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AGARWOOD RESIN INDUCEMENT METHOD USING MYCOTOXIN-CONTAINING EXTRACTS OF SELECTED FUNGAL SPECIES IN *AQUILARIA CRASSNA*

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The current study for the first time showed that agarwood resin formation in *Aquilaria* species could be induced by the mycotoxin-containing aqueous extracts of certain fungi. Volumes of 25, 50, and 100 mL of mycotoxin-containing extracts from the ASP-U strain of *Aspergillus niger* and the FUS-U strain of *Fusarium solani* were inoculated into *Aquilaria crassna* trees at 1 m intervals with three replicates. Resin production due to the extracts of ASP-U and FUS-U strains was restricted to ± 20 cm and ± 60 cm, respectively from the inoculation point after seven months and the 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 of the resin extracts detected 27 constituents responsible for the characteristic aroma of agarwood resin. Among them were phenyl butanone, agarofuran, agarospirol, β-caryophyllene, alloaromadendrene oxide and (-) guaiene-1(10),11-diene-15-ol were found in all extracts. These compounds were common in commercially available agarwood induced by live fungal species. Hence, the study demonstrated that mycotoxin extracts from specific fungal strains could be used for agarwood production in *Aquilaria*.

Keywords: Mycotoxins, *Fusarium solani, Aspergillus niger*, Agarwood, *Aquilaria, Gyrinops*

INTRODUCTION

Agarwood is 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 at the commercial scale. *Aquilaria* species are abundant from northern India to China, Indonesia and Malaysia and Vietnam. *Gyrinops* species are distributed mainly in eastern Indonesia and Sri Lanka (Subasinghe et al. 2012). Agarwood resin was commonly used in Chinese medicine as an analgesic, to treat stomach problems and as an expectorant (Blanchette 2003). It is used as a key perfumery agent, known as agar or oud in the Middle East and India. Agarwood has also gained popularity among Western perfumery and other cosmetic industries (Naziz et al. 2019).

Agarwood resins are produced as defense mechanisms to mimic internal tissue damage and to activate the wound healing process. Volatile compounds such as terpenoids, volatile organic constituents, and non-volatile compounds mainly 2(2-phenylethyl) chromones and fatty acids are commonly found in agarwood resins (Wetwitayaklung et al. 2009). Studies conducted to determine the effectiveness of inoculation with certain fungi for forming agarwood in *Aquilaria* species (Mohamed et al. 2014, Nobuchi, 2016, Faizal et al. 2017, Subasinghe et al. 2019) found several fungal species growing in infected agarwood. Since the natural process of agarwood formation is slow, high-quality artificially formed agarwood is popular in the market. Therefore, the inoculation of agarwood-producing trees with suitable fungal species is a common practice at a commercial scale (Mohamed et al. 2014).

Fungal genera such as *Aspergillus, Chaetomium, Fusarium, Lasiodiplodia*, and *Penicillium* are known to cause pathogenesis in living organisms (Sangareswari et al. 2016) through their secondary

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metabolites, which are involved in the pathogenic process. Therefore, it could be assumed that agarwood formation could be induced by the mycotoxins formed as secondary metabolites in certain fungal species. It was found that mycotoxins produced by different strains of the same fungal species vary in their composition, characteristics and functions (Hitokoto et al. 1980).

A previous study confirmed that living cultures of both *Aspergillus niger* and *Fusarium solani* were capable of forming agarwood in *Gyrinops walla* growing in Sri Lanka (Subasinghe et al. 2019). Dias (2016) optimized a liquid culture media of the above two species to be used 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 previous findings, the current study was designed to identify the ability of mycotoxins to form agarwood and the quantitative as well as qualitative differences among the produced resins. Therefore, mycotoxin-containing aqueous extracts of living fungal strains previously used by us to induce agarwood resin formation in *Aquilaria crassna* and *Gyrinops walla* were used. Based on our literature survey, this is the first study of its kind conducted to investigate mycotoxin-induced agarwood resin production. Upon the successful use of toxins, difficulties faced by *Aquilaria* tree growers with inoculating living fungi-containing media to the trees in the natural environment could be avoided while increasing the efficiency of agarwood production.

