Presence of Actinomycetes in agarwood tissues of *Aquilaria crassna*: A preliminary study

A.N.G.C.K. Vidurangi[1], D.S. Manamgoda[2], S.M.C.U.P. Subasinghe[1]*

1. Centre for Forestry and Environment, Department of Forestry and Environmental Science, University of Sri Jayewardenepura, Nugegoda, Sri Lanka

2. Department of Botany, University of Sri Jayewardenepura, Nugegoda, Sri Lanka

Date Received:14th August 2022 Date Accepted: 28th September 2022

Abstract

Agarwood is a valuable resin produced inside certain tree species of the family Thymalaeaceae distributed in the Asian region. Agarwood production occurs as a defense mechanism when the trees are under physical or biological stresses. However, the formation of agarwood resins in significant extractable quantities due to natural stress conditions is rare; therefore, the farmers use various methods to induce its formation artificially. Certain fungal species such as *Fusarium* and *Aspergillus* become more popular among them to produce high-quality agarwood. However, studies are rare on using other microbial organisms such as Actinomycetes, which exhibit properties of both bacteria and fungi. Among the agarwood-producing species, *Aquilaria crassna* is one of Asia's most commonly planted species for agarwood production. This species was introduced to Sri Lanka in 2012 for mid and lower elevations of the wet zone. Due to the lack of studies on agarwood resin formation by non-fungal microbial methods, the present study attempted to identify the presence of Actinomycetes species in agarwood resinous tissues of *A. crassna*. Agarwood resinous tissue samples were collected from four plantations in the wet zone of the country. Surface sterilized, small sized tissues were placed on starch casein agar medium and incubated at room temperature for ten days. Using the morphological and microscopic characteristics, it was possible to identify *Nocardia, Psuedonocardia*, and three *Streptomyces* species with varying abundance. The species level should be confirmed using molecular analysis, and their potential for agarwood resin formation inducement should be tested by re-inoculating to the healthy *A. crassna* trees.

Keywords: *Aquilaria crassna*, Agarwood, Isolation, Actinomycetes species

1. Introduction

Aquilaria crassna of the family Thymalaeaceae is commercially the most common agarwood-forming species in Asia. Though it is not native, plantation establishment using *A. crassna* in the low country wet and intermediate zones of Sri Lanka started in 2012 as a short rotation crop. The agarwood, which is dark resinous heartwood forms in this species, fetches a high value in the market as an ingredient for Chinese medicine, perfume, and incense manufacturing (Blanchette, 2003). The quality and the unique characteristics of agarwood resins vary due to its inducement method, region of growth and age of trees. The resin formation within trees due to natural causes is rare; therefore, the demand is higher than the available quantity on a commercial scale (Akter et al., 2013).

Correspondence: upuls@sjp.ac.lk
ISSN 2235-9362 Online

Agarwood resin is not present inside the tree during its growth, and its formation occurs due to pathological or non-pathological damage to the stem and roots. Therefore the *A. crassna* plantations owners use different methods such as wounding to artificially induce agarwood resin formation (Liu et al., 2013). However, those non-pathological inducement methods are not capable of producing good quality and large quantity of agarwood (Cheong and Choi, 2013). Therefore, using biological methods such as certain fungal species became common among them to induce agarwood resin formation (Subasinghe et al., 2019). Those fungal species help to spread the induction through their hyphal growth in the tree to other regions of the stem and roots (Turjaman et al., 2016). In its natural surroundings, *A. crassna* is highly exposed to a diverse group of microorganisms (Nimnoi et al., 2011), and therefore it is essential to identify the potential endophytic microorganisms growing with *Aquilaria*. Endophytes are described as microorganisms that establish an endo-symbiotic relationship with plants, whereby plants receive ecological benefits from the presence of the symbionts, such as the ability to increase tolerance to stresses or plant growth promotion (Hasegawa et al., 2006).

