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Induction of Agarwood Olfactory Sesquiterpenes from *Aquilaria filaria* Callus Culture

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Aquilaria filaria is a native tree species from the East Indonesian forests, whose heartwood produces an aromatic resin which is widely used as the fragrance raw material. This oleoresin known as "agarwood" is rich in unique olfactory molecules and exhibits great industrial demands with high market values globally. However, as a consequence of non-sustainable harvesting in the past, *A. filaria* has been included in the IUCN (International Union for Conservation of Nature) red list of threatened species. In an effort to meet the demand for the fragrance compounds from the valuable agarwood using plant biotechnology, this research aimed to evaluate the potential of *A. filaria* callus culture to produce volatile fragrance sesquiterpenes (SQTs) under the stimulation of selected biotic and abiotic elicitors. SQTs emitted from the callus cultures were extracted using a stir bar sorptive extraction (SBSE) method and the components were identified using GC-MS. The results revealed that the production of sixteen fragrance SQTs previously identified as the olfactory compounds of agarwood were highly inducible in *in vitro A. filaria* cultures. Some of those agarwood compounds were jinkohol, acorenone, α-agarofuran, dehydrojinkoh-eremol, spathulenol, α-guaiene, and α-cedrene. It was concluded that callus cultures of *A. filaria* have the potential to be developed as a bioreactor for the agarwood fragrance compounds production.

Keywords: callus culture; fragrance oleoresin; SBSE; sesquiterpenes

I. INTRODUCTION

Aquilaria filaria is a native tropical forest tree species distributed in the eastern part of Indonesia. The genus *Aquilaria* belongs to the family Thymelaeaceae of the order Malvales that produces impregnated fragrant resin called agarwood. Agarwood has been used since the past by the Chinese, Indians and Arabs as a fragrance, incense in religious ceremonies, aromatherapy, and consumed as an ingredient of the Ayurvedic, Tibetan and East Asian traditional medicine (Damania, 2001; Eurlings & Gravendeel, 2005; Persoon, 2007). Trading of agarwood has been known since 900 BC (Chakrabarty *et al*., 1994). To date, agarwood remains as an economic commodity exported from Southeast Asia to the Far East, Middle East and European countries as well as USA (Antonopoulou *et. al*., 2010; Lim & Anak, 2010).

Numerous studies on agarwood oil have confirmed that the distinctive scent of agarwood resin was due to the complex mixture of numerous compounds mainly comprised of sesquiterpenes (SQTs). Agarofuran, agarospirol, jinkoh-eremol, jinkohol, kusunol, guaiene, humulene and butanone are some types of aromatic SQTs detected from agarwood (Ishihara & Tsuneya, 1993a; 1993b; Naef, 2011; Nakanishi *et. al*., 1983; Tajuddin & Yusoff, 2010). Moreover, in-depth investigations on agarwood oil from three *Aquilaria* species revealed that the most important aroma-impact SQTs contributing to

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the typical agarwood scent are β-agarofuran, 4-phenyl-2-butanone, α-bergamotene, α-humulene, α-bulnesene (δ-guaiene), jinkohol, α-agarofuran, jinkoh-eremol, kusunol (valerianol), acorenone, and eremophiladien (Nakanishi *et. al.*, 1981; Pripdeevech *et. al.*, 2011; Thuy *et al.*, 2019).

The production of aromatic secondary metabolites by plants is well known as a response of plant protection mechanism against pathogens or herbivores, a stress response to the environment, or a mechanism to attract plant pollinators. There was evidence of mechanical wounding, herbivore oral secretion, pathogens, and biotic and abiotic stress during growth acting as triggers for the defence gene expression (Kant *et. al.*, 2009; Maes & Debergh, 2003; Pichersky & Gershenzon, 2002; Vickers *et al.*, 2009).

Natural agarwood formation is a slow process that occurs as a result of wounding or pathogen infection (Burfield & Kirkham, 2005). Aromatic resin could be found in the younger two-decade old agarwood-producing trees, albeit agarwood of high quality and grade is expected to be harvested from trees that had grown for at least half a century. The quality and grade of agarwood is reflected in the oleoresin content, types of fragrances released, medicinal properties, colours and the formation process (Damania, 2001). Agarwood fragrance is dominated by the woody character types with extra notes of warm, peppery, sweet, nutty or spicy. These fragrances correspond to the SQT contents in the agarwood (Pripdeevech *et al.*, 2011). Fragrance industries pay a lot of attention on the woody and ambery notes from natural resources. Woody and ambery scents were the major impact notes used as the basic aroma in most contemporary fragrance products (Naef, 2011; Panten *et al.*, 2004).

