J. Agrobiotech. **Vol 6,** 2015, p. xx–xx.

ISSN 1985-5133 (Press)
ISSN 2180-1983 (Online)

Agarwood Inducement Technology: A Method for Producing Oil Grade Agarwood in Cultivated *Aquilaria malaccensis* Lamk.

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ABSTRACT

Agarwood is the fragrant resin impregnated wood derived from the wounded *Aquilaria* trees. Agarwood is priceless due to its oleoresin content. Under natural conditions, oleoresin can only be produced by natural wounding such as injury by lightning or wounding by animals. However, the natural process of oleoresin accumulation is a time consuming process. The concept of plantations for *Aquilaria* trees in combination with artificial agarwood inducement methods serves as an alternative way to supply agarwood and conserve the wild *Aquilaria* stock. Our study evaluated a novel technique for producing oil grade agarwood in cultivated *Aquilaria* trees. *Aquilaria malaccensis* was used for the agarwood inducement study. For *A. malaccensis* trees treated with this inducement technique, resin was formed and spread throughout the xylem cell from the transfusion point in the trunk. Agarwood yield per tree reached approximately 3-4 kg. Furthermore, the agarwood derived from the induction was found to have a similar quality to the wild agarwood. This indicates that this inducement technology may have commercial potential. Inducement of cultivated agarwood by using this method could satisfy the significant demand for agarwood, while conserving and protecting the remaining wild *Aquilaria* trees.

Keywords: *Aquilaria malaccensis* Lamk., inducement technology, resin formation

ABSTRAK

Gaharu ialah kayu wangian beresin yang berasal dari pokok *Aquilaria* (Karas) iaitu akibat daripada kecederaan. Nilai kayu gaharu amat tinggi disebabkan oleh kandungan resinnya. Dalam keadaan semulajadi, oleoresin hanya akan dihasilkan

melalui kecederaan semula jadi seperti kilat atau haiwan. Tambahan pula, proses pengumpulan semula jadi resin adalah satu proses yang sangat memakan masa. Konsep perladangan pokok karas dengan kombinasi kaedah inokulasi gaharu buatan adalah bertindak sebagai satu cara alternatif untuk membekalkan sumber kayu gaharu dan memulihara stok *Aquilaria* liar. Kajian kami bertujuan untuk menilai satu teknik baru untuk menghasilkan kayu gaharu bergred minyak di dalam pokok karas di ladang. *Aquilaria malaccensis* telah digunakan dalam kajian ini. Bagi pokok *Aquilaria malaccensis* yang dirawat dengan teknik inokulasi ini, resin gaharu adalah terbentuk dan tersebar melalui sel xilemnya dari titik inokulasi dalam batang. Hasil kayu gaharu yang terbentuk bagi setiap pokok adalah dalam anggaran 3-4 kg. Tambahan pula, resin gaharu yang diperolehi dari inokulasi ini mempunyai kualiti yang sama dengan kayu gaharu liar. Ini menunjukkan teknologi inokulasi ini mempunyai potensi dagangan yang tinggi. Teknologi inokulasi gaharu ini dapat memenuhi permintaan kayu gaharu yang tinggi dalam pasaran, di samping dapat memulihara dan melindungi pokok karas liar.

Kata kunci: *Aquilaria malaccensis* Lamk., teknologi inokulasi, pembentukan resin

INTRODUCTION

Agarwood is a valuable non-timber forest product which has many usages due to its fragrance. The fragrant agarwood has been used for centuries as incense in religious ceremonies. It is also used as medicine for its effects as a sedative and carminative. The agarwood essential oil is a highly demanded ingredient in perfumery for its earthy and unique balsamic notes. The most important source of agarwood is the *Aquilaria* spp. tree from the Thymelaeaceae family (Rogers, 2009). Agarwood-producing species are found in the areas ranging from India eastwards throughout Southeast Asia, as well as in southern China. Indonesia and Malaysia are the two major producing countries as the origin for agarwood. *Aquilaria malaccensis* is the main source of agarwood production in Malaysia.