Some endophytic microorganisms have developed the ability to produce the same or similar bioactive substances such as pathogenesis-related enzymes or what their host plants produce that may be involved in a symbiotic association (Strobel and Daisy, 2003). Changes in the activity of various enzymes in naturally infected trees indicate that they may be involved in the infection process and developing disease symptoms in agarwood trees. Among the Actinomycetes species associated with wood-forming areas, a few could exhibit pathogenesis while others seem to be saprophytic. Many actinomycetes such as *Strptomyces* promote plant growth and increase the biomass (Lin and Xiu, 2013). They also improve the soil condition by enhancing the nutrient recycling (AbdElgawad et al., 2020). The morphology of Actinomycetes is a fungi-like bacteria forming long filaments stretching through the substrate, which have an extensive impact on the environment by decomposing and transforming a wide variety of complex organic residues (Malvia et al., 2014). Endophytes such as *Actinomycetes* colonize within plants and usually confer nutrition to the host plants and also prevent host plants being attacked by plant pathogens by producing a variety of lytic enzymes (Nimnoi et al., 2010). They can also benefit plants by producing phytohormones, siderophores, antibiotics, and fixing nitrogen (Bailey et al., 2006).

Though there are many studies in the literature on agarwood resin inducement by fungal species, the ability of Actinomycetes species for the same is not commonly studied. Therefore, the present study aimed to identify the Actinomycetes species associated with the agarwood resinous tissues of *A. crassna* to provide preliminary recommendations that can be used to develop artificial inducers of agarwood resin formation at a commercial scale.

2. Materials and Methods

2.1 Study sites and sampling of plant materials

A. crassna plantations established between 2012-14 in four locations (Handapangoda, Horana, Ingiriya, and Mathugama) of the low country wet zone were selected for collecting samples (Figure 1). All plantation locations are located in Kalutara administrative district of the western province of Sri Lanka. Handapangoda, Horana and Mathugama plantations are located in the Wet-Low 1a agro-ecological zone and Ingiriya plantation is located in the Wet-Low 1b zone. The average annual rainfall of those zones are 2,800-3,200 mm and the average temperature is 25^{o}-28^{o} C. The soil type is red yellow podzolic. All plantations were established on lands with minimal slopes and the average diameter and height growth of the trees in those plantations are given in Table 1. The ground was found to be covered with native grasses. Young and mature *Aquilaria* plantations were available only in the wet zone of Sri Lanka and therefore,

sampling was restricted that zone. Sample collection was carried out during dry periods. Those plantations have been managed under minimal treatments such as occasional weeding and application of organic fertilizer.

Table 1: Average growth values ±SE of *A. crassna* trees in the selected plantations.

Location	Diameter (cm)	Height (m)
Handapangoda	20.8±1.0	10.6±0.2
Horana	17.6±0.4	9.0±0.3
Ingiriya	15.0±0.9	9.6±0.3
Mathugama	20.1±0.7	11.8±0.6

Agarwood resinous tissues formed due to abrasions and broken branches were collected from three trees of each plantation in a non-destructive manner. After identification of the wounds in the stem, a small sample was first tested to confirm the presence of agarwood resin which produces a strong pleasant aroma when burning. Then those tissues were extracted using a sterilized sharp knife and a chisel. Collected samples were sealed in sterilized polythene bags packed in a cool bag adjusted to 4-5^{o}C temperature and then stored at the laboratory at 4±1^{o}C until further analysis.

