al., 2011; Ismaiel et al., 2015). Fungal species such as *Aspergillus*, *Chaetomium*, *Fusarium*, *Lasiodiplodia* and *Penicillium* are known to cause pathogenesis in living organisms (Sangareswari et al., 2016) through their enzymes, which are involved in the pathogenic process. Therefore, it can be assumed that agarwood formation can be induced by the mycotoxins formed as secondary metabolites in certain fungal species.

Aflatoxins, citrinin, ergot alkaloids, fumonisins, ochratoxins, patulin, trichothecenes and zearalenone are the common mycotoxin classes produced by different fungal species (Dalie et al., 2010; Alimentarius, 2011). It has also been found that mycotoxins produced by different strains of the same fungal species vary in their composition, characteristics and functions (Hitokoto et al., 1980).

Aflatoxin is the most widespread mycotoxin and is produced by a polyketide pathway by *Aspergillus* species (Klich et al., 2000). These mycotoxins occur in several chemical forms, viz., aflatoxin B1, B2, G1, G2, and M1. Citrinin, a polyketide mycotoxin, is produced mainly by *Aspergillus* and *Penicillium* species. Ergot alkaloids are indole alkaloids derived from a tetracyclic ergoline ring system that are produced mainly by *Claviceps* species (Bennett and Bentley, 1999). Fumonisins are structural mycotoxins produced by *Fusarium* species (Bottone et al., 1998). Among them, fumonisin B1 is considered to be the most abundant and the most toxic (Sweeney et al., 2000). Unlike other mycotoxins, fumonisins are highly water soluble and do not bear aromatic structures or unique chromophores that facilitate analytical detection (Manthey et al., 2004).

Studies have revealed that a variety of fungal species, including *Aspergillus carbonarius*, *A. niger* and *Penicillium verrucosum* (Bannett, 1987), are able to produce ochratoxins. Ochratoxin A was classified as the most toxic mycotoxin in this group. Patulin is also produced by *Penicillium* species (Trucksess and Tang 2001), and trichothecenes are produced mainly by species of *Fusarium*, *Myrothecium*, *Phomopsis*, *Stachybotrys*, *Trichoderma*, and *Trichothecium* (Cole and Cox, 1981; Chu, 1998).

A previous study conducted at the University of Sri Jayewardenepura, Sri Lanka, confirmed that both *Aspergillus niger* and *Fusarium solani* are capable of forming agarwood in *Gyriopsis walla* (Subasinghe et al., 2019). Dias (2016) optimized a liquid culture media of the above two species for use as a successful inoculum for agarwood resin formation. Withanage (2017) found that some strains of the same fungal species induced agarwood formation significantly better than other strains.

Based on the above findings, the current study was designed to identify the ability of selected mycotoxins to form agarwood and the quantitative and qualitative differences among the produced resins. For this reason, fungal species and strains previously used by us to induce agarwood resin formation in *Aquilaria crassna* and *Gyriopsis walla* were used. Based on our literature survey, this is the first study of its kind conducted to investigate mycotoxin-induced agarwood resin production.

## Materials And Methods

### Selection of the fungal strains

Withanage (2017) studied the differences in the agarwood resin contents and constituents formed in *Gyriopsis walla* through artificial inoculation with two strains of *Aspergillus niger* and two strains of *Fusarium solani*. Those findings demonstrated that one strain of each fungal species, viz. ASP-U of *A. niger* and FUS-U of *F. solani*, was more capable of forming agarwood in *G. walla* than the others. Therefore, the same two strains, viz. ASP-U and FUS-U, were selected to identify the ability of their mycotoxins to form agarwood resins in *Aquilaria crassna* trees in the present study.

The results of DNA sequencing of those strains were analyzed using NCBI database of National Center for Biotechnology Information of United States National Library of Medicine to confirm the strains (ASP-U: accession no. AY373852.1; FUS-U accession no. MT107081.1). Both strains were deposited at USJCC (University of Sri Jayewardenepura Culture Collection) under the codes of USJCC-0059 (ASP-U) and USJCC-0060 (FUS-U).

### Preparation of plate cultures of selected fungi

Pure cultures of the ASP-U strain of *A. niger* and the FUS-U strain of *F. solani* maintained at the Department of Forestry and Environmental Science, University of Sri Jayewardenepura, Sri Lanka, were separately transferred to sterilized potato dextrose agar (PDA) media autoclaved at 121°C under a steam pressure of 1.055 kg cm<sup>-2</sup>. After cooling to room temperature, gentamicin was added as an antibiotic to prevent bacterial growth. Those cultures were then incubated at 25°C in a culture room until optimum growth was attained.