The healthy wood of *Aquilaria* trees is without oleoresins. Under natural conditions, oleoresin can only be produced by natural wounding such as injury by lightning or wounding by animals, typically around wounded or rotting parts of the trunk (Pojanagaroon & Kaewrak, 2006; Blanchette & Heuveling, 2009). However, the natural process of oleoresin accumulation is a time consuming process due to the fact that agarwood formation occurs slowly and infrequently in old trees. Thus the supply of agarwood from wild sources is far less than the market demands

Because of its immense value and rarity, agarwood resources in Malaysian forests is facing threats of over-exploitation by illegal trading, harvesting and smuggling of agarwood. The agarwood poaching activities began in Malaysia

during the 19th century and early 20th century by the local Orang Asli. From 1990 onwards, foreign agarwood poachers from Thailand, Indonesia, Philippines and Cambodia were actively operating in Peninsular Malaysia and Borneo.

During these illegal poaching activities, trees of all sizes, from small saplings upward, were felled without sufficient regard to conserving stocks and this has caused major destruction of the *Aquilaria* population in Malaysia (Mah *et al.,* 1983). As a result, the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) Appendix II was implemented to the agarwood-producing taxa especially the *Aquilaria* spp. since 2004 to show concern over the effect trade has had on this genus and to ensure that the trade is well-regulated, and that it proceeds under a system of permits based on conditions of legality and sustainability (Lim *et al.*, 2010).

Efforts have been made to preserve natural *Aquilaria* populations (Soehartono & Newton, 2001) and to increase agarwood supply. This includes developing the cultivation of *Aquilaria* species and to intentionally injuring the cultivated trees to produce agarwood. In Indonesia, Cambodia, Thailand, Vietnam and some other countries, *Aquilaria* plantations have been established (Barden *et al.*, 2007). In Malaysia, over one million *Aquilaria* spp. trees are widely cultivated in Peninsular and Borneo Malaysia.

The existing artificial agarwood inducement methods include bark removal as well as axe and nail wounding methods, the burning method, and the fungi infection method (CITES, 2004; CITES, 2005a; CITES, 2005b; Pojanagaroon & Kaewrak, 2006; Barden *et al.*, 2007; IUCN, 2009). These methods require a long time for agarwood formation, and produce a low agarwood yield. To date, some comparatively new and efficient methods have been developed such as the cultivated agarwood kits (CA-Kits) by Blanchette from the University of Minnesota (Blanchette & Heuveling, 2009) and the whole-tree agarwood-inducing technique (Agar-Wit) by the Chinese Academy of Medical Sciences and Peking Union Medical College (Liu *et al.*, 2013).

Our lab also has developed a similar technology in inducing the agarwood formation. It is a simple, fast and efficient method to induce agarwood formation. We drill several small holes in spiral at the trunk and apply agarwood inducer into the xylem part of *Aquilaria* trees through these holes. Due to water transportation, the inducer is transported to the whole trunk, thus forming an overall wound in the tree, and as a result, agarwood is finally formed in the *Aquilaria* trees in a short period of time. To evaluate the agarwood quantity and quality induced by our agarwood inducement technology, the harvested agarwood was analyzed and measured by thin layer chromatography and gas chromatography mass spectrometer.

MATERIALS AND METHODS

Plant Material Treatment

The experiment of this study was carried out in an *Aquilaria* spp. plantation at the Malaysian Nuclear Agency (Nuclear Malaysia), Malaysia. Five-year-old *Aquilaria malaccensis* trees with similar trunk girth were chosen as experimental materials. Two techniques, including the agarwood inducement method (AINM) developed by Nuclear Malaysia and the fungi infection method (FI) were applied to induce resin formation.

Several small holes deep into the xylem were drilled in spiral from the ground of the main trunk by an electric drill (Fig. 1). The agarwood inducer (AINM) or the basidiomycetes fungi solution (FI) was slowly injected into the xylem tissues through a wash bottle. Each agarwood inducer was tested in three trees respectively. The composition of the two agarwood inducers remains a technical secret. A pure water treatment was taken as the negative control (NC), and healthy wood as a blank control (BC). The treated *A. malaccensis* trees were harvested 18 months later for the quantity and quality analysis.