MATERIALS AND METHODS

Selection of the fungal strains

Withanage (2017) studied the differences in the agarwood resin contents and constituents formed in *Gyrinops walla* through artificial inoculation with two live strains of *Aspergillus niger* and two live strains of *Fusarium solani*. The findings confirmed that one strain of each fungal species, namely 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, ASP-U and FUS-U were selected to identify the ability of their mycotoxin extracts to form agarwood resins in *Aquilaria crassna* trees in the present study. The results of DNA sequencing of those strains were analysed using NCBI database of the 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 the 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 sterilised potato dextrose agar media autoclaved at 121 °C under a steam pressure of 1.055 kg cm^{-2}. After cooling to room temperature, gentamicin was added as an antibiotic to prevent bacterial growth. The cultures were then incubated at 25 °C in a culture room until optimum growth was attained.

Extraction of mycotoxins

We examined the effectiveness of the mytoxin-containing extracts of the selected fungi in agarwood formation. Therefore, after reaching the optimum growth in potato dextrose agar medium, three agar plugs of the ASP-U strain were transferred to Czapek-Dox broth medium and incubated at room temperature for 14 days. Three agar plugs of the FUS-U strain were transferred to yeast extract glucose broth medium and incubated for 10 days (Withanage 2017). Both media were then filtered using sterilised filter papers and a series of ceramic filters of descending pore sizes (Zheng et al. 2010). The filtrate was finally collected by filtering through 0.22 µm membrane filter discs to remove all microbial cells. The process was capable of retaining the conidia of *A. niger* (Gugnani, 2003, Reddy et al. 2010) and *F. solani* (Chandran & Kumar 2012). The mycotoxin extracts of ASP-U and FUS-U were also inoculated to potato dextrose agar and yeast extract glucose broth media, respectively to confirm the absence of spores or mycelia.

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Confirmation of extracts toxicity

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. The leaf tissue under the drop of the filtrate was then slightly damaged using a sterilised needle. Six leaves were used for each strain and sterilised liquid media were used as controls. The lesion diameter under each drop was measured in two perpendicular directions after 48 hours.

Production of mycotoxin extracts for the inoculations

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

Inoculation into *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 1/3rd of the tree diameter using a 16 mm electric drill powered by a portable generator. A brass connector was then inserted into the hole and a 1.5 m long with 16 mm diameter transparent rubber tube was attached to the brass connecter. 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 entire volume of toxin extracts was absorbed by the tree, the tubes and connectors were removed and the holes were sealed using sterilised seasoned clay.

Sample collection

All *A. crassna* trees were cut down seven months after inoculation, following the work of Mohamed et al. (2014) and Subasinghe et al. (2019). 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 agarwood formation were calculated. The agarwood tissues were then carefully removed, air-dried for 72 hours and stored in sealed polythene bags. The colors of the resinous tissues and extracted resins were identified using a Munsell soil color chart.

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 added with 10 mL of dichloromethane. The flask was placed on a mechanical shaker operated at 100 rpm and the extract was collected after 12 hours. The process 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. Subesequently, the weight of the vials with the resin extract was measured to calculate the weight of the resin (Subasinghe & Hettiarachchi 2013).

Analysis of the resin constituents

The solid resin extract was weighed and dissolved in anhydrous acetone to produce a 10 µg mL^{-1} solution. Constituent analysis was conducted by a gas chromatograph-mass spectrometer equipped with an HP-5 MS 5% phenyl methyl siloxane capillary column (30 m × 0.25 mm × 0.25 µm). The oven temperature was increased from 50 °C to 220 °C at 5 °C min^{-1} and held for 10 min at 220 °C while keeping the injector temperature at 250 °C. The helium carrier gas was kept at a flow rate of 2 mL min^{-1}. The interface temperature was set to 250 °C. The split ratio was set to 1:10 and 0.1 µL resin solution was injected into the

column using the autosampler. The constituents were quantified by the area normalisation 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 & Hettiarachchi 2013).

RESULTS

Toxicity of the extracted mycotoxins from the fungal strains

Mycotoxin extracts of ASP-U and FUS-U strains inoculated to culture media did not show any form of growth, confirming that spores or mycelia were not present in the extract. The average lesion diameters caused by the mycotoxin extracts of the ASP-U and FUS-U fungal strains were 0.73 ± 0.05 cm and 0.95 ± 0.03 cm, respectively. The control had a lesion diameter of 0.10 cm, which was the initial size of the damage caused by the needle when the toxin was introduced into the leaves. The observations confirmed that the mycotoxins produced by both strains were capable of causing damage to living plant tissues.