### Extraction of mycotoxins

After reaching the optimum growth in PDA medium, three agar plugs of the ASP-U strain were transferred to Czapek-Dox broth (CDB) medium and incubated at room temperature for 14 days. Three agar plugs of the FUS-U strain were transferred to yeast extract glucose broth (YEGB) medium and incubated for 10 days (Withanage, 2017). Both media were then filtered using Whatman No. 1 filter papers and a series of ceramic filters of descending pore sizes. The filtrate was finally collected by filtering through 0.45 µm membrane filter discs to remove all microbial cells.

### Confirmation of toxicity of the extracts

The strength of the mycotoxins produced by the selected strains was first assessed via a bioassay using *A. crassna* leaves. Six drops of 20 µl of each filtrate were placed at six positions on *A. crassna* leaves kept in a humid chamber. Then, the leaf tissue under the drop of filtrate was slightly damaged using a sterilized needle. Sterilized liquid media were used as controls. Six leaves were used for each strain. The lesion diameter under each drop was measured in two perpendicular directions after 48 hours.

### Production of mycotoxins for the inoculations

After confirming the toxicity of the mycotoxins produced by both fungal strains using the above method, a 2 L volume of mycotoxin was separately produced using ASP-U in CDB medium and FUS-U in YEGB medium. The filtrates were concentrated five times by freeze-drying at -40°C under vacuum pressure.

### Inoculation into live *Aquilaria crassna* trees

A ten-year-old *A. crassna* plantation located in Mathugama, Kalutara district, Sri Lanka, was selected for the inoculation experiments. The diameter of the selected *A. crassna* trees varied from 17.0 to 21.0 cm, and their height varied from 8.5 to 11.0 m. Inoculations of 25, 50 or 100 ml were performed on the selected trees, with three inoculation replicates for each strain. The first inoculation was made on two opposite sides of the stem at 1 m above the ground. The second and third inoculations were made at 1 m intervals from the first inoculation point. Inoculations of only the medium were performed as controls, with three replicates.

Inoculation holes were made at the selected points on the stem to up to 1/3<sup>rd</sup> of the tree diameter using a 16 mm electric drill powered by a portable generator. Then, a brass connector was inserted into the hole, and a 1.5 m long, 16 mm diameter transparent rubber tube was attached to the brass connector. The selected mycotoxin volume was then carefully poured into the tube, and the top end of the tube was attached to the stem with a rubber strap. After the tree absorbed the entire volume of toxins, the tubes and connectors were removed, and the holes were sealed using sterilized, seasoned clay.

### Sample collection

All *A. crassna* trees were cut down seven months after inoculation. Each tree was then cut into 20 cm sections above and below the inoculation points. These sections were cleaned, and the areas of the cross-section and of agarwood formation were calculated. Then, the agarwood tissues were carefully removed, air-

dried for 72 hours and stored in sealed polythene bags. The colors of the resinous tissues and extracted resins were identified by a Munsell soil color chart. The strength of the tissues was determined by bending the wood with both hands until it broke.

#### **Analysis of the resin content**

Resin content analysis was performed by the solvent extraction method with three replicates. One gram of resinous agarwood tissue was reduced in size with a sharp edge cutter and placed in a 100 ml conical flask, and 10 ml of dichloromethane was added. The flask was placed on a mechanical shaker operated at 100 rpm, and the extract was collected after 12 hours. This was repeated twice, and the total extract was collected in amber-colored glass vials of known weight. The vials were kept at room temperature to evaporate the dichloromethane. Then, the weight of the vials with the resin extract was measured to calculate the weight of the resin (Subasinghe and Hettiarachchi, 2013).

#### **Analysis of the resin constituents**

The solid resin extract was weighed and dissolved in anhydrous acetone to make a  $10 \mu\text{g ml}^{-1}$  solution. Constituent analysis was conducted by a gas chromatograph-mass spectrometer (GC 7890 MSD 5975 Agilent Technologies®, USA) equipped with an HP-5 MS (Agilent Technologies®, USA) 5% phenyl methyl siloxane capillary column ( $30 \text{ m} \times 0.25 \text{ mm} \times 0.25 \mu\text{m}$ ). The oven temperature was increased from  $50^\circ\text{C}$  to  $220^\circ\text{C}$  at  $5^\circ\text{C min}^{-1}$  and held for 10 min at  $220^\circ\text{C}$  while keeping the injector temperature at  $250^\circ\text{C}$ . Helium, the carrier gas, was kept at a flow rate of  $2 \text{ mL min}^{-1}$ . The interface temperature was set to  $250^\circ\text{C}$ . The split ratio was set to 1:10, and  $0.1 \mu\text{L}$  resin solution was injected into the column using the autosampler. The constituents were quantified by the area normalization method using ChemStation® software. Mass spectra were recorded at 70 eV, and the mass range was considered from the mass-to-charge ratio ( $m/z$ ) with 33 to 550 atomic mass units. Constituents were identified by mass fragmentation pattern qualification based on the National Institute of Standards and Technology 2008 Library, which was further verified by published data on agarwood (Subasinghe and Hettiarachchi, 2013).