High-quality agarwood is formed over a long process in the trees of the Thymelaceae family which includes many agarwood-producing species listed on Appendix II of the Convention on International Trade in Endangered Species of Wild Flora and Fauna (Schmidt, 2011). In addition, the growing demand for environmentally friendly products motivated the chemical industries including perfumeries to use renewable sources with sustainable development known as “green chemistry” in their production process (Panten *et al.*, 2004).

Plant biotechnology with the application of plant cell cultures offers a means to produce naturally occurring valuable compounds which could not be synthesised chemically or by bacterial culture (Vijaya Sree *et al.*, 2010). In principle, it is feasible to commercially produce plant secondary metabolites through large-scale plant cell cultures. The development in plant tissue culture techniques such as the screening of high production cell lines, as well as optimisation of culture growth, medium and processing methods could improve the production of metabolites by more than 20-fold (Verpoorte *et al.*, 2002). Moreover, higher production is attainable with the application of elicitation strategy using biotic or abiotic elicitors (Chattopadhyay *et. al.*, 2002; Verpoorte *et al.*, 2002). The production of plant metabolites through plant cell cultures has achieved commercial success in the cases of shikonin from *Lithospermum erythrorhizon*, paclitaxel from *Taxus* sp., ginsenoside from *Panax ginseng* and purpurin from *Rubia akane* cell cultures (Dong & Zhong, 2002; Vasconsuelo & Boland, 2007).

For the above reasons, along with the prospective application of biotechnology in the production of valuable secondary metabolites, this research aimed to study the potential of *Aquilaria filaria* callus culture to produce high-value SQTs which are usually produced naturally. Several biotic and abiotic elicitors were evaluated for their ability to induce the production of SQTs in *A. filaria* callus culture.

II. MATERIALS AND METHODS

A. Plant Materials

Plant materials in the form of *A. filaria in vitro* plantlets were obtained from *in vitro* culture collection of the Biotechnology Laboratory, Faculty of Agriculture, University of Mataram, Lombok, Indonesia. The *A. filaria* material used in this research originated from Sorong, West Papua, and was obtained from West Papua Provincial Forestry Service. Callus stock cultures were induced and propagated from *A. filaria* plantlet leaves (Figure 1a) on the MS solid medium (Murashige & Skoog, 1962). The medium consisted of 4.4 g/L MS medium (Duchefa Biochemie, Netherlands), 20 g/L sucrose (Duchefa Biochemie, Netherlands), 0.6 g/L MES (Duchefa Biochemie, Netherlands), 1 mg/L and 2 mg/L

phytohormones BAP and IBA (Duchefa Biochemie, Netherlands), and 8 g/L agar (Duchefa Biochemie, Netherlands) (Listiana *et al.*, 2018). Callus cultures were incubated in the dark at 27°C and sub-cultured fortnightly (Figure 1b).

B. Elicitation

Different types of elicitors were tested for their ability to induce the secretion of SQTs from *A. filaria* callus on solid medium. The elicitation experiment was conducted using MS solid medium supplemented with 20 g/L sucrose, 1 mg/L BAP, 2 mg/L IBA and additional biotic or abiotic elicitor. Applied at a concentration of 1 g/L, yeast extract (Carl Roth GmbH & Co, Germany) was used as a biotic elicitor to mimic fungal infection *Aquilaria* trees which was assumed contribute to the natural formation of agarwood (Burfield, 2005). The biotic elicitor laminarin (Sigma-Aldrich, UK) containing β-1-3-glucan capable of inducing plant defence reaction was tested at a concentration of 50 mg/L (Aziz *et. al.*, 2003; Xin *et al.*, 2019). Abiotic elicitors, silver nitrate ($AgNO_3$, Duchefa Biochemie, Netherlands) and copper sulphate ($CuSO_4$, Carl Roth GmbH & Co, Germany), were applied at a concentration of 5 mg/L. Silver ions were proven to have an effect on plant secondary metabolites synthesis (Ashtiani *et. al.*, 2010, Farrell *et al.*, 2017), while $CuSO_4$ was shown to increase secondary metabolites production in plant cell cultures (Hernandez *et. al.*, 2005; Bota & Deliu, 2011). A gram of callus is used to initiate culture on 50 mL agar medium containing elicitor in a glass Petridish of 9 cm diameter. The negative control treatment was made by culturing callus in medium without the addition of elicitors and the blank control comprised of medium without callus initiation. The treatment and control cultures were prepared in triplicate. The cultures were incubated in the dark at 27°C up to 20 weeks. The cultures were subjected to volatile compound extraction from headspace, agar media and cells. Measurements of the fresh and dry weight of callus were taken. Callus was freeze-dried for 24 h for dry weight measurement.