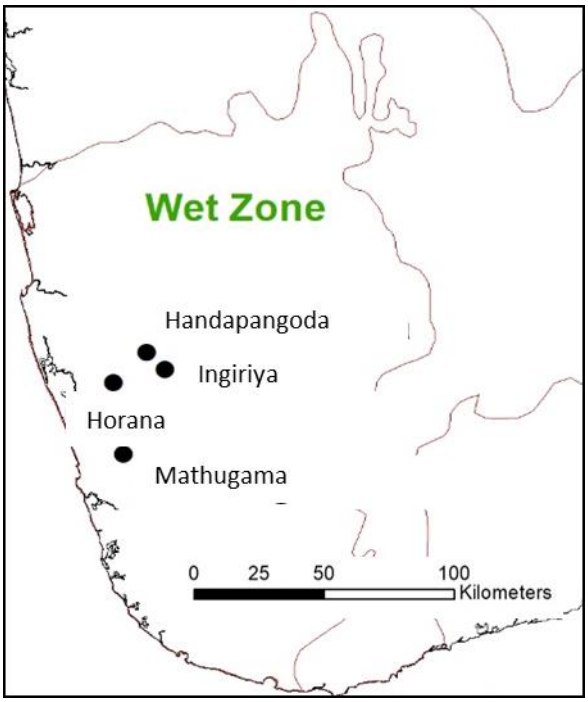


Figure 1: Locations of the selected plantations for sample collection.

2.2 Isolation of Actinomycetes species

The tissues were reduced to about 1×1×0.5 cm using sterilized sharp-edge cutters in the laboratory. They were surface-sterilized by placing in 10% sodium hypochlorite for 2 min followed by in 70% alcohol for

another 2 min. Then the samples were rinsed in sterilized distilled water twice (Laurence, 2013). Those were placed on a starch casein agar (SCA) medium with 100 µg ml^{-1} of nystatin and cycloheximide to inhibit the fungal growth (Wang et al., 1999). Three replicates were prepared for each sample and incubated at 29±1^{o} C for ten days under aseptic conditions to obtain the maximum colony appearances. Actinomycetes colonies of different morphology were transferred to separate SCA plates to isolate the pure Actinomycetes cultures. Microscopic examinations were used to identify the species level of the isolated Actinomycetes species.

2.3 Morphological observations and estimation of relative abundance

Observations of the growth of different Actinomycetes colonies were carried out periodically for ten days using sub-cultures in SCA medium. The morphological characteristics, viz., the colour of aerial mycelium and substrate and mycelium pattern, were visually studied. Hyphae and conidia's shapes were identified using the Trinocular Microscope by preparing slide cultures for each isolation. For this, mycelia were carefully scraped to avoid damage to hyphae structures. The mycelia were then stained by methylene blue, and a cover glass was carefully placed on top to avoid bubble formations (Nimnoi et al., 2010). Relative abundance was calculated as a percentage of the total number of individual colonies belonging to one taxon divided by the total number of colonies belonging to all taxa (Gong and Guo, 2009).

3. Results

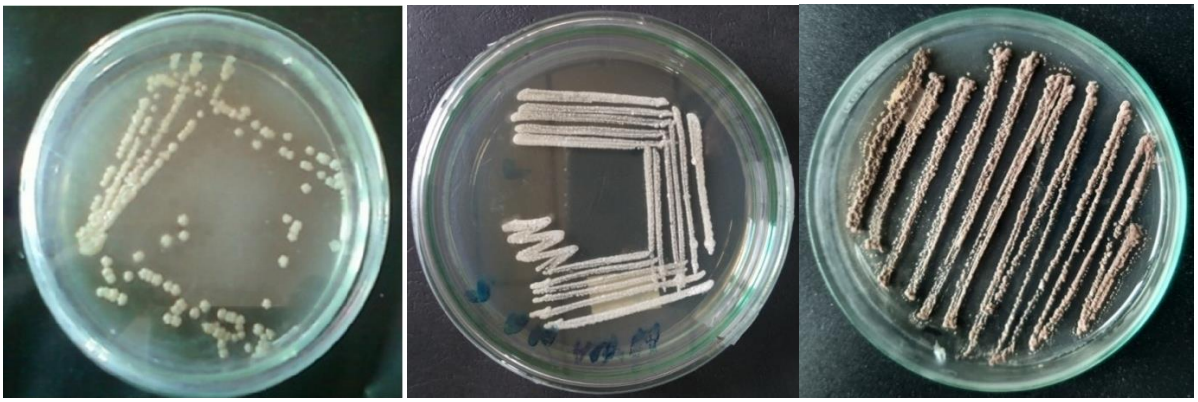
3.1 Morphological observations

The results of the visual and microscopic morphological observations were used to identify the endophytic Actinomycetes in the agarwood resinous tissue samples. Altogether, it was possible to identify five isolates belonging to three genera (Table 2). Though the characteristics of *Streptomyces* sp2 and 3 are similar in Table 2, the colony appearance was different (Figure 2).

