Thai J. For. 9 : 163-171 (1990) วารสารวนศาสตร์ 9 : 163-171 (2533)

ROLES AND ACTIVITIES OF FUNGI ASSOCIATED WITH AGARWOOD AND KRITSANA TREE IN THAILAND

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บทคัดย่อ

การสำรวจและการทดลองเพื่อต้องการทราบถึงอิทธิพลและกิจกรรมของเชื้อราที่ทำให้เกิดไม้หอมกับต้นกฤษณา (*Aquilaria crassna* Pierre ex H. Lec.) นั้นได้ดำเนินการวิจัยในบริเวณท้องที่อุทยานแห่งชาติเขาใหญ่ จังหวัดนครราชสีมา และในท้องที่จังหวัดอื่น วิธีการศึกษาได้ทำการทดลองโดยใช้วิธีเปลือยรอยบาดแผล (Open wounding) และวิธีควบคุมรอยบาดแผลเพื่อป้องกันเชื้อราเข้าทำลาย (Controlled infection of fungi) ทำการแยกเชื้อราที่เกิดกับรอยแผล และตรวจพิสูจน์ชนิดของเชื้อราที่แยกได้จากรอยบาดแผลที่ทำการทดลอง นอกจากนั้นยังได้ทำการตรวจแยกเชื้อราจากไม้หอมกฤษณาที่เกิดขึ้นเองตามธรรมชาติ สำรวจเชื้อราที่ทำให้ไม้กฤษณาผุ โรคของเมล็ดและกล้าไม้กฤษณา และเชื้อราไมคอร์ไรซาของต้นไม้กฤษณา ผลการวิจัยพบว่าการเกิดไม้หอมกฤษณา (Agar or oleoresin deposit) นั้นมิได้มีความสัมพันธ์กับอิทธิพล และกิจกรรมของเชื้อราซึ่งเชื่อถือกันมาแต่อดีตอันยาวนานแต่อย่างใด แต่เป็นที่ปรากฏแน่ชัดว่าไม้หอมกฤษณานั้นเกิดจากปฏิกิริยาโต้ตอบขึ้นภายในของต้นไม้เอง โดยเกิดจากรอยบาดแผลบนต้นไม้ อันเนื่องมาจากขบวนการทางกลวิธี ทางฟิสิกส์ ทางเคมี หรือทางชีววิธี อย่างไรก็ดีผลอันเกิดจากปฏิกิริยาโต้ตอบในการป้องกันอันตรายจากรอยบาดแผล หรือจากผลของการกระทำทางชีววิธีต่อไม้กฤษณานั้นคาดว่าเป็นสาเหตุที่สำคัญต่อการเกิดน้ำยางหอม (oleoresin deposit) ขึ้นในลำต้นไม้กฤษณา การพบเชื้อราในเนื้อไม้กฤษณานั้นเป็นสาเหตุทำให้ไม้หอมกฤษณาด้อยคุณภาพ และน้ำหนักลดน้อยลง

ABSTRACT

Survey and experiments to determine the roles and activities of fungi in the formation of incence aloes-wood or agarwood on Kritsana tree (*Aquilaria crassna* Pierre ex H. Lec.) were carried out at Khaoyai National Park, Nakhon Ratchasima, and other provinces. Open wounding and controlled infection of fungi were studied. Many fungi infected in the artificial woundings, fungi colonized on naturally kritsana trees, wood – rotting fungi, seed – borne and seedling diseases and mycorrhizal association with kritsana or agarwood threes were isolated and identified. Results revealed that kritsana or agar (oleoresin deposit) formation did not correlate with the roles or activities of specific fungi, as was previously believed, but it was obviously

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formed by the interaction of the tree host through injuries or woundings which might be derived from the mechanical, physical, chemical or biological processes. However the result of a protective reaction of the tree host to injuries, woundings or biological invasions to form oleoresin deposits in kritsana trees was expected as the main causes, the fungal infection in agarwood would be degraded to the quality and reduced in weight of agar or kritsana.