C. Extraction of Volatile Compounds

A stir bar sorptive extraction (SBSE) technique using Twister, a 20 mm long stir bar with a 1 mm polydimethylsiloxane (PDMS) coating (Gerstel GmbH & Co. KG, Germany), was employed to extract the volatile compounds emitted by *A. filaria* callus (Figure 1c). The produced compounds were extracted from the headspace, medium and callus of the cultures (Hoffmann *et al.*, 2000). Headspace extraction was conducted by placing Twister inside the lid of the culture Petri dish which was placed upside down for 6 h. For extraction from agar medium, the medium was mixed to homogeneity and Twister was embedded in the agar for 3 h. Compound extraction from the callus was conducted on callus ground using mortar and pestle. The callus paste was diluted with the addition of 1 mL double-distilled water per gram of callus, and the callus solution was stirred with Twister for 2 h. Prior to preservation in enclosed glass vial, the Twister was rolled on a piece of Whatman filter paper to dry up the remaining water. The vial with the Twister was then placed in the fridge before gas chromatography–mass spectrometry (GC-MS) analysis.

D. Analysis of Extracted Volatile Compounds

Elucidation of volatile compounds secreted by the plant callus culture was conducted using the procedure modified from Hoffmann *et al.* (2000). Analysis of SQTs employed a GC-MS system equipped with thermal desorption unit (TDU). Compounds bound to the Twister were desorbed in the Gerstel TDU (Gerstel GmbH & Co. KG, Germany) on an Agilent Technologies 7890A GC coupled to a mass

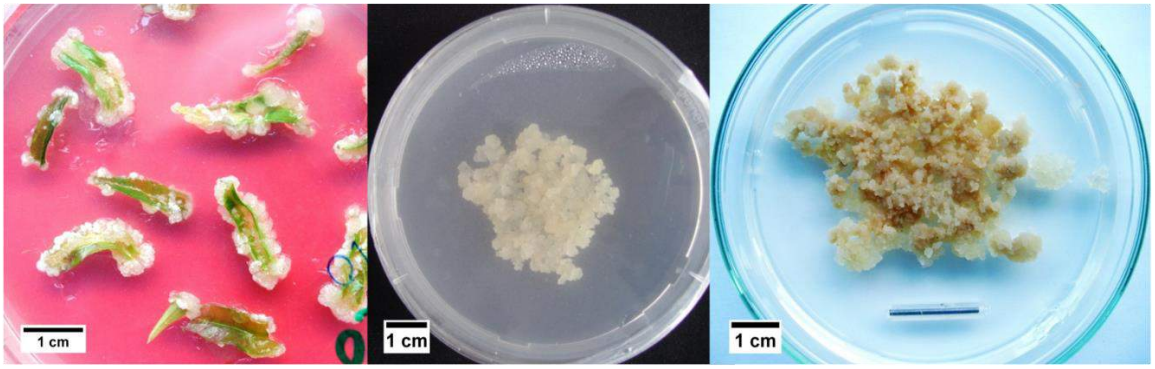

Figure 1. (a) Callus derived from the leaves of *Aquilaria filaria in vitro* plantlet used in the experiment. (b) Propagation and maintenance of *A. filaria* callus. (c) Extraction of volatile compounds from *A. filaria* callus treated with biotic and abiotic elicitor using the SBSE/Twister method.

spectrum detector (Agilent Technologies 5975C inert XL MSD with triple axis detector) with a fused silica capillary column (DB-1, 60 m × 250 µm inner diameter, 0.25 µm) (Agilent Technologies, USA). The mass spectrometry analysis used an electron impact ionisation mode at 1835 eV, mass range of 25–550 m/z and detector temperature of 150°C. Identification of compounds was carried out by comparison of the spectra of analysed samples to the spectra developed from pure compounds in the Symrise inhouse library (Adams, 2017; Nakanishi *et al*., 1981). The peak area of each volatile compound detected from headspace, medium and callus was estimated and considered as the amount emitted from *A. filaria* callus culture.