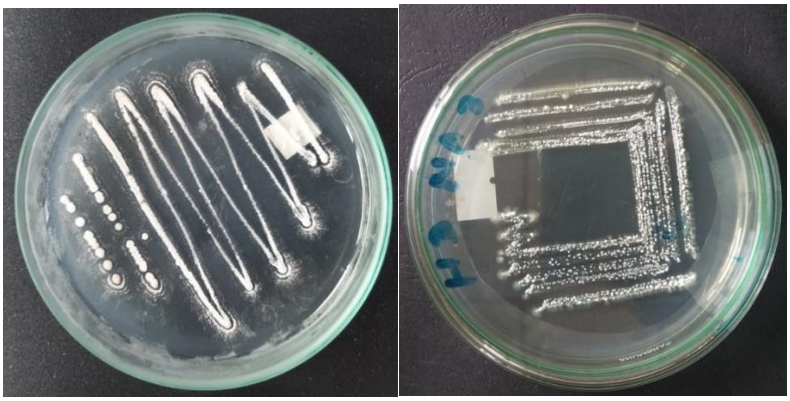
Table 2: Colony and microscopic characteristics of Actinomycetes isolates.

Colony characteristics				Microscopic characteristics	Isolate name
Diameter (mm)	AM	SM	PG		
3	Ivory	White	-	Branched and rod-shaped fragments	*Nocardia* sp.
3	Dusty gray	Yellow	-	Long chains of spores	*Pseudonocardia* sp.
5	Brown	Black	Brown-black	Spiral and long chains of spores	*Streptomyces* sp1.
4	Ivory cream	Cream-yellow	-	Spiral and long chains of spores	*Streptomyces* sp2.
5	Ivory cream	Cream-yellow	-	Spiral and long chains of spores	*Streptomyces* sp3

AM=aerial mycelium colour; SM= substrate mycelium colour; PG=diffusible pigments released into the medium.



(a) *Pseudonocardia* sp (b) *Nocardia* sp. (c) *Streptomyces* sp1



(c) *Streptomyces* sp2 (d) *Streptomyces* sp3

Figure 2: Pure cultures of Actinomycetes colonies of different genera.

3.2 Abundance of Actinomycetes species and distribution in agarwood resinous tissues in different plantations

A total of 27 Actinomycetes colonies were isolated from 144 resinous tissues collected from *A. crassna* trees of four locations. However, the abundance of Actinomycetes species in agarwood resinous tissues of *A. crassna* varied in four locations (Figure 3). Among them, *A. crassna* in Ingiriya had the highest number (3) of Actinomycetes species, and the lowest number was recorded in Mathugama (1). Nocardia and Psuedonocardia were found only in Horana and Handapangoda plantations respectively, while Streptomyces sp1 was found only in Ingiria plantation. Streptomyces sp2 was found as the most common which was in three plantations, viz. Horana, Handapangoda and Ingiriya and sp3 was found in Ingiriya and Mathugama. Further studies are needed, however, to identify the potential of those Actinomycetes species to induce agarwood resin formation, which should be done by re-inoculating those species separately to healthy *A. crassna* trees.

