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ORIGINAL PAPER

Use of two fungal species to induce agarwood resin formation in *Gyrinops walla*

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Abstract *Gyrinops walla* Gaertn. is the only species growing in Sri Lanka that belongs to the agarwood family, Thymelaeaceae. Although agarwood resin induction and extraction from *Aquilaria* species of the same family have been practised for many decades in Southeast Asian region, the ability of producing agarwood resins in *G. walla* was discovered recently. Since previous studies were on agarwood resins formed due to natural causes, the present study was conducted to identify the potential fungal species that are capable of artificially inducing agarwood resin formation in *G. walla*. Since this is the first ever study conducted on artificial inducement of agarwood resin formation in *G. walla*, *Aspergillus niger* and *Fusarium solani* were selected owing to their high abundance in the naturally formed agarwood resinous tissues collected from 25 *G. walla* trees. Both fungal species were separately grown in yeast extract glucose agar and used to inoculate healthy *G. walla* trees under aseptic conditions. Three holes were made for each tree and 2 g of fungal culture including the medium were placed in each hole. Tissue discoloration, characteristic aroma, resin content and resin constituents were checked at 10 cm intervals above and below the inoculation points for a period of 1 year. Results revealed that tissue discoloration and resin content were higher in the trees inoculated with *A. niger*. Other than at 10 cm above and below the inoculation points, samples collected at all locations had significantly higher resin contents when inoculated with *A. niger* compared to *F. solani*. Sixteen agarwood resin constituents, which were also recorded in *Aquilaria* species, were identified from the discolored tissues using GC–MS analysis. Jinkohol, agarospirol and 2(2-phenyl) chromone derivatives were found in all discolored tissues collected at 10-cm intervals of the trees inoculated with both fungi. β-Seline, γ-eudesmol and valerenal were found in nine of 10 sample points on the stem. γ-Elemene was recorded only in one sample. The characteristic aroma during burning was stronger for dark-colored tissues than the light-colored ones. The present study confirmed the potential use of certain fungal species to induce agarwood resin in *G. walla* and that *A. niger* is more effective than *F. solani*.

Keywords *Gyrinops walla* · Agarwood resin · *Aspergillus niger* · *Fusarium solani* · Inoculation

Introduction

Agarwood is a dark-colored, resinous substance formed in stems, branches and roots of certain species of family Thymelaeaceae (Chen et al. 2012). It is highly fragrant and therefore extensively used as incense, perfumes and traditional medicine, especially in Asia, Europe and Middle East (Okudera and Ito 2009). Most species of *Aquilaria* and a few species of *Gyrinops*, *Aetoxylon* and *Gonystylus* are

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capable of producing agarwood (Blanchette 2003). These species are naturally distributed in at least 12 countries in Southeast Asia (Bangladesh, Bhutan, Cambodia, India, Indonesia, Laos, Malaysia, Myanmar, Philippines, Thailand, Vietnam and Papua New Guinea; Blanchette 2003).

Agarwood forms as a result of a self-defense mechanism against a physical, chemical or biological stress (Mohamed et al. 2014; Ng et al. 1997; Tamuli et al. 2005; Zhang et al. 2012). Three hypotheses have been proposed for agarwood formation resulting from pathological invasion, wounding/pathological and/or no pathological processes (Ng et al. 1997). As a response to those causes, the tree produces a resin, which is high in volatile organic compounds that aid in suppressing or retarding the growth of fungi or any other microorganisms. The natural formation of agarwood is rare and takes a long time to produce an extractable amount (Chen et al. 2011; Chong et al. 2015) and is commonly found in trees of about 20 years or older (Blanchette 2003). Therefore, the use of artificial methods to induce the resin formation process is popular, especially for *Aquilaria* species as such methods require comparatively much shorter periods of time (Chen et al. 2011; Tamuli et al. 2005). In addition, agarwood resin formation can be artificially induced even in smaller trees about 4–5 years old (Mohamed et al. 2014). It is also believed that artificial inducements can yield agarwood 10 times faster than natural formation (Persoon 2007). Although agarwood had been traditionally induced by creating open wounds on the tree trunk (Blanchette 2003), many artificial methods have been reported, including wounding of trees, use of chemicals and biological agents such as fungi (Tabata et al. 2003). These injuries provide suitable sites for microbial infection and also stress the trees, which help in spreading the microbes (Barden et al. 2000).