III. RESULTS

A. Fresh and Dry Weight of A. filaria *Callus Cultures*

Figure 2 showed the fresh and dry weight of *A. filaria* callus obtained from elicitors treated cultures. Abiotic elicitors $CuSO_4$ and $AgNO_3$ resulted in an improvement in the growth of *A. filaria* callus. The callus growth in $CuSO_4$-treated culture exceeded that of the control treatment. There was no significant difference in the weight of *A. filaria* callus harvested from the control and $AgNO_3$-treated cultures. In contrast, biotic elicitors, yeast extract and laminarin, repressed the *A. filaria* callus growth in terms of both fresh and dry weight of the callus. It is important to note that the application of 1 g/L of yeast extract elicitor in the culture medium reduced the fresh weight of *A. filaria* callus from 1 g used to initiate the culture to 0.68 g.

B. SQTs Emitted from A. filaria *Callus Cultures*

SQTs were detected not only from the elicitor-treated cultures of *A. filaria*, but also from the control culture without elicitor added to the medium. Sixteen SQTs produced by the *in vitro A. filaria* callus cultures were identified, namely jinkohol, columellarin, dehydrojinkoh-eremol, α-agarofuran, spathulenol, rotundon, α-guaiene, α-humulene, prezizaene, α-cedrene, α-cucurmene, eudesmane, longipinene, eremophilene, α-copaene and β-cedrene (Figure 3). In contrast, no SQT was detected from the blank medium (data not shown). Some prevalent SQTs emitted from the *A. filaria* callus cultures were jinkohol, α-humulene, prezizaene and α-cedrene.

The highest concentration of SQTs was detected from the $AgNO_3$-treated and yeast extract-treated cultures. The yeast extract-treated culture secreted only two types of SQTs: spathulenol and eudesmane. Of the *A. filaria* callus cultures, eudesmane SQTs were only detected in the yeast extract-treated culture. Meanwhile, $AgNO_3$-treated culture produced jinkohol and prezizaene at concentration higher than spathulenol and eudesmane in yeast extract-treated culture.

It was shown in Figure 3 that each type of elicitor induced different blend of SQTs. Laminarin-treated culture produced three types of SQTs. Six types of SQTs were detected from the $CuSO_4$-treated culture at low concentration, including jinkohol, spathulenol, rotundon, α-humulene, prezizaene and α-cedrene. Eight types of SQTs were emitted from the $AgNO_3$-treated culture, and

ten SQTs from the control culture without elicitor. Jinkohol, columellarin, dehydrojinkoh-eremol, α-agarofuran, α-copaene and β-cedrene were among the SQTs produced in the control culture, all at a rather low concentration.

IV. DISCUSSION

SQTs found contributing to the natural agarwood scent in previous studies (Nakanishi *et. al.*, 1983, Pripdeevech *et. al.*, 2011, Thuy *et al.*, 2019) were emitted from solid callus cultures of *A. filaria* in this study. The SQTs include α-agarofuran and dehydrojinkoh-eremol which were produced in the control culture; jinkohol and α-humulene detected in all cultures, except for those treated with yeast extract; and eremophilene which was detected in trace amount in the $AgNO_3$-treated culture. This is the first report on the production of valuable SQTs from callus culture of agarwood plant *Aquilaria filaria* to our knowledge. This study confirmed that plant bioreactor technique by means of *in vitro A. filaria* callus or cell cultures is an efficient system in producing natural agarwood SQTs. The *in vitro A. filaria* callus was competent to synthesise stress-induced high-impact fragrant SQTs in a manner similar to the well-differentiated cells in *Aquilaria* tree that produced natural agarwood. Vasconsuelo and Boland (2007) reported the association between biotic and abiotic stress and the synthesis of plant secondary metabolites as a plant defence mechanism. In plant callus or cell culture system, biotic and abiotic elicitors could be applied as inducers of plant