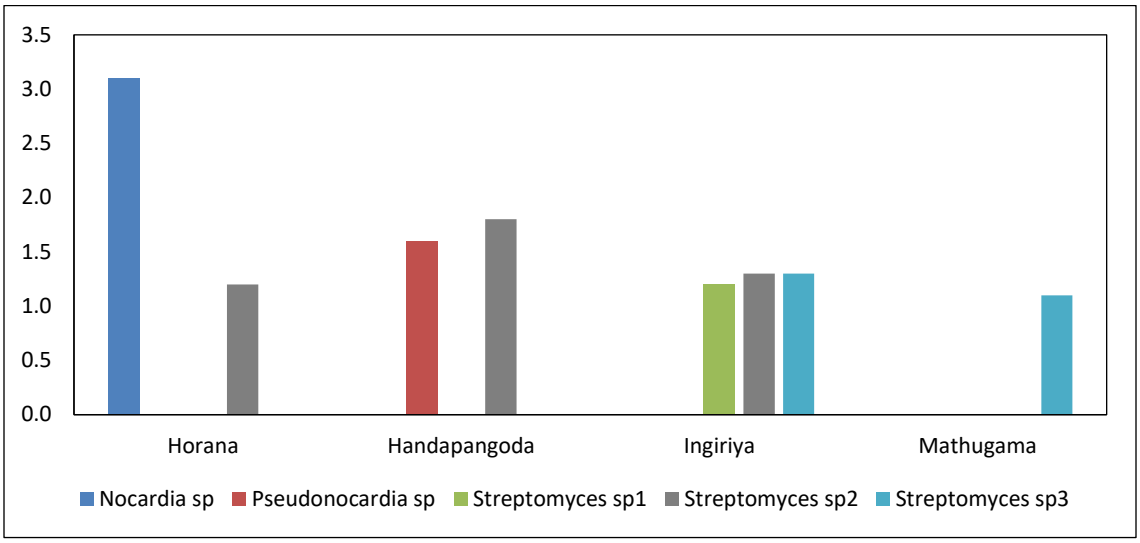


Figure 3: Abundance percentage of Actinomycetes in the resinous tissues of *A. crassna*.

4. Discussion

We selected the starch casein agar medium for culturing Actinomycetes species, which is capable of providing the saccharolytic organisms enough nutrients to produce their proteins and carbohydrates (Hasegawa et al., 2006). These high nutrient media is essential to isolate Actinomycetes species from terrestrial sources. Though Bredholdt et al. (2007) stated that the Actinomycetes are one of the significant microbial dominant groups showing the capacity to survive in extreme habitats, their presence in agarwood resinous tissues of *A. crassna* was limited compared to the fungal communities. It could be due to the ability of the identified isolates to induce agarwood resin formation. Else, it could be due to suppressing their growth by the fungi belonging to genera of *Aspergillus*, *Cunninghamella*, *Curvularia*, *Fusarium*, *Lasiodiplodia*, *Xylaria* etc. which are commonly found in agarwood resinous tissues in many countries (Cui et al. 2013, Mohamed et al., 2014, Subasinghe et al., 2019). The present study identified the Actinomycetes up to the genus level, which should be further identified by molecular analysis. The essential next step would be to test their ability to form agarwood resins in healthy *A. crassna* trees grown in diverse locations in Sri Lanka.

References

Akter, S., Islam, M.T., Zulkefeli, M. and Khan, S.I., 2013. Agarwood production-a multidisciplinary field to be explored in Bangladesh. *International Journal of Pharmaceutical and Life Sciences*, 2(1): pp.22-32.

Bailey, B.A., Bae, H., Strem, M.D., Roberts, D.P., Thomas, S.E., Crozier, J., Samuels, G.J., Choi, I.Y. and Holmes, K.A., 2006. Fungal and plant gene expression during the colonization of cacao seedlings by endophytic isolates of four Trichoderma species. *Planta*, 224(6): pp.1449-1464.

Bredholdt, H., Galatenko, O.A., Engelhardt, K., Fjærvik, E., Terekhova, L.P. and Zotchev, S.B., 2007. Rare actinomycete bacteria from the shallow water sediments of the Trondheim fjord, Norway: isolation, diversity and biological activity. *Environmental microbiology*, 9(11): pp.2756-2764.

Blanchette, R.A., 2003. Deterioration in historic and archaeological woods from terrestrial sites. *Art, biology, and conservation: biodeterioration of works of art. The Metropolitan Museum of Art, New York, NY*: pp.328-347.