Agarwood resin formation by mycotoxin extracts

Agarwood formation was clearly visible in *A. crassna* cross-sections at seven months after inoculation (Figure 1). The colour of the resinous agarwood tissue samples varied from yellowish-brown to black (darkest), while the colour of the resins extracted from those tissues varied from pale yellow to dark yellowish-brown. However, only the trees inoculated with FUS-U strain produced black colored tissues, viz. 25 mL solution at 40 cm and 60 cm above the inoculation point and 50 mL and 100 mL solutions at the inoculation point. No possible relationship between the resin tissue color and strength and the resin extract color was established.

Agarwood resin formation was restricted to within ±20 cm of the inoculation point in the trees inoculated with mycotoxin extracts of the ASP-U strain (Figure 2). Resin formation was not observed at +20 cm even in the trees inoculated with 100 mL of mycotoxin extract 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 ± 60 cm from the inoculation point in the trees inoculated with the mycotoxin extracts of the FUS-U strain (Figure 3). The largest

Figure 1 Resinous agarwood tissues formed due to the mycotoxins of ASP-U (left) and FUS-U (right)

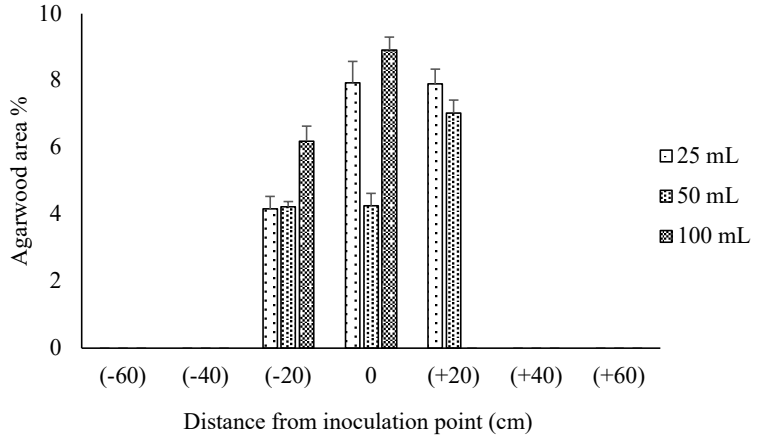

Figure 2 Variation in agarwood resinous area (+SE) along the stem due to the mycotoxin extracts of the ASP-U strain

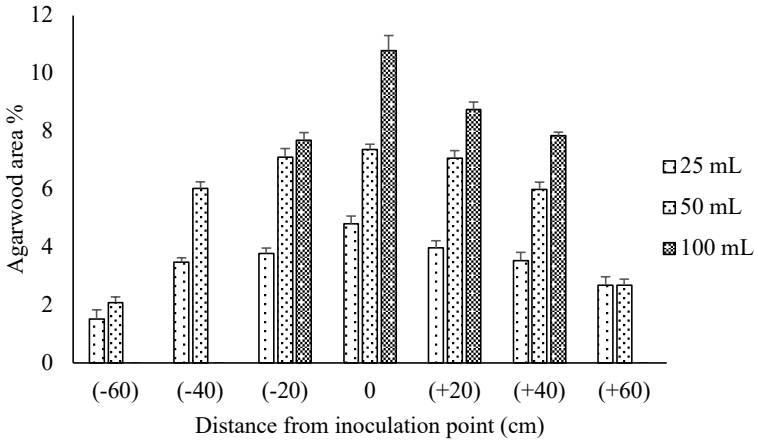

Figure 3 Variation in agarwood resinous area (+SE) along the stem due to the mycotoxin extracts of the FUS-U strain.

resinous area formed due to the mycotoxin extracts 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 mycotoxin extracts, resinous tissues in trees inoculated with 100 mL of FUS-U strain mycotoxin extracts were not observed at the -40 cm, -60 cm and +60 cm points (Figure 3).