INTRODUCTION

Kritsana is the tree, classified in the genus *Aquilaria* (Family : Thymelaeaceae). The tree species naturally occur in India, Bangladesh, China, Burma, and extend through Southeast Asia to the Philippines (Ding Hou , 1960, 1964). Only 3 out of 15 species of the plant have known to distribute in Thailand:- *Aquilaria crassna* Pierre ex H. Lec., *A. malaccensis* Rosb. (Syn. *A. agallocha* Roxb.), and *A. subintegra* Ding Hou (Siripatanadilok, 1982).

Kritsana or agarwood tree is a large evergreen tree, 18-21 m in height and 1.5-1.8 m in girth, with a moderately straight and often fluted stem. The sound wood is soft, light and elastic. It is white to pale yellowish white and has no characteristic odor. Anatomically it is featured by the absence of growth rings, medium sized to small vessels borne mostly in radial rows of 3-4 and numerous longitudinal interxylery strands of included phloems consisting of soft bast and some fibers. The heartwood is not distinct (Prachakul, 1989).

The agarwood is known as Agar (Hindi), Sasi (Assamese), Indian Eaglewood (Trade) and kritsana (Thai). This wood contains patches of oleoresin deposits. It has been entered into trade of Thailand since 600-700 years ago (Anuwatvanarak, 1942) under the name Agar or kritsana (incence or fragrant wood). This has been prized since time immemorial in Egypt, Arabia and throughout the East for use as incence, perfumes, drugs, stimulant, cordial tonic, carminative and enters into several compound preparations (Atal and Kapur, 1982).

Previously scientists believed that agar was regarded as a pathological product formed as the result of a fungus disease which had been identified the fungus mycelium present in the cells of the diseased wood as belonging to the group Fungi Imperfecti (Jalaluddin, 1977). But recently Rahman and Basak (1980), Rahman and Khisa (1984), and Gibson (1977) reported that there was no primary role of any specific fangus in the formation of agar as was previously believed.

Agar consists of irregular patches of dark wood highly charged with oleoresin, within the trunk or branch. The agar-bearing trees have a somewhat like diseased appearance, and the oleoresin is usually found where the branches loosen out from the stem. Agar is found frequently in young trees about 20 years old, but the tree about 50-60 years have the highest agar concentration (Gibson, 1977; Rahman and Khisa, 1984).

Agar is found in the market in the form of chips and splinters and also with blocks. Agar has a slightly bitter aromatic taste and peculiar odor comparable to that of sandalwood or of ambergris. It burns with a bright frame or smoulders giving off a pleasant odor of volatile oil. True agar is hard, heavy, brown and rich in resin. Black and heavy agar commands the highest price in the market. A softer and yellowish white variety is of comparatively inferior quality. It is used of volatile oil distillalion cottage industry called *agar attar* (Rao and Bhatia, 1959).

This study aims to investigate and examine the role and activities of fungi in the formation of oleoresin within Kritsana tree *(Aquilaria crassna)*. Fungi, as wood-rotting fungi, diseases and mycorrhizal association were also studied.

METERIALS AND METHODS

Infection of Fungi in Artificial Wounding

Experiments to determine the role and activities of fungi infected in the artificial woundings and agar formation in Kritsana tree *(Aquilaria crassna)* were conducted at Khao Yai National Park, Nakhon Ratchasima Province, Central Thailand. Four sizes of Kritsana trees; GBH 69, 100, 100 and 108 cm were selected. The individual tree was wounded by a sharp-edged chisel for making square holes of 2.5×2.5 cm. Three replications were assigned for each of five treatments in a tree; open wounding (control), Santar SM surfaced paint, Santar Sm + surfaced oil paint, Santar A surfaced paint, and Santar A + surface oil paint. The experiment was lasted for 2 months. After 2 months, the upper and lower parts of wounding holes were removed out at a depth of 2.5 cm for drawing of test-block samples. The test blocks then quickly transferred to keep in cleaned plastic bags for isolating fungi and examining the agar formation in the laboratory. All test-block samples were first examined for surface discoloration. If contamination was suspected, it was flamed immediately before incubating. Small pieces of test blocks were drawn aseptically with 4 replicates from wounded wood under cut surface including the zone of dark-brown and white areas, then were incubated in sterile humid chambers (plastic boxes) and petri-dishes by blotter and PDA agar method (Neergaard, 1977) for 5-7 days under dark/near ultraviolet light (NUV) for 12/12 hr cycles at room temperature (30° ± 3° C). After fungi sporulating over the test samples, their characteristics on genera and species were examined and identified in precentage occurrence.