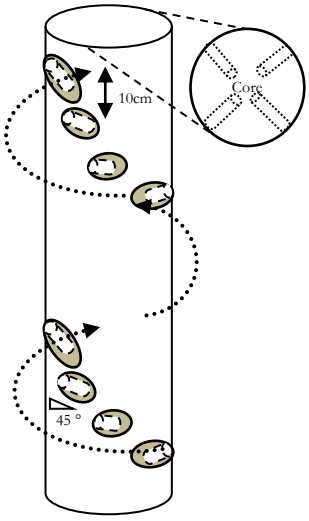

Fig. 1. The inducement method used in this experiment where holes were drilled inside the trunk.

Agarwood Yield Estimation

As agarwood was formed inside the whole tree, to accurately measure the agarwood yield per tree, we had to separate agarwood resin completely from the white wood and air dry it for 14 days to weight for the agarwood yield per tree.

Material Processing

To evaluate agarwood quality, the resinous wood from each inducement technique, as well as the wild agarwood as control and also the wood samples of NC and BC, were grinded into powder.

Quality and Quantity Analyses

i) Essential oil

The essential oil was extracted using the hydro-distillation method. All the agarwoods of AINM, FI, NC and BC were grinded into powder. Agarwood powder (50 g) was extracted in water for 24 hours. The essential oil was collected in a tube in which the aqueous portion of the distillate is automatically separated and returned to the distilling flask. The extracted essential oil was isolated and stored in a sealed bottle at room temperature until analysis.

ii) Thin-layer chromatography (TLC)

The quality analysis was conducted using the Thin-Layer Chromatography (TLC) method for the chemical compound profiling and patterning. Approximately, 1 µL of the extracted essential oil was dropped onto the TLC plate (TLC Silica gel 60 F254, 20 × 20 cm, Merck). Benzene:acetone (9.5:0.5, v/v) solvent was used as the mobile phase. The TLC plate was stained with 5% vanillin-hydrochloric acid and heated at 100 °C (Zweig & Sherma, 1980).

iii) Gas chromatography mass spectrometer (GC-MS)

The chemical constituents in the essential oil were identified using the Gas Chromatography Mass Spectrometer (GC-MS) method (Xie *et al.*, 2013). The Agilent 7890A/5975C was used in this study. The GC-MS conditions are listed in Table 1.

Table 1. GC-MS conditions

Gas Chromatography Mass Spectrometer	Agilent 7890A/5975C
Capillary column	HP-5MS
Oven Program	
Initial Temperature	60 °C
Initial Time	10 min.
Rate	3 °C/min.
Final Temperature	230 °C
Final Time	1 min.

RESULTS AND DISCUSSION

Agarwood Formation

The agarwood inducer was injected into the drilled hole of *Aquilaria malaccensis* trees through a wash bottle. The inducer was spread through xylem due to water transpiration pressure and induced the agarwood formation. Agarwood resin formed over several months throughout the trunk as well as branches of the tree (Fig. 2). A ring shape dark area containing agarwood resin was observed on the cross sections from the branches three months after the inducement (Fig. 3). The trunk was cut down from 10 cm above the ground at harvesting time. Agarwood resin was separated from the white wood by carving. The agarwood resin was mostly found in the whole tree stem after we harvested and processed the tree.

Agarwood resin formed slowly after inducement by the agarwood inducer AINM. The agarwood resin that accumulated in the wood over the time appeared as a light brown ring in the first three months (Fig. 3). A thick resinous layer was observed all over the trunk after twelve months, indicating the agarwood resin was actively secreted after being triggered by the inducer (Fig. 4). The color of the resin formed correlated to the amount of the resin that accumulated. The darker the resin, the more the resin accumulate. After 18 months from the agarwood inducer AINM inducement, dark brown resinous wood formed inside the whole tree (Fig. 5). The cross sections of the trees induced by AINM, FI, NC and BC are shown in Figure 6.

All the induced trees were harvested after 18 months for the yield estimation. All of the trees induced with the agarwood inducers AINM developed a thick layer of dark brown agarwood resin and spread throughout the trunk forming pieces of agarwood (Fig. 7). However, the trees induced with agarwood inducer FI only developed a thin layer of light brown agarwood resin just at the drilled site. This result showed that the agarwood inducer FI did not spread throughout the trunk due to the fungi inducer not penetrating through the plant cell thus failing to grow inside the tree (Fig. 6B). On the other hand, the NC remained as white wood, with no resin formation, this was the same for the BC, indicating that pure water cannot induce agarwood resin formation. Therefore, agarwood inducers play an important role in inducing the formation of agarwood resin.