Cheong, J.J. and Do Choi, Y., 2003. Methyl jasmonate as a vital substance in plants. TRENDS in Genetics, 19(7): pp.409-413.

Cui, J., Guo, S., Fu, S., Xiao, P. and Wang, M., 2013. Effects of inoculating fungi on agilawood formation in Aquilaria sinensis. Chinese Science Bulletin, 58(26): pp.3280-3287.

Gong, L. and Guo, S., 2009. Endophytic fungi from *Dracaena cambodiana* and *Aquilaria sinensis* and their antimicrobial activity. African Journal of Biotechnology, 8(5): pp.731-736.

Hasegawa, S., Meguro, A., Shimizu, M., Nishimura, T. and Kunoh, H., 2006. Endophytic actinomycetes and their interactions with host plants. Actinomycetologica, 20(2): pp.72-81.

Laurence, W.V.A., 2013. Isolation and characterization of endophytes isolates from akar gaharu. Research Dissertation, Universiti Malaysia Sarawak, Malaysia.

Liu, Y., Chen, H., Yang, Y., Zhang, Z., Wei, J., Meng, H., Chen, W., Feng, J., Gan, B., Chen, X. and Gao, Z., 2013. Whole-tree agarwood-inducing technique: an efficient novel technique for producing high-quality agarwood in cultivated Aquilaria sinensis trees. Molecules, 18(3): pp.3086-3106.

Malviya, N., Yandigeri, M.S., Yadav, A.K., Solanki, M.K. and Arora, D.K., 2014. Isolation and characterization of novel alkali-halophilic actinomycetes from the Chilika brackish water lake, India. Annals of microbiology, 64(4): pp.1829-1838.

Mohamed, R., Jong, P.L. and Kamziah, A.K., 2014. Fungal inoculation induces agarwood in young Aquilaria malaccensis trees in the nursery. *Journal of forestry research*, 25(1): pp.201-204.

Nimnoi, P., Pongsilp, N. and Lumyong, S., 2010. Endophytic actinomycetes isolated from Aquilaria crassna Pierre ex Lec and screening of plant growth promoters production. *World Journal of Microbiology and Biotechnology*, 26(2): pp.193-203.

Nimnoi, P., Pongsilp, N. and Lumyong, S., 2011. Actinobacterial community and diversity in rhizosphere soils of Aquilaria crassna Pierre ex Lec assessed by RT-PCR and PCR-DGGE. Biochemical systematics and Ecology, 39(4-6): pp.509-519.

Strobel, G. and Daisy, B., 2003. Bioprospecting for microbial endophytes and their natural products. Microbiology and molecular biology reviews, 67(4): pp.491-502.

Subasinghe, S.M.C.U.P., Hitihamu, H.I.D. and Fernando, K.M.E.P., 2019. Use of two fungal species to induce agarwood resin formation in Gyrinops walla. *Journal of Forestry Research*, 30(2): pp.721-726.

Turjaman, M., Hidayat, A. and Santoso, E., 2016. Development of agarwood induction technology using endophytic fungi. In Agarwood (pp. 57-71). Springer, Singapore.

Lin, L. and Xu, X., 2013. Indole-3-acetic acid production by endophytic Streptomyces sp. En-1 isolated from medicinal plants. Current Microbiology, 67(2): pp.209-217.

AbdElgawad, H., Abuelsoud, W., Madany, M.M., Selim, S., Zinta, G., Mousa, A.S. and Hozzein, W.N., 2020. Actinomycetes enrich soil rhizosphere and improve seed quality as well as productivity of legumes by boosting nitrogen availability and metabolism. Biomolecules, 10(12): p.1675.

Wang, Y., Zhang, Z.S., Ruan, J.S., Wang, Y.M. and Ali, S.M., 1999. Investigation of actinomycete diversity in the tropical rainforests of Singapore. Journal of Industrial Microbiology and Biotechnology, 23(3), pp.178-187.