## **Results**

#### **Toxicity of the extracted mycotoxins from the fungal strains**

The average lesion diameters caused by the extracted mycotoxins of the ASP-U and FUS-U fungal strains were  $0.73 \pm 0.05 \text{ cm}$  and  $0.95 \pm 0.03 \text{ cm}$ , respectively. The control had a lesion diameter of  $0.10 \text{ cm}$ , which was the initial size of the damage caused by the needle when the toxin was introduced into the leaves. This confirmed that the mycotoxins produced by both strains are capable of causing damage to living plant tissues.

#### **Agarwood resin formation by mycotoxins**

Agarwood formation was clearly visible in *A. crassna* cross-sections at 7 months after inoculation (Fig. 1). The color of the resinous agarwood tissue samples varied from yellowish brown to black, while the color of the resins extracted from those tissues varied from pale yellow to dark yellowish brown (Table 1). However, it was not possible to establish a relationship between the resin tissue color and strength and the resin extract color.

Table 1 Variation in the strength and color of resinous agarwood tissue and agarwood extracts.

Strain	Volume (ml)	Height (cm)	Tissue strength	Tissue color	Extract color
FUS-U	25	-80	Strong	Dark yellowish brown	Pale yellow
		-60	Strong	Very dark gray	Pale yellow
		-40	Moderately strong	Very dark gray	Pale yellow
		-20	Strong	Very dark gray	Pale yellow
		0	Moderately strong	Very dark gray	Yellow
		+20	Strong	Dark gray	Pale yellow
		+40	Strong	Black	Pale yellow
		+60	Strong	Black	Pale yellow
FUS-U	50	-60	Strong	Dark yellowish brown	Dark yellowish brown
		-40	Moderately strong	Dark yellowish brown	Dark yellowish brown
		-20	Weak	Dark gray	Pale yellow
		0	Weak	Black	Dark yellowish brown
		+20	Weak	Dark yellowish brown	Dark yellowish brown
		+40	Strong	Dark yellowish brown	Yellow
		+60	Moderately strong	Very dark grayish brown	Dark yellowish brown
FUS-U	100	-20	Strong	Yellowish brown	Pale yellow
		0	Weak	Black	Dark yellowish brown
		+20	Moderately strong	Very dark gray	Dark yellowish brown
		+40	Moderately strong	Dark yellowish brown	Pale yellow
ASP-U	25	-20	Moderately strong	Gray	Yellow
		0	Weak	Very dark grayish brown	Yellow
		+20	Weak	Yellowish brown	Pale yellow
ASP-U	50	-20	Weak	Dark grayish brown	Yellow
		0	Moderately strong	Gray	Yellow
		+20	Moderately strong	Dark gray	Yellow
ASP-U	100	-20	Moderately strong	Gray	Pale yellow
		0	Strong	Brown	Dark yellowish brown
Control		0	Moderately strong	Gray	Pale yellow
		+20	Moderately strong	Dark grayish brown	Pale yellow

0=inoculation point; +=above the inoculation point; -=below the inoculation point.

Agarwood resin formation was restricted to within  $\pm 20$  cm of the inoculation point in the trees inoculated with mycotoxins of the ASP-U strain (Fig. 2). Resin formation was not observed at +20 cm even in the trees inoculated with 100 ml of mycotoxin of the same strain. This could be due to the destruction of cell structure by the large volume of toxins, which would not allow adequate time to transport the toxin via the xylem tissues. However, the resinous area grew to  $\pm 60$  cm from the inoculation point in the trees inoculated with the mycotoxins of the FUS-U strain (Fig. 3). The largest resinous area formed due to the mycotoxins of FUS-U was observed at the inoculation point, and the resinous area gradually decreased in size along the vertical axis from that point. Similar to the resinous tissues in trees inoculated with ASP-U mycotoxins, resinous tissues in trees inoculated with 100 ml of FUS-U strain mycotoxins were not observed at the -40 cm, -60 cm and +60 cm points (Fig. 3).