Fig. 2. Agarwood resin formed at the branches of the tree.

Fig. 3. A ring shape agarwood resin was observed three months after inducement.

Fig. 4. A resinous layer was observed all over the trunk twelve months after inducement.

Fig. 5. A skilled worker carved the tree trunk to obtain the dark brown resinous wood 18 months after inducement.

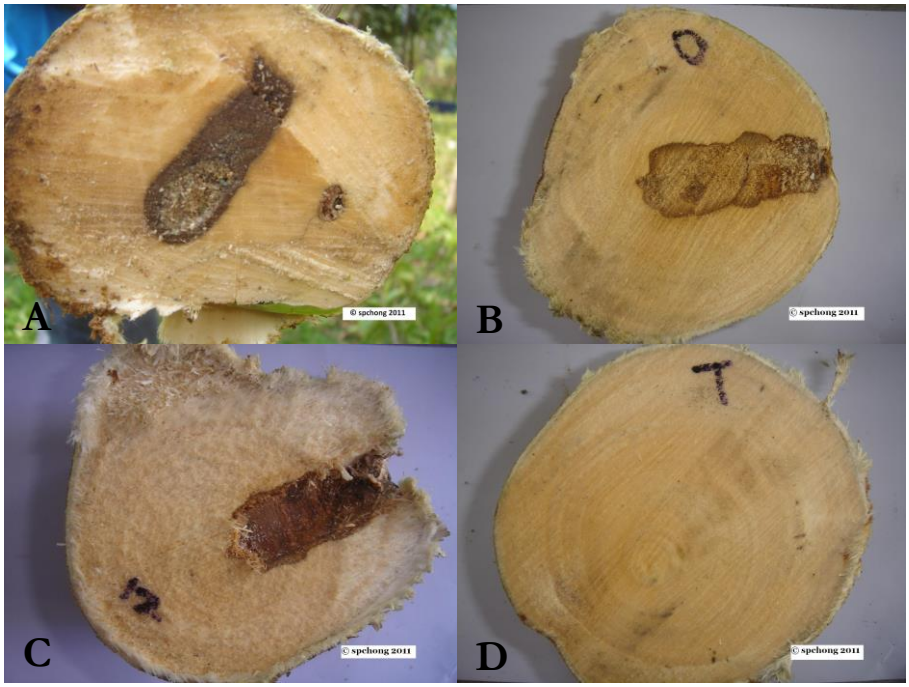

Fig. 6. Cross sections of the trees induced by the AINM (A), FI (B), NC (C) and BC (D) methods. Results were observed 18 months after inducement.

Fig. 7. Pieces of agarwood carved from the tree induced with the AINM method.

Agarwood Percentage Yield

In this experiment, the agarwood resin was separated and carved from the white wood to produce the agarwood pieces. The pieces of agarwood produced from each inducement were weighed and measured and the percentage yield of the agarwood produced was compared to the tree mass. The result showed a significant difference in percentage yield produced by AINM inducement compared to the other inducers (Fig. 8). The agarwood with inducer AINM showed significantly better results in terms of the average yield, roughly 3 to 4 kg per tree. The FI inducement method also induced agarwood formation, but resin was only found around the wound sites and with a very low yield. The average agarwood percentage yield per tree induced with different inducement methods was measured to be 42.42% for AINM inducement and 3.30% for FI inducement. The yield per tree by AINM was 12.9 times higher than the FI. No resin was formed in the NC and BC samples.

The results showed both AINM and FI inducement methods were able to produce agarwood resin. However, the fungi-based FI inducement method was significantly lower in yield than the AINM method due to the environmental factors such as the changing of moisture or temperature which might slow down the growth of fungi or cause fatality. The inconsistency of the FI method in resin inducement performance directly reduces the efficiency of this method.