However, over the years, agarwood inducement practices have been expanded to include modern methods (Laurence 2013; Mohamed et al. 2014). Among them, inoculation with a fungus is effective, especially for *Aquilaria* species (Laurence 2013). Naturally occurring microorganisms can enhance agarwood production, and the product extracted from natural forests are much higher in quality than wood from young plantations (Jensen 2005). The fungal infection induces resin formation after the fungal inocula are introduced to the stem by drilling at regular intervals. Suitable fungal inocula for this purpose can be prepared by isolating and growing the fungi on a selective medium (Mohamed et al. 2014). *Fusarium* spp., *Trichoderma* spp., *Curvularia* spp. and *Cunninghamella* spp. are common fungi that can be used to induce agarwood resin in *Aquilaria* species (Blanchette 2003; Mohamed et al. 2010).

Aquilaria, *Gonystylus* or *Aetoxylon* species have not been found in Sri Lanka; *Gyrinops walla*, a medium-tall tree to 15 m tall, is the only agarwood-producing species growing in the country (Subasinghe and Hettiarachchi 2013). It has been recorded in the lower elevations of the wet zone of Sri Lanka (Dassanayake and Fosberg 1981) where the annual rainfall is above 2000 mm.

Until agarwood resin-producing ability was first scientifically proven by Subasinghe et al. (2012) and Subasinghe and Hettiarachchi (2013), *G. walla* had not been considered a valuable tree species. These authors confirmed that the quality of agarwood formed in *G. walla* from natural causes is similar to the agarwood produced commercially using *Aquilaria* species. However, previous studies focused on agarwood resins that formed due to natural causes in *G. walla* tree trunks (Subasinghe and Hettiarachchi 2013, 2015; Subasinghe et al. 2012), so it is now imperative to devise methods to induce resin formation in this species for commercial production. Therefore, this study investigated the ability of two fungal species that inhabit *G. walla* to induce agarwood resin formation.

Materials and methods

Isolation and identification of fungal species inhabiting agarwood tissues

Since this is the first report on artificial inducement of agarwood resin formation in *G. walla*, we decided to use the most abundant fungal species in naturally formed agarwood tissues of living trees. Dark-colored agarwood tissues that had formed due to natural damages in the stems were collected from 25 healthy *G. walla* trees growing on private lands in Mathugama Divisional Secretariat (6°23′15″N, 80°7′08″E), a low country wet zone in Sri Lanka, where the average annual rainfall is 3000 mm and average temperature is 26 °C. The tissues were thoroughly washed using deionized water and cut into 1.0 cm × 0.5 cm × 0.2 cm pieces, which were then surface-sterilized in 5% sodium hypochlorite for 2 min, then 70% ethanol for 2 min and rinsed in distilled water (Laurence 2013). They were then placed on yeast extract glucose agar (YEGA) containing antibiotics under aseptic conditions and incubated at 27 °C.

After 1–2 weeks, pure cultures were prepared from distinguished colonies that grew on the YEGA based on the physical characteristics, growth pattern and abundance in the culture plate. Following the work of Mohamed et al. (2014), different fungal species were identified using colony characters and morphological characteristics of hyphae and reproductive structures.

Preparation of fungal inocula and inoculation of *G. walla* trees

Due to their abundance in agarwood samples collected from all 25 *G. walla* trees, *Aspergillus niger* (ANS1) and *Fusarium solani* (FSS1) were selected as potential inocula in this study. Their identity was confirmed by comparison with authenticated fungal specimens preserved at the Rubber Research Institute of Sri Lanka. Other fungal species identified were not used because they were not present in most of the agarwood tissues.

Both *A. niger* and *F. solani* were separately grown on YEGA at 27 °C until sporulation, then used in separate inoculations of 20 *G. walla* trees growing on a private land of the Mathugama Divisional Secretariat. Three spirally arranged inoculation holes (1 cm × 1 cm × 2.5 cm) were aseptically cut at 30-cm intervals in each tree stem, keeping the lowest point 30 cm above ground level. A mycelial disk equivalent to 2 g was cut from the culture (Laurence 2013) and aseptically placed in each hole within a minimal time. Then the hole was sealed using sterile cotton wool moistened with sterile distilled water to prevent contamination. An uninoculated disk of YEGA of the same size was used as the control.