The resin content formed due to inoculation with 100 ml of FUS-U mycotoxin at the inoculation point was significantly higher than that formed due to inoculation with the ASP-U strain mycotoxin ( $T=-10.77$ ;  $p=0002$ ). The resin contents formed by the mycotoxins of the two strains were not significantly different between any other inoculation volumes.

The average agarwood resin content resulting from the mycotoxin of the ASP-U fungal strain varied from 0.89% to 4.44% (Fig. 4), and that resulting from the FUS-U strain mycotoxin varied from 1.24% to 9.20% (Fig. 5). The average resin contents of the individual trees inoculated with mycotoxins of the ASP-U strain varied from  $0.89\pm 0.35\%$  to  $3.24\pm 0.86\%$  for a volume of 25 ml, from  $0.89\pm 0.36\%$  to  $4.44\pm 1.50\%$  for 50 ml and from  $1.91\pm 0.10\%$  to  $2.20\pm 0.46\%$  for 100 ml.

The agarwood resin contents extracted from the trees inoculated with mycotoxins of the ASP-U strain at 20 cm from the inoculation point varied significantly ( $F=3.18$ ,  $p=0.026$ ). The resin content formed at -20 cm from the inoculation point by 50 ml was significantly higher than that formed at +20 cm and -20 cm by 50 ml and 25 ml, respectively. However, the resin contents were not significantly different among other distances and treatments. Furthermore, a relationship between the resinous area and resin content could not be established.

The agarwood resin contents of the individual trees inoculated with different volumes of the FUS-U strain mycotoxin varied from  $1.61\pm 0.18\%$  to  $3.74\pm 0.11\%$  for 25 ml, from  $1.24\pm 0.29\%$  to  $8.71\pm 0.60\%$  for 50 ml and from  $1.62\pm 0.65\%$  to  $9.20\pm 0.52\%$  for 100 ml (Fig. 5).

The resin contents formed by different inoculation volumes of the FUS-U strain mycotoxin were significantly different ( $F=17.27$ ;  $p=0.000$ ). The resin contents formed by 100 ml of mycotoxin at 20 cm above the inoculation point (+20 cm) and by 50 ml at +40 cm were the highest and were significantly higher than the resin contents formed by other volumes at different points, except 50 ml at -40 cm (Fig. 5).

#### **Variation in agarwood resin constituents formed due to mycotoxins**

Gas chromatography-mass spectrometry analysis of the agarwood resins formed due to the mycotoxins of the ASP-U and FUS-U strains confirmed the availability of several constituents that are common to commercially available agarwood produced in *Aquilaria* trees. Of these constituents, phenyl butanone, agarofuran, agarospirol,  $\beta$ -caryophyllene, alloaromadendrene oxide and (-)guaiene-1(10),11-diene-15-ol were found in all resinous samples formed due to the mycotoxins of both fungal strains (Table 2,3). Although cloveone was common to all resin samples formed due to the ASP-U strain (Table 2), it was not detected in some of the resin samples formed due to the FUS-U strain (Table 3). The 2-(2-phenyl)-chromone derivative is another common constituent of commercially available agarwood resins. However, it was not found in some of the agarwood samples tested in this study. Baimuxinic acid was the least common recorded constituent, as it was found only in one sample formed by the ASP-U strain and in three samples formed by the FUS-U strain. Although  $\beta$ -ionone (isomers) and octadecenoic acid were found only in two samples formed by the ASP-U strain (Table 2), both of these constituents were commonly observed in the resins formed by the mycotoxins of the FUS-U strain (Table 4). Octadec-11-enoic acid was found only in six samples of agarwood resins formed by the FUS-U strain, while 2(1H)-naphthalenone, 3,5,6,7,8,8a-hexahydro-4,8a-dimethyl-6-(1-methylethenyl) and tetradecanoate were found in seven and eight samples, respectively (Table 3).

Table 2 Presence of chemical constituents in agarwood resins formed by the mycotoxins of the ASP-U strain.