The AINM inducement method based on non-living substance successfully induced the agarwood resin to produce high yield compared to the FI method and the control. The AINM method showed consistency in agarwood resin production in all the specimens and its performance was not affected by environmental factors. The advantage factors in the AINM method helped to increase the efficiency of agarwood resin production.

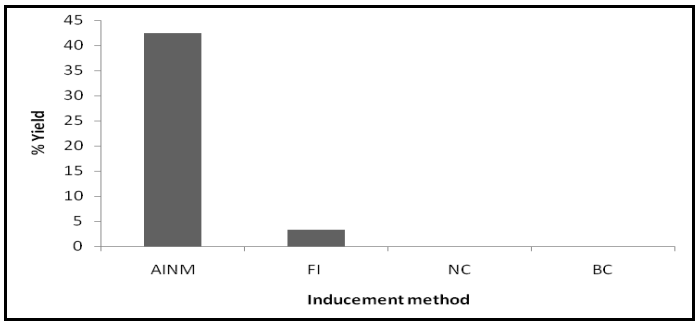

Fig. 8. Agarwood percentage yield with different inducement techniques. Agarwood inducement Nuclear Malaysia (AINM), fungi infection method (FI), negative control (NC) and blank control (BC).

Agarwood Chemical Compound Fingerprinting by TLC

The TLC fingerprint profiles of agarwood oil samples including A as induced by AINM, B as induced by FI, C as negative control (NC) and D as blank control (BC); were analyzed and compared with E, the pure agarwood oil extracted by Kedaik Agarwood Sdn. Bhd. as the standard agarwood oil. TLC chromatograms of all the samples are shown in Figure 9. Eight common spots (Rf = 0.17, 0.43, 0.54, 0.60, 0.67, 0.84, 0.88 and 0.91) were detected among the samples A, B and E when the TLC plate was stained with 5% vanillin-HCL and heated for 10 min at 100 °C. These results demonstrated that the agarwood oil obtained by ANIM has chemical compounds similar to the wild agarwood oil. Moreover, the deeper the pink color, the better the corresponding agarwood, which showed that the agarwood oil produced by AINM has a better quality than the agarwood oil produced by FI. As shown in Figure 9, the negative control and blank control exhibited the same results.

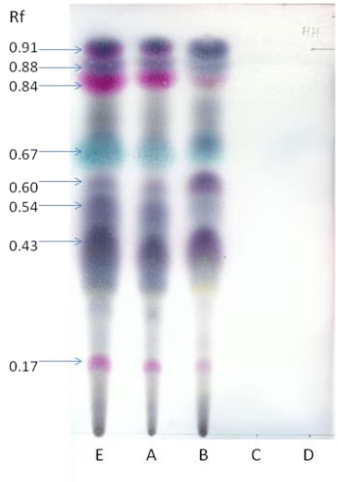

Fig. 9. TLC chemical compounds fingerprinting of the agarwood oils induced by AINM (A), induced by FI (B), negative control (C), blank control (D) and stardard agarwood oil from Kedaik Agarwood Sdn. Bhd. (E).

The pattern of chemical compounds in the TLC chromatograms showed that the agarwood oil induced by the AINM method shared the same pattern as the standard agarwood oil. In other words, this means a similar group of compounds from the standard agarwood oil was detected in the agarwood oil induced by the AINM method. The agarwood oil induced by the FI method showed a slightly different pattern from the standard agarwood oil which indicates that its agarwood oil consisted of a different group of compounds. This might be due to the

differential compositions between the monoterpene, sesquiterpene and diterpene groups. A further study to characterize the composition of agarwood oil was conducted using GC-MS.

Essential Oil Contents

Agarwood oil is a complex mixture of aromatic terpene compounds including monoterpenes, sesquiterpenes and diterpenes (Naef, 2011; Chen *et al.*, 2012). The main compounds present in agarwood oil have been identified as the sesquiterpenes. In this preliminary study, the agarwood oil extracted from the induced agarwood with AINM and FI methods were analyzed by GC-MS and compared to the standard agarwood oil from Kedaik Agarwood Sdn. Bhd. (Fig. 10).