The resin content formed due to inoculation with 100 mL of FUS-U mycotoxin extract 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 mycotoxin extracts of the two strains were not significantly different from any other inoculation volumes. The average agarwood resin content resulting from the mycotoxin extract of the ASP-U fungal strain varied from 0.89% to 4.44% (Figure 4) and resulted from the FUS-U strain mycotoxin extract varied from 1.24% to 9.20% (Figure 5). The average resin contents of the individual trees inoculated with mycotoxin extracts of the ASP-U strain varied from 0.89 ± 0.35% to 3.24 ± 0.86% for a volume of 25 ml, from 0.89 ± 0.36% to 4.44 ± 1.50% for 50 ml and from 1.91 ± 0.10% to 2.20 ± 0.46% for 100 mL.

The agarwood resin contents extracted from the trees inoculated with mycotoxin extracts 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

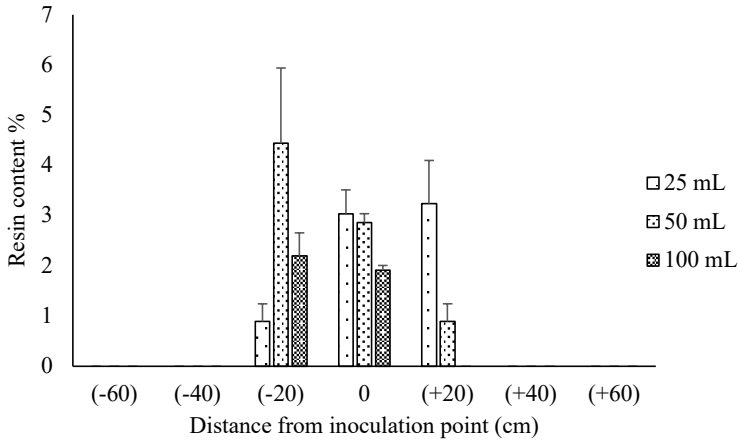

Figure 4 Variation in agarwood resin content (+SE) along the stem with different volumes of the ASP-U strain mycotoxin extract.

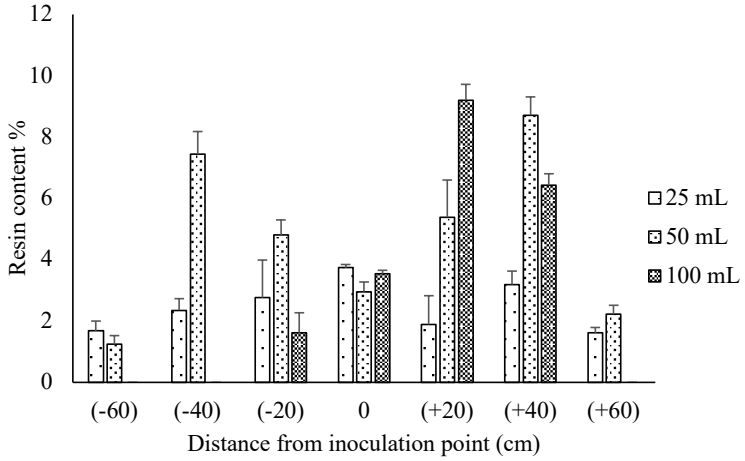

Figure 5 Variation in agarwood resin content (+SE) along the stem with different volumes of the FUS-U strain mycotoxin extract.

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 extract varied from 1.61 ± 0.18% to 3.74 ± 0.11% for 25 mL, from 1.24 ± 0.29% to 8.71 ± 0.60% for 50 mL and from 1.62 ± 0.65% to 9.20 ± 0.52% for 100 mL (Figure 5).

The resin contents formed by different inoculation volumes of the FUS-U strain mycotoxin extract were significantly different (F = 17.27; p = 0.000). The resin contents formed by 100 mL of mycotoxin extract 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 (Figure 5).

Variation in agarwood resin constituents formed due to mycotoxins

Gas chromatography-mass spectrometry analysis of the agarwood resins formed due to the mycotoxin extracts of the ASP-U and FUS-U strains confirmed the availability of several constituents that were common to commercially available agarwood produced in *Aquilaria* trees. Constituent such as phenyl butanone, agarofuran, agarospirol, β-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. Although cloveone was common to all resin samples formed due to the ASP-U strain, it was not detected in some of the resin samples formed due to the FUS-U strain. The 2-(2-phenyl)-chromone derivative was 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 β-ionone (isomers) and octadecenoic acid were found only in two samples formed by the ASP-U strain, both of these constituents were commonly observed in the resins formed by the mycotoxins of the FUS-U strain. 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.