Constituent	(-)20 cm			0			+20 cm	
	25	50	100	25	50	100	25	50
Phenyl butanone	0.93	1.08	1.31	0.85	1.32	1.05	2.10	0.93
Agarofuran	3.17	4.36	3.47	4.56	2.18	3.66	4.01	3.23
Agarospinol	ND	ND	2.22	1.45	0.98	1.12	3.25	ND
Baimuxinol	ND	1.60	ND	1.89	1.01	0.84	0.40	ND
$\beta$ -Guaiene	ND	0.82	0.91	0.57	ND	1.24	ND	ND
Baimuxinic acid	ND	1.63	ND	ND	ND	ND	ND	ND
$\delta$ -Selinene	ND	ND	0.90	ND	ND	3.11	2.43	4.35
$\delta$ -Cadinane	ND	ND	ND	4.57	2.25	3.64	5.91	ND
Decahydro-2,2,4,8,-tetramethyl-4,8-Methanoazulen-9-ol	2.23	3.11	ND	3.96	4.01	1.87	ND	2.30
2(1H)-Naphthalenone, 3,5,6,7,8,8a-hexahydro-4,8a-dimethyl-6-(1-methylethenyl)	ND	ND	0.58	ND	0.87	0.24	ND	ND
$\beta$ -Ionone (isomers)	ND	ND	1.74	ND	ND	ND	0.70	ND
Cloveone	5.50	0.68	5.61	6.47	2.87	5.44	2.45	7.88
Tetradecanoate	7.80	ND	8.54	4.24	8.74	ND	ND	11.10
3,5,6,7,8,8a-Hexahydro-4,8a-dimethyl-6-(1-methylethenyl)2(1H) Naphthalen5.63one	ND	ND	ND	0.82	ND	ND	1.04	ND
$\alpha$ -Guaiene	8.50	3.24	0.77	4.65	5.68	5.12	5.20	4.33
$\beta$ -Caryophyllene	4.18	5.83	4.45	8.64	6.55	3.48	6.25	10.15
Alloaromadendrene oxide	10.20	5.60	4.59	3.65	2.87	4.11	3.12	11.80
2,4,6,7,8,8a-hexahydro-3,8-Dimethyl-4-(1-methylethylidene)-;(8S-cis)5(1H)-Azulenone	ND	ND	1.54	ND	1.02	ND	2.62	ND
(-)-Guaiene-1(10),11-diene-15-ol	5.30	2.43	6.80	5.43	7.14	5.10	4.38	5.30
4,4a,5,6,7,8-hexahydro-4a,5-Dimethyl-3-(1-methylethylidene)-(4ar-cis)2(3H)-Naphthalenone	ND	1.30	3.05	ND	0.98	1.621	1.57	ND
Spatulenol	1.66	2.09	ND	ND	3.04	ND	ND	3.76
Palmitate	0.53	ND	2.60	ND	0.46	1.65	2.18	0.30
Palmitic acid	1.70	ND	1.81	2.16	1.44	2.08	ND	1.81
2-(2-phenyl)-Chromone derivative	ND	0.90	0.64	1.54	0.62	1.09	4.66	0.62
Oleic acid	0.20	ND	0.48	ND	ND	0.22	1.04	0.40
Octadec-11-enoic acid	ND	ND	ND	2.98	ND	ND	11.72	ND
Octadecenoic acid	ND	2.16	ND	1.36	ND	ND	ND	ND

ND=Not Detected

Table 3 Presence of chemical constituents in agarwood resins formed by the mycotoxins of the FUS-U strain.