Examination of agarwood resin formation

Holes of 1 cm × 1 cm × 2.5 cm at 10, 20, 30, 40 and 50 cm above and 10 and 30 cm below the inoculation sites on the tree stems were made to collect samples and check for agarwood resin formation and analyze content and constituents at monthly intervals for the first 4 months and at bi-monthly intervals for the next 8 months. The amount of tissue discoloration was also recorded using the Munsell soil color chart (Munsell Soil Color Charts 1994). The aroma produced from burning air-dried tissue samples was also tested by a sensory panel.

Determination of resin content and constituents

A collected tissue sample equivalent to 2 g was placed in a scintillation glass vial, and 20 mL of dichloromethane was added. The vial was shaken on a mechanical shaker at a low speed to maximise resin extraction. The extract was collected at 12-h intervals for 36 h by renewing the solvent. The bulk extract was then evaporated under a low-pressure stream of nitrogen at room temperature and stored away from light (Subasinghe and Hettiarachchi 2013, 2015).

A known mass of each extract was dissolved in dehydrated acetone at 100 ng mL^{-1} for gas chromatography-mass spectroscopy analysis of the resin constituents using HP 6790 GC and HP 5971 auto sampler (Agilent Technologies, USA). l-Menthol (100 ng mL^{-1} dehydrated acetone) was used as the internal standard. The capillary column of 95% phenylsiloxane was 30 m long with 0.25 mm of inner diameter and 0.25 μm film thickness (AT-5MS, Alltech, USA). The injector was kept at 220 °C and the oven was programmed for 10 °C min^{-1} gradient temperature from 100 to 200 °C. Spectra for resin constituents were identified by comparison with published spectra and an online database library (NIST 5, NIST, USA; Wiley 275, John Wiley and Sons Inc. USA) (Subasinghe and Hettiarachchi 2015).

Re-isolation of *A. niger* and *F. solani* from the inoculated tissues

Following a method similar for fulfilling Koch's postulates, 10 tissue samples were randomly collected from the sampling sites to confirm the presence of *A. niger* and *F. solani* in the inoculated *G. walla* trees. The tissues were surface sterilized and placed on YEGA medium under aseptic conditions and incubated at 27 °C. Fungi were identified using the morphological characteristics (Mohamed et al. 2014).

Results

Abundance of fungal species in naturally formed agarwood resinous tissues

Four *Fusarium* species, four *Trichoderma* species, three *Aspergillus* species, one *Diplocladium* species and one *Mucor* species were isolated from agarwood tissues collected from *G. walla* stems. Only *Aspergillus niger* and *Fusarium solani* were found in all tissue samples cultured.

Discoloration of inoculated wood

Tissues were more discolored closer to the inoculation points and lighter as the distance increased (Table 1). No discoloration was present 40 and 50 cm above the inoculation sites with *A. niger* within the first 2 months, but present from the third month on. No discoloration was found 30, 40 and 50 cm above sites inoculated with *F. solani* until the third month. Moreover, at 30 cm below the inoculation site with *F. solani*, tissues did not become discolored until the second month (Table 1). Discoloration increased at each time point within the 12-month study. Other than tissues 50 cm above the inoculation point, tissues at all other sites became black within 12 months in *G. walla* trees inoculated with *A. niger*. However, only the tissues sampled at 10 and 20 cm above and 10 cm below became black in trees inoculated with *F. solani* (Table 1). Burnt tissues produced a pleasant woody aroma that was

Table 1 Discoloration of wood tissues after inoculation over time

Species	Month	Distance from inoculation point (cm)						
		10a	20a	30a	40a	50a	10b	30b
A. niger	1	DRB	DB	DB	0	0	DB	DRB
	2	DB	DB	DRB	0	0	DB	DRB
	3	VDB	VDB	VDB	DRB	DRB	VDB	DRB
	4	B	VDB	VDB	DRB	DRB	VDB	DB
	6	B	B	VDB	DRB	DRB	B	VDB
	8	B	B	B	VDB	DRB	B	VDB
	10	B	B	B	VDB	DRB	B	B
	12	B	B	B	B	VDB	B	B
F. solani	1	DRB	DRB	0	0	0	DRB	0
	2	DB	DRB	0	0	0	DRB	DRB
	3	DB	DB	DRB	DRB	DRB	DRB	DRB
	4	VDB	DB	DB	DRB	DRB	DB	DRB
	6	VDB	VDB	DB	DRB	DB	VDB	DRB
	8	B	VDB	DB	DB	DB	VDB	DB
	10	B	B	VDB	DB	DB	B	DB
	12	B	B	VDB	DB	VDB	B	VDB

a above inoculation site, *b* below inoculation site, *DRB* dark reddish brown, *DB* dark brown, *VDB* very dark brown, *B* black, *0* no color change

stronger as tissue darkening increased. The aroma was not produced by tissues that were not discolored.