DISCUSSION

Previous studies showed the ability to artificially develop agarwood resin in *Aquilaria* species using fungi, especially *Fusarium* species (Mohamed et al. 2010, Sangareswari et al. 2016) and also using *A. niger* (Hitihamu et al. 2014). *A. niger* and *F. solani* were also known to produce several types of mycotoxins (Sweeney et al. 2000, Alimentarius 2011) as secondary metabolites. These toxins were 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 mycotoxin extracts of certain fungal species could successfully be used to produce agarwood resins in *A. crassna* trees. However, the study did not characterise the mycotoxins produced by both fungal strains at this stage. The mycotoxins will be isolated and characterised in future studies.

The selected fungal strains used for this study were both first cultivated in potato dextrose agar medium for the preparation of inocula because their growth rates were found to be higher than in other media (Dias 2016). The present study identified a unidirectional radial growth pattern for both strains, similar to the observations of Dias (2016) and the 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 showed 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 previous 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, the previous study identified agarwood resin formation only by different fungal strains, while the current study tested on the potential to induce agarwood formation with mycotoxin extracts produced by the two strains. Nor-Azliza et al. (2014) also found that different strains of *F. solani* showed different morphological characteristics and growth rates in potato dextrose agar 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 were suggested to characterise 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.

Mohamed et al. (2014) reported discoloured agarwood tissues along 1.17 cm and 1.70 cm lengths in the stems of *Aquilaria malaccensis* after three and six months of fungal inoculation respectively. Subasinghe et al. (2019) reported *Gyrinops walla* stem tissue discoloration within 50 cm above and 30 cm below the inoculation points after 6 months of inoculation with *A. niger* and *F. solani*. Based on these two earlier studies, the present study also observed agarwood formation after seven months of inoculation with mycotoxin-containing extracts ASP-U and FUS-U strains.

The formation of agarwood resins in *A. crassna* trees inoculated with mycotoxin-containing extracts of ASP-U occurred at shorter distances from the inoculation point (± 20 cm) than that in trees inoculated with the FUS-U strain (± 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 plant cells especially those in the xylem

tissues were damaged by the toxins, transport may have been affected which prevented the solutions from moving upward or downward in the trees. The contents of agarwood resin produced by mycotoxin extracts of the FUS-U strain within a distance of ± 20 cm from the inoculation point were mostly higher than those produced in the same region by mycotoxins of ASP-U. However, the present study could not establish a clear pattern of agarwood resin content by distance from the inoculation point.

The value and quality of agarwood resin were determined by the presence of certain resin constituents (Subasinghe & Hettiarachchi 2015). Subasinghe et al. (2019) identified 16 constituents in agarwood resins of *G. walla* trees after six months of direct inoculation with *A. niger* and *F. solani* fungal cultures. However, the current study managed to identify 27 constituents. Chemical analysis on agarwood resins from *Aquilaria* species 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 identified in agarwood resins included α-agarofuran, 3-agarofuran, 10-epi-γ-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, which proved the ability of mycotoxins to induce commercially viable agarwood resin formation. Since the present study was conducted in the wet zone of Sri Lanka lowlands, we recommend to test the effectiveness of the same inocula on *A. crassna* growing in the other climatic regions.

CONCLUSION

The current study showed for the first time that agarwood resin formation could be induced in *Aquilaria* trees by using mycotoxins produced from 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 studies in the past. The current study also indicated that FUS-U strain was better for agarwood resin production than the ASP-U strain.

Identification of toxins capable of inducing agarwood formation in *Aquilaria* species, could encourage specific toxins to be artificially produced through biotechnological methods and to be used as potential and reliable source of inoculation for uniform and economical agarwood production in the future. The use of such synthetic toxins could eliminate the difficulties of growing and maintaining fungi at the commercial scale as artificial inoculants for agarwood formation. The development could greatly help *Aquilaria* forest plantation managers to increase agarwood production in an effective way as well as to generate higher profits.

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