Constituent	(-)60 cm		(-)40 cm		(-)20 cm			0			20 cm			40 cm			60	
	25	50	25	50	25	50	100	25	50	100	25	50	100	25	50	100	25	50
Phenyl butanone	1.43	1.22	1.10	1.06	1.13	1.13	0.47	0.98	1.57	1.38	1.23	1.02	1.07	1.56	1.06	1.17	1.2	1.2
Agarofuran	0.48	0.75	0.98	9.46	0.84	5.35	1.85	1.47	2.26	2.08	1.21	2.38	1.92	0.89	8.96	3.86	1.7	1.7
Agarospinol	0.58	0.49	0.69	ND	0.42	0.28	0.31	0.57	1.01	ND	0.94	0.66	ND	0.51	0.52	1.50	0.5	0.5
Baimuxinol	0.53	0.68	0.59	ND	0.32	ND	ND	0.34	0.66	0.41	0.28	0.34	0.41	0.27	ND	0.40	0.5	0.5
β-Guaiene	ND	0.08	0.11	ND	ND	0.13	ND	0.24	0.10	0.17	ND	0.45	0.08	ND	ND	ND	ND	ND
Baimuxinic acid	ND	ND	1.30	ND	ND	1.08	ND	ND	ND	ND	1.25	ND	ND	ND	ND	ND	ND	ND
δ-Selinene	1.65	2.01	ND	ND	1.43	1.51	ND	1.45	ND	1.31	2.25	3.14	3.25	2.48	ND	ND	3.6	3.6
δ-Cadinane	0.56	0.78	0.59	2.40	0.41	ND	ND	1.58	0.52	0.84	1.02	0.49	ND	0.82	ND	ND	0.6	0.6
Decahydro-2,2,4,8,- tetramethyl-4,8- Methanoazulen-9-ol	ND	1.64	ND	1.75	0.23	0.47	1.65	2.01	1.84	0.88	0.38	ND	0.34	0.12	1.75	1.50	ND	ND
2(1H)-Naphthalenone, 3,5,6,7,8,8a-hexahydro- 4,8a-dimethyl-6-(1- methylethenyl)	1.42	ND	1.56	ND	0.86	ND	ND	ND	ND	1.57	1.77	ND	ND	0.94	ND	ND	1.6	1.6
β-Ionone (isomers)	0.95	ND	4.65	ND	0.61	ND	ND	0.91	0.44	0.73	0.69	0.61	ND	0.81	ND	ND	4.6	4.6
Cloveone	0.56	1.85	ND	4.90	ND	2.61	4.20	ND	2.18	3.56	ND	2.68	3.34	ND	3.43	4.80	ND	ND
Tetradecanoate	0.56	ND	0.79	ND	0.41	ND	ND	ND	0.41	ND	1.04	ND	ND	0.85	6.10	ND	0.9	0.9
3,5,6,7,8,8a-Hexahydro- 4,8a-dimethyl-6-(1- methylethenyl) 2(1H)Naphthalen5.63one	ND	ND	1.20	ND	ND	0.84	1.24	1.87	0.84	ND	2.30	ND	1.17	1.56	ND	ND	ND	ND
α-Guaiene	4.51	3.58	ND	ND	4.45	3.11	ND	ND	2.87	ND	5.63	3.42	ND	ND	ND	ND	0.2	0.2
β-Caryophyllene	3.63	4.47	6.02	4.48	5.30	2.51	1.46	4.02	5.81	4.11	6.14	4.18	3.62	5.74	3.08	2.10	4.3	4.3
Alloaromadendrene oxide	1.11	1.98	6.18	7.13	0.89	3.28	5.23	1.64	1.05	1.47	0.96	1.96	1.44	2.68	6.36	2.30	1.7	1.7
2,4,6,7,8,8a-Hexahydro- 3,8-dimethyl-4-(1- methylethylidene)-,(8S- cis)5(1H)-Azulenone	ND	1.42	ND	9.00	0.62	0.35	0.84	0.41	0.87	0.25	0.21	5.11	4.38	0.25	8.42	7.45	0.1	0.1
(-)Guaiene-1(10),11- diene-15-ol	2.23	2.66	2.89	2.60	6.58	5.09	2.40	1.84	3.31	2.56	3.09	3.01	1.14	1.84	3.40	9.80	4.1	4.1
4,4a,5,6,7,8-Hexahydro- 4a,5-dimethyl-3-(1- methylethylidene)-(4ar- cis)2(3H)-Naphthalenone	3.02	ND	5.76	ND	ND	ND	0.60	ND	0.87	ND	0.35	ND	0.71	ND	0.50	ND	ND	ND
Spatulenol	ND	0.65	ND	0.93	ND	1.37	1.70	1.06	0.81	1.24	0.84	ND	ND	1.14	1.56	1.45	ND	ND
Palmitate	ND	0.85	0.70	0.25	1.55	ND	1.80	ND	ND	ND	ND	1.36	0.56	1.12	0.25	0.90	0.9	0.9
Palmitic acid	ND	1.41	ND	0.85	ND	2.42	3.45	1.44	ND	3.02	ND	0.92	ND	2.08	1.60	ND	1.4	1.4
2-(2-phenyl)-Chromone derivative	0.89	1.12	0.56	ND	ND	2.27	2.10	1.47	1.08	1.94	ND	1.54	2.43	ND	ND	4.05	ND	ND
Oleic acid	ND	2.13	ND	0.65	0.81	0.84	0.96	0.51	0.43	0.82	ND	ND	1.22	ND	ND	ND	ND	ND
Octadec-11-enoic acid	ND	1.51	ND	ND	0.98	ND	ND	ND	ND	1.17	ND	0.24	0.87	ND	ND	ND	ND	ND
Octadecenoic acid	ND	ND	ND	4.15	ND	ND	2.00	2.21	1.85	2.09	ND	ND	2.65	ND	4.30	ND	ND	ND

ND=Not Detected

## Discussion

The ability to artificially develop agarwood resin in *Aquilaria* species using fungi, especially *Fusarium* species, has been studied in the past (Bhuiyan et al., 2008; Mohamed et al., 2014, 2010; Sangareswari et al., 2016), and this ability using *A. niger* was proven recently (Hitihamu et al., 2014). *A. niger* and *F. solani* are also both known to produce several types of mycotoxin (Sweeney et al., 2000; Alimentarius, 2011) as secondary metabolites. These toxins are also capable of inducing a variety of physiological and cytological abnormalities in plants (Manthey et al., 2004; Warzecha et al., 2011; Ismaiel et al., 2015). The



present study confirmed for the first time that the mycotoxins of certain fungal species can successfully be used to produce agarwood resins in *A. crassna* trees.