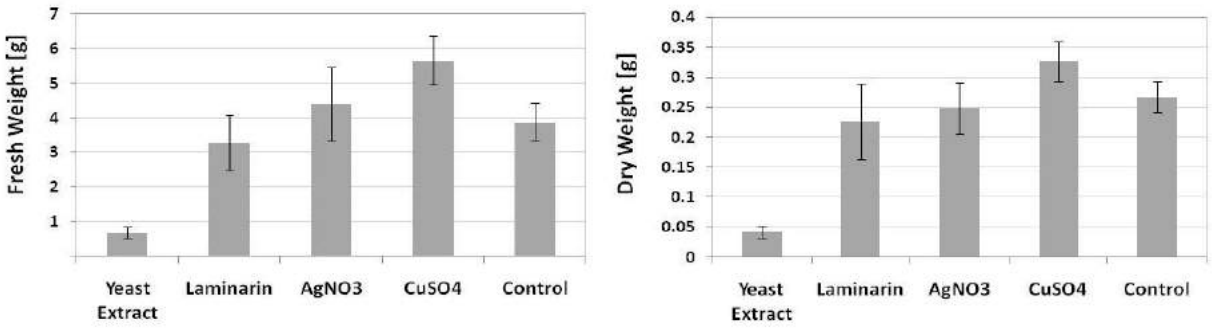

Figure 2. Fresh and dry weight of *Aquilaria filaria* callus cultures treated by biotic (yeast extract, and laminarin) and abiotic ($CuSO_4$ and $AgNO_3$) elicitors, expressed as mean and standard deviation of three replicates.