Variation in resin content

Average resin contents were higher in trees inoculated with *A. niger* than with *F. solani* for all locations sampled (Fig. 1). The highest average resin contents in trees inoculated with *A. niger* were recorded at 10 cm above (1.65%), 10 cm below (1.31%) and 20 cm above (1.27%). The same locations had higher resin contents in trees that were inoculated with *F. solani*, proving that resin concentration was higher closer to the inoculation sites.

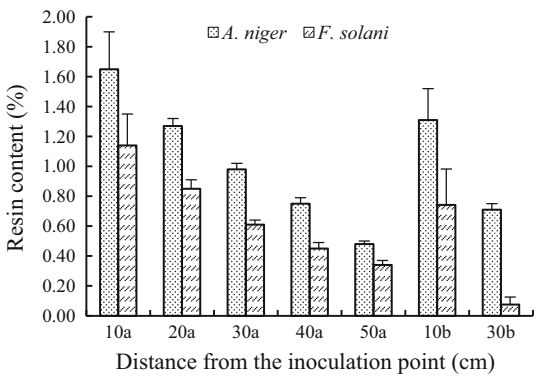

Fig. 1 Average (+ SE) resin content with respect to the height from the inoculation point. *a* above inoculation site; *b* below inoculation site

Statistical analysis revealed that there was no significant difference in the resin content at 10 cm above ($F_{1,28} = 0.17$, $p = 0.358$) and 10 cm below ($F_{1,28} = 0.21$, $p = 0.556$) the inoculated points for *A. niger* and for *F. solani*. However, the resin contents differed significantly for *A. niger* and for *F. solani* at 20 cm above ($F_{1,16} = 18.75$, $p = 0.000$), 30 cm above ($F_{1,16} = 57.35$, $p = 0.000$), 40 cm above ($F_{1,16} = 15.62$, $p = 0.000$), 50 cm above ($F_{1,16} = 22.14$, $p = 0.001$) and 30 cm below ($F_{1,16} = 37.54$, $p = 0.000$) from the inoculation sites.

Variation in resin constituents

Sixteen resin constituents were identified from agarwood resinous tissues induced by *A. niger* and *F. solani* (Table 2). They were also found in the agarwood resins of *Aquilaria* species. However, not all 16 constituents were recorded at a single sampling site (Table 2). The highest number of constituents, viz. 15 of 16, was recorded at 10 cm above the inoculation site of the trees inoculated with *A. niger*. Jinkohol, agarospirol and 2(2-phenyl) chromone derivatives were detected in tissue samples from all sites on trees inoculated with both fungal species. β-Seline, γ-eudesmol and valerenol were detected in samples from all sites on trees inoculated with *A. niger*, but in only 4 of 5 sites on trees inoculated with *F. solani* (Table 2). Of the 16 compounds, γ-elemene was detected in the fewest sites, only at 10 cm above the site inoculated with *A. niger*.

Table 2 Average resin constituents at different distances from the inoculation sites on trees inoculated with *A. niger* or *F. solani*

Constituent	Distance from *A. niger* site (cm)					Distance from *F. solani* site (cm)				
	10a	30a	50a	10b	30b	10a	30a	50a	10b	30b
3-Phenyl butanone	3.41	1.54	0.52	188	0.04	2.14	–	1.85	0.02	1.15
Azulenone	2.47	3.15	1.82	–	–	0.99	–	0.58	0.58	–
β-Seline	1.07	0.98	0.01	0.10	0.07	1.17	0.01	–	0.26	0.05
Isopropyl napthaline derivative	0.03	5.44	2.58	0.06	–	0.01	0.08	–	1.02	0.45
Jinkohol	0.08	0.54	0.08	0.04	0.77	0.06	1.10	0.18	0.07	0.09
Agarospirol	0.65	0.62	0.04	0.96	0.34	0.05	0.04	0.03	4.22	2.02
γ-Eudesmol	4.42	0.08	1.46	7.41	2.17	–	0.07	2.51	5.86	0.68
Valerenol	1.12	1.11	0.05	1.65	0.01	0.11	–	0.91	1.26	–
γ-Elemene	5.01	–	–	–	–	–	–	–	–	–
2,2,6,8-Tetramethyl biyclo undece-7-en-3-ol	–	4.58	0.11	–	0.04	4.12	–	–	0.94	0.06
Hexadecanoic acid	0.54	–	2.10	0.07	0.84	0.02	0.33	–	0.12	–
Camphene	1.95	–	–	–	–	–	0.42	–	6.55	–
6-Acetyl-7-hydroxy-2,2-dimethyl benzopyran	1.85	–	–	–	–	–	0.57	–	–	–
Valerenal	7.31	3.68	0.05	0.03	3.46	3.54	0.04	3.14	–	2.98
9-Octadecanoic acid	1.82	2.01	–	–	–	2.02	–	–	0.08	–
2(2-Phenyl) chromone derivatives	0.33	0.18	1.02	1.41	5.51	2.55	0.04	0.11	0.02	2.01