The selected fungal strains used for this study were both first cultivated in PDA medium for the preparation of inocula because their growth rates were found to be higher in PDA than in other media (Dias, 2016). Our study identified a unidirectional radial growth pattern for both strains, similar to the observations of Dias (2016); this pattern could be due to the presence of self-inhibitory compounds produced by the hyphae (Bottone et al., 1998).

Different strains of the same fungal species can show distinct differences in growth, secondary metabolite production and bioactivities (Dresch et al., 2015). Withanage (2017) identified the potential of different strains of *A. niger* and *F. solani* to induce agarwood formation. Out of the four selected strains for that study, more agarwood production was shown by the ASP-U strain than by the ASP-N strain of *A. niger* and by the FUS-U strain than by the FUS-N strain of *F. solani*. However, that study identified agarwood resin formation only by different fungal strains, while our study tested the potential to induce agarwood formation with the mycotoxins produced by those two strains. Nor Azliza et al. (2014) also found that different strains of *F. solani* showed different morphological characteristics and growth rates in PDA medium. The ability to induce agarwood formation with the ASP-U strain of *A. niger* and the FUS-U strain of *F. solani* could also differ among other strains of the same species. Therefore, further studies are suggested to characterize the particular strains used in the present study using molecular identification and phylogenetic analysis while comparing them with other fungal strains identified from agarwood tissues.

The formation of agarwood resins in *A. crassna* trees inoculated with mycotoxins of ASP-U occurred at shorter distances from the inoculation point ( $\pm 20$  cm) than that in trees inoculated with the FUS-U strain ( $\pm 60$  cm). This could be due to the rapid damage caused to living tree tissues by the mycotoxins of ASP-U. Similarly, the trees inoculated with 100 ml of FUS-U mycotoxin did not form agarwood beyond -20 cm and +40 cm from the inoculation point. Once the cells, especially those in the xylem tissues, were damaged by the toxins, transport may have been affected, which would have prevented the solutions from moving upward or downward in the trees.

The contents of agarwood resin produced by mycotoxins of the FUS-U strain within a distance of  $\pm 20$  cm from the inoculation point were mostly higher than those produced in the same region by mycotoxins of ASP-U. However, we could not establish a clear pattern of agarwood resin content by distance from the inoculation point.

The value and quality of agarwood resin are determined by the presence of certain resin constituents (Subasinghe and Hettiarachchi, 2015). Chemical studies on agarwood resins from *Aquilaria* species have reported the presence of several sesquiterpenes, sesquiterpene alcohols, oxygenated sesquiterpenes, hydrocarbons and aromatic acids (Ishihara et al., 1993; Tamuli et al., 2005; Nor Azah et al., 2008). Some of the constituents that have been identified in agarwood resins included  $\alpha$ -agarofuran, 3-agarofuran, 10-epi-y-eudesmol, agarospirol, jinkohol, jinkohol II and valerianol (Ishihara et al., 1993; Nor Azah et al., 2008). Gas chromatography-mass spectrometry analysis of the resins produced in this study also reported the presence of the main important agarwood compounds, successfully proving the ability of mycotoxins for to induce commercially viable agarwood resin formation.

After identifying mycotoxins capable of inducing agarwood formation in *Aquilaria* species, these mycotoxins can be synthesized to achieve uniform and economical inoculation. The use of synthetic mycotoxins will eliminate the difficulties of growing and maintaining fungi at the commercial scale for use as artificial inoculants for agarwood formation. This development will greatly help *Aquilaria* forest plantation managers increase agarwood production in an effective manner, which could generate higher profits.

We proved for the first time that agarwood resin formation can be induced in *Aquilaria* trees by using mycotoxins produced as secondary metabolites of fungal species. The agarwood resins formed due to inoculation with toxins extracted from ASP-U and FUS-U fungal strains had key chemical constituents similar to those in agarwood resins formed by natural and various artificial methods reported by many authors in the past. In the current study, the FUS-U strain was indicated to be better for agarwood resin production than the ASP-U strain.

## Declarations

### Author contributions

The author contributions are listed below.