According to the GC-MS analysis data, the main chemical compounds were found between the retention times from 26.0 to 56.0 and basically belong to the compounds in terpene group listed in Table 2. The agarwood oil marker compounds such as the agarospirol, agarofuran, guaiene and hinesol in sesquiterpene group had been detected in these agarwood oils (Pant *et al.*, 1980; Nakanishi *et al.*, 1983; Ishihara *et al.*, 1991; Ishihara *et al.*, 1993; Näf *et al.*, 1995). In the control agarwood oil, marker compounds like the agarofuran and guaiene were found. On the other hand, agarofuran and agarospirol which served as the marker compounds were found in the agarwood oil induced by the AINM method. However, in the agarwood oil induced by the FI method, only hinesol was found.

Based on the terpene compounds found in the agarwood oils, the control agarwood oil contained 84.6% of sesquiterpenes, 7.7% of monoterpenes and 7.7% of diterpenes. Meanwhile the agarwood oil induced by the AINM method contained 93.3% of sesquiterpenes and 6.7% of monoterpenes. The agarwood oil induced by the FI method contained 78.6% of sesquiterpenes, 14.3% of monoterpenes and 7.1% of diterpenes. The agarwood oils from the control and AINM showed higher level of sesquiterpenes content compared to the agarwood oil from the FI, with lower sesquiterpenes content but higher monoterpene content.

The difference between agarwood oils from the control and AINM compared to the agarwood oil from FI might be due to the different responses of the plant reacting to the different inducement methods. The plant defense system against pathogen attack such as the penetration of fungi will trigger the accumulation of monoterpenes. Although both volatile sesquiterpenes and monoterpenes served as defenses against herbivores and pathogens, the monoterpene compounds act more specifically as toxins to fungal pathogens (Blanchette, 1992).