a above inoculation site, *b* below inoculation site

Isolation and distribution of *A. niger* and *F. solani* in the inoculated trees

Both *A. niger* and *F. solani* grew from cultured resinous tissues excised at 4 months of inoculation. No other fungal species were isolated from these samples, and none grew from the noninoculated controls. All inoculated *G. walla* trees showed healthy growth and appeared disease free.

Discussion

Selection of the two fungal species, *A. niger* and *F. solani*, for inoculating healthy *G. walla* trees was based on their abundance in all tested agarwood resinous tissues that had formed after natural damage. Although several other species of *Fusarium*, *Trichoderma*, *Aspergillus*, *Diplocladium* and *Mucor* were isolated, their abundance varied. Mohamed et al. (2010) found *Fusarium*, *Trichoderma*, *Curvularia*, and *Cunninghamella* species as common fungi in the agarwood tissues of *Aquilaria malaccensis*. *Fusarium* species were successful in inducing agarwood resins in *Aquilaria* species (Karlinasari et al. 2015; Laurence 2013; Tabata et al. 2003). Molecular identification of 36 strains of potential agarwood-inducing fungi collected from 17 provinces in Indonesia also revealed that all the isolates belonged to *Fusarium*, mostly *F. solani* (Akter et al. 2013). Moreover, *Xylaria* and *Lasiodiplodia* were successfully used for resin induction in *A. sinensis* (Cui et al. 2013). Reports on the ability of inducing agarwood resin formation by *Aspergillus* species in *Aquilaria* trees, however, are not common. However, the results of the present study revealed that *A. niger* is more effective in inducing agarwood resins in *G. walla.*

As shown in the present study, stem tissue discoloration provides remarkable evidence of agarwood formation. Although sonic and ultrasonic wave methods have recently been developed to detect agarwood in trees, still the common practice is to obtain the inner stem tissues to examine the colour change, aroma, resin content and quality (Chen et al. 2012; Cui et al. 2013; Mohamed et al. 2010).

Cui et al. (2013) reported a marked agarwood resin formation in *Aquilaria* species after 2 months of fungal inoculations. Mohamed et al. (2014) observed tissue discoloration at 1.17 and 1.70 m along the stem at 3 and 6 months, respectively, after inoculation of *A. malaccensis* with ascomycetes fungi. Tian et al. (2013) found that stem tissues of *Aquilaria* trees inoculated with *Fusarium* species changed color from pale yellow to red at 7–21 months after inoculation. The present study also revealed an increasing formation of agarwood resin over time with the continuous growth of the fungi inside the tree stem. Agarwood tissue colors were darker and resin content was higher closer to the inoculation sites, which could be due to the impact of high growth of fungi in that area (Mohamed et al. 2014).

The resin constituents identified in this study were also identified by Subasinghe and Hettiarachchi (2015) from agarwood resins formed in response to natural damage of *G. walla* stems. Thus, there is no difference between the constituents of the agarwood resins formed due to natural causes and after inoculation with a fungus. The 16 constituents identified in the current study were also recorded in *Aquilaria* species (Mei et al. 2013; Nor Azah et al. 2008; Wetwitayaklung et al. 2009). Although *A. niger* and *F. solani* can be pathogenic to certain crops, disease symptoms were not detected on *G. walla* trees after inoculation with the two fungal species.

Conclusion

Though there were no considerable differences in the resin constituents of agarwood tissues induced by *A. niger* and *F. solani*, *A. niger* induced more resin and tissue discoloration than *F. solani*. Therefore, *A. niger* is the preferred candidate or inducing agarwood resin formation in *G. walla*.

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