S.M.C.U.P. Subasinghe: Inoculation methodology development, manuscript preparation, proofreading

R.A.P. Malithi: Mycotoxin inoculation, resin quantity and quality analysis

S.W. Withanage: Mycotoxin preparation, testing

T.H.P.S. Fernando: Mycotoxin extraction methodology development

D.S. Hettiarachchi: GC-MS method development, constituent identification, proof reading

### Ethics declaration and consent to participate

Not applicable

### Consent for publication

Not applicable

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## Competing interests

The authors declare that they have no competing interests.

## Availability of data and materials

The datasets supporting the conclusions of this article are included within the main text and the additional files as well as in Tables 1, 2 and 3 and Figs. 1, 2, 3, 4 and 5. Both ASP-U and FUS-U strains were deposited at USJCC under the codes of USJCC-0059 and USJCC-0060 respectively.

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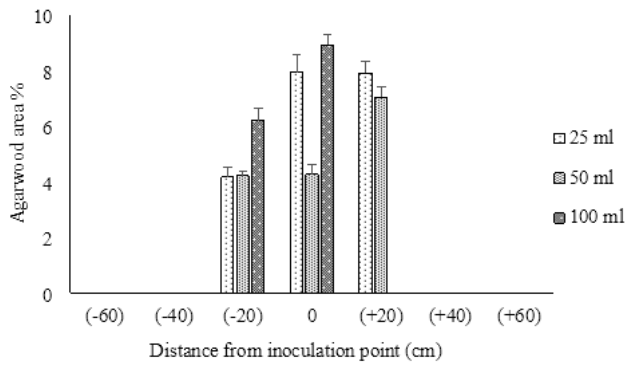
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## Figures



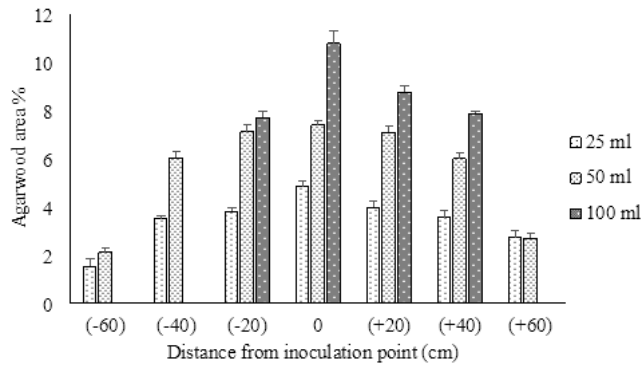
Figure 1

Resinous agarwood tissues formed due to the mycotoxins of ASP-U (left) and FUS-U (right).



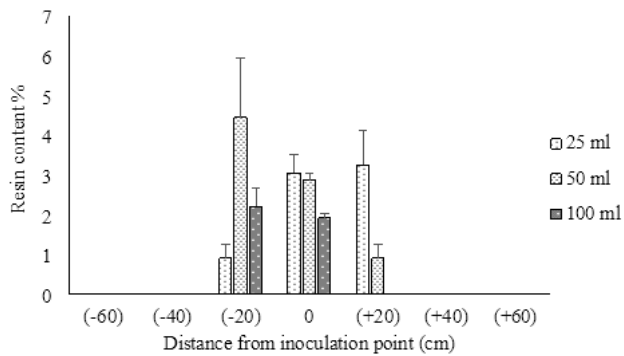
**Figure 2**

Variation in agarwood resinous area (+SE) along the stem due to the mycotoxins of the ASP-U strain.



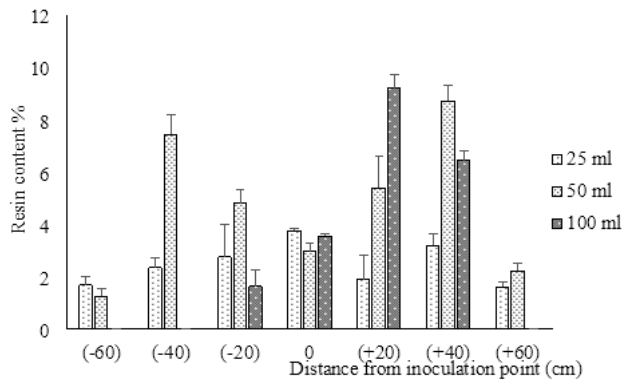
**Figure 3**

Variation in agarwood resinous area (+SE) along the stem due to the mycotoxins of the FUS-U strain.



**Figure 4**

Variation in agarwood resin content (+SE) along the stem with different volumes of the ASP-U strain mycotoxin.



**Figure 5**

Variation in agarwood resin content (+SE) along the stem with different volumes of the FUS-U strain mycotoxin.