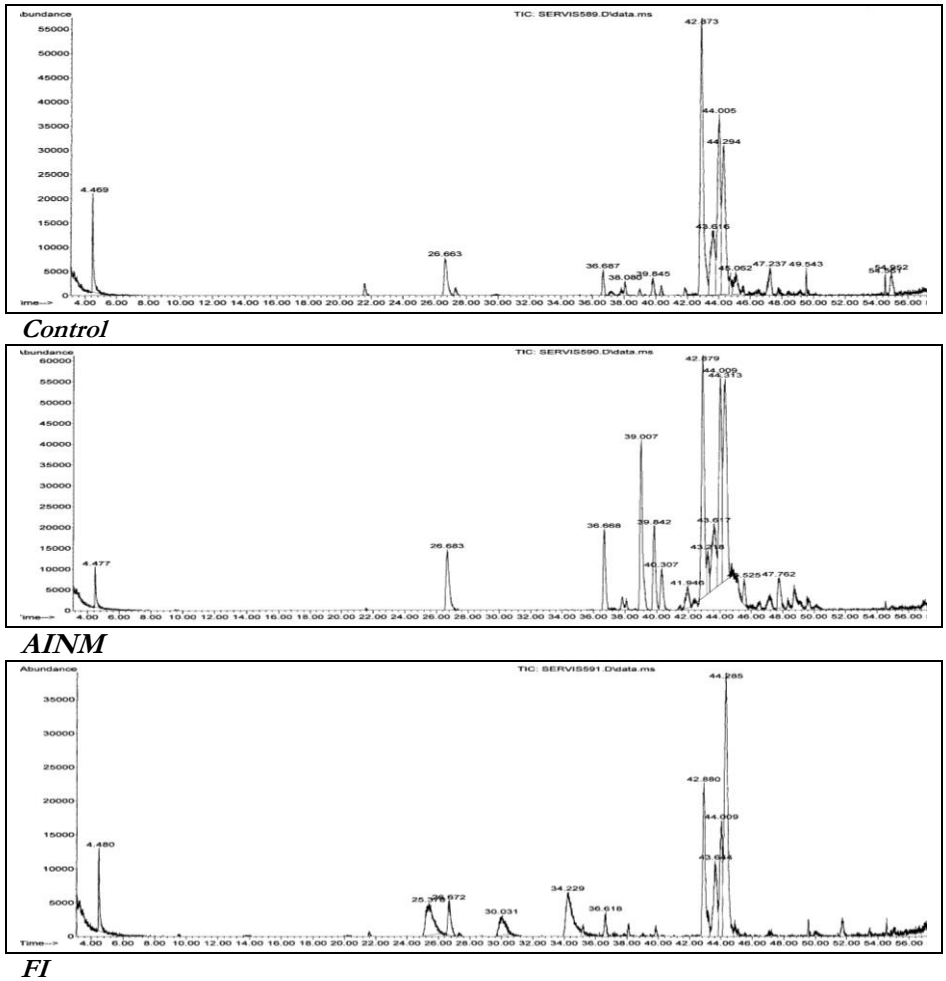

Fig. 10. GC-MS chromatogram for the control (standard agarwood oil from Kedaik Agarwood Sdn. Bhd.) and the induced agarwood oil by AINM and FI methods.

Table 2. Comparison of the composition of agarwood oil induced by AINM and FI methods to the control standard agarwood oil from Kedaik Agarwood Sdn. Bhd.

Composition of the Agarwood Oil	Control	AINM Method	FI Method	Terpene Group
Muurolene	+	-	-	Sesquiterpene
Eudesmol	+	+	+	Sesquiterpene
Valencene	-	-	+	Sesquiterpene
Guaiene	+	-	-	Sesquiterpene
Zonarene	-	+	-	Sesquiterpene
Cadinene	-	+	-	Sesquiterpene
Columellarin	+	-	-	Sesquiterpene
Allo-cedrol	+	-	+	Sesquiterpene
Liguloxide	+	-	-	Sesquiterpene
Presilphiperfolan-8-ol	+	+	+	Sesquiterpene
Dihydroagarofuran	+	-	-	Sesquiterpene
Anethole	+	+	-	Monoterpene
Italicene ether	+	+	-	Sesquiterpene
Agarofuran	+	+	-	Sesquiterpene
Gurjunene	+	-	+	Sesquiterpene
Pseudo phytol	+	-	+	Diterpene
Kessane	-	+	-	Sesquiterpene
Hedycaryol	-	+	-	Sesquiterpene
Maaliol	-	+	-	Sesquiterpene
Patchoulene	-	+	-	Sesquiterpene
Agarospirol	-	+	-	Sesquiterpene
Mustakone	-	+	-	Sesquiterpene
Cubebol	-	+	-	Sesquiterpene
Eremophilone	-	+	+	Sesquiterpene
Anethole	-	-	+	Monoterpene
Linalool acetate	-	-	+	Monoterpene
Longipinene	-	-	+	Sesquiterpene
Hinesol	-	-	+	Sesquiterpene
Longiborneol	-	-	+	Sesquiterpene
Rosifoliol	-	-	+	Sesquiterpene
Liguloxide	-	-	+	Sesquiterpene

CONCLUSION

The AINM method is proven in this study as an efficient technology to induce agarwood production especially in cultivated *Aquilaria* trees. This non-living based inducement technique successfully triggered the plant metabolism system to produce the secondary metabolite in agarwood which accumulated in the plant cells. This technology combines well with the principle of water transpiration in the plant to transport the AINM inducer throughout the trunk and up to the branches of the whole tree. The whole inducement took only 18 months to obtain agarwood closely resembling wild agarwood in terms of quality.

This AINM technology has many advantages especially in the effectiveness of its inducer which assures consistency in producing agarwood and is not influenced by any environmental factors. Besides, this technology is an environmentally safe and friendly method, harmless to our ecosystem. It is a simple, low cost and time effective technology which is commercially feasible to be applied by farmers. The idea of implementing this technology in cultivated *Aquilaria* plantations in Malaysia will meet the high demand of agarwood from the local and international markets. Moreover, this technology helps to sustain the agarwood resources and to conserve the wild *Aquilaria* trees.

ACKNOWLEDGEMENTS

This project was funded by the Malaysian Nuclear Agency R&D Program (MINT-R&D-06-18-02). This work was supported by the Terengganu State Forestry Department, the Forestry Department of Peninsular Malaysia and the Forest Research Institute of Malaysia (FRIM). Special thanks to the local agarwood industry stakeholders; Kedaik Agarwood Sdn. Bhd. and Legenda Yakin Sdn. Bhd. for providing the standard agarwood and agarwood oil for the quality evaluation.

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