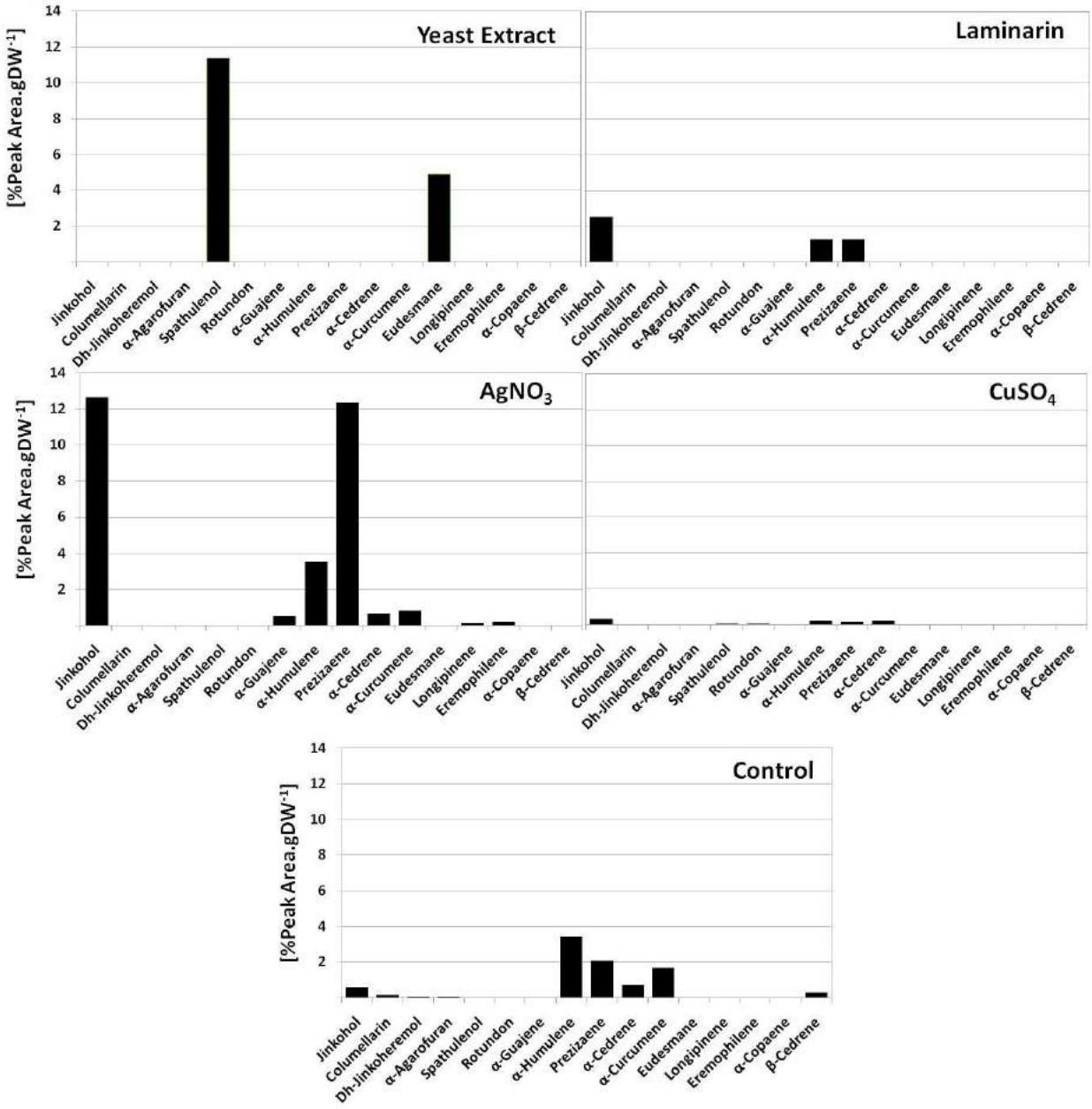

Figure 3. Volatile compounds emitted from the *Aquilaria filaria* callus cultures induced by biotic (yeast extract, laminarin) and abiotic ($CuSO_4$, $AgNO_3$) elicitors against control culture without elicitor.

defence metabolites. Moreover, Namdeo (2007) mentioned that both biotic and abiotic elicitors induced secondary products accumulation not only in intact plants or plant organs, but also in plant cell cultures as a result of their defensive, protective or offensive reactions. The emission of SQTs from non-treated cultures of *A. filaria* could be explained as a stress response to the *in vitro* controlled condition for culturing the plant callus.

Shukor *et al.* (2013) stated that application of biotic and abiotic elicitors in culture medium improved the variety as well as the amount of several secreted compounds. In agreement with their conclusion, the types and quantity of SQTs emitted from *A. filaria* callus culture in this study was found to be greatly dependent on the types of elicitors employed.

Yeast extract and $AgNO_3$ seemed to stimulate SQT production through different mechanisms. The component of yeast extract that functions as the elicitor was suggested to be low molecular weight peptides (Ferrari, 2010). Based on the study by Roat and Ramawat (2009), yeast extract added to the culture medium of plant cell cultures not only elicited secondary metabolites synthesis, but also functioned as a nitrogen source for plant growth. In contrast, yeast extract used in this study boosted the

synthesis of specific SQTs but reduced the fresh and dry weight of callus. Our finding was in line with the results from a study by Chen and Chen (2000) where they found yeast extract increased the production of secondary metabolites through a decrease in primary metabolite synthesis and cell biomass.

$AgNO_3$ improved the cell biomass as opposed to the yeast extract treatment. It was suggested that $AgNO_3$ promoted cell regeneration as the mechanism for eliciting secondary metabolites titre. Xu *et al.* (2007) and Kumar *et al.* (2009) thought that $AgNO_3$ stimulated the production of secondary metabolites in plant cell cultures by controlling the ethylene action on plant cells. Furthermore, Ferante and Francini (2006) stated that ethylene in plant caused necrosis and chloroplast degradation in leaf cells which led to cell death. Silver ions blocked the action of ethylene and promoted auxin efflux which in turn promoted an increase in cell biomass (Strader *et al.*, 2009).

Laminarin is a type of β-glucan from algae. Glucan is a polysaccharide present in the plant cell walls which will be released following a fungal attack (Siddiqui *et al.*, 2013). However, the addition of laminarin to the *A. filaria* culture medium did not elicit the production of secondary metabolites as well as the yeast extract elicitor treatment did. Similarly, although cupric ions were shown to increase secondary metabolites production in plant cell cultures of many species (Ramakrishna & Ravishankar, 2011), the SQTs produced in *A. filaria* cell cultures induced by $CuSO_4$ were minute in amount.

V. CONCLUSION

In vitro callus cultures of *A. filaria* can be used as bioreactor for agarwood fragrant SQTs production. Different types of elicitors induced the production of specific types and amount of agarwood SQTs in the *A. filaria* callus culture.

VI. ACKNOWLEDGEMENTS

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