Infection of Fungi in Naturally Formed Agarwood

During study period, survey of Kritsana or agarwood in natural occurrence had been attempted. Agarwood samples were collected and studied. Fungi associated with agarwood samples were investigated and identified using the methods described in 1. Natural agarwood were collected from various locations : such as Khao Yai National Park at Nakhon Ratchasima and Prachin Buri Provinces; Khao Soi Dao Wildlife Sanctuary at Chanthaburi Province; and Trang Wildlife Sanctuary at Trang Province. Immediately after collection, all samples were examined for the presence of fungi colonized in agarwood. The methods of Neergaard (1977) as detailed in 1 were employed for direct incubation and isolation of associated fungi. Identification of fungi was reported in percentage occurrence.

Survey was made to investigate the occurence of wood rotiting fungi decayed in agarwood of *Aquilaria spp.* The higher fungi in the group of Basidiomycotina (Hymenomycetes), and Ascomycotina infected in standing trees, decayed on tree trunks, wood, timbers, branches and barks were collected, idenfified and recorded.

Seed-Borne and Seedling Diseases

Fruits and seeds of *Aquilaria crassna* were collected from Khao Yai National Park and Khao Soi Dao Wildlife Sanctuary. Fruits were broken out and then the internal seeds were taken off. Two hundred seeds were placed in the petridish with 10 seeds each and then incubated using the blotter and PDA agar methods as described by Neergaard (1977). The presence of associated fungi was isolated and identified after 5-7 days incubation under 12/12 hr NUV light/dark cycles at $30° \pm 3°$ C.

Mycorrhizal Association

Mycorrhiza colonized on tree roots of *Aquiaria spp.* has played the vital role to the growth and nutrient uptake of the plant. Roots and soils at 10-20 cm depth along the rhizosphere of Agar tree were collected from Khao Yai Natinal Park. The soils mixed with tree roots were brought to extract in the laboratory by wet sieving and decanting techniques as described by Gerdemann and Nicolson (1963). The lateral and short roots were seperated from the soils, cleaned and fixed in 3% glutaraldehyde. The techniques for clearing and staining roots and assessement of mycorrhizal roots were using the techniques of Phillips and Hayman (1970). Mycorrhizal association on tree roots was visualized and examined by stereo-and compound microscopes. Azygospores and chlamydospores of endomycorrhizal (vesicular – arbuscular) fungi were isolated and identified.

RESULTS AND DISCUSSION

Infection of Fungi in Artificial Wounding on Kritsana Tree

In Table 1, the occurrence of agarwood and fungal infection in Kritsana tree *(Aquilaria crassna)* were recorded to determine the role and activities of fungi by woundings in 4 sizes of selected Kritsana trees; DBH 69, 100, 100 and 108 cm; treated by 5 treatments: control (open-wounding), Santar SM surfaced paint only, Santar SM + surfaced oil paint, Santar A surfaced paint only, and Santar A + surfaced oil paint. Results found that there were fungal infections in the treatments of control (open wounding alone), Santar SM surfaced paint only and Santar A surfaced paint only; but no incidence of fungal infection in the treatments of Santar SM + surfaced oil paint and Santar A + surfaced oil paint. As illustrated in Table 1 it was indicated that whether fungal infection or not, the agar formation in Kritsana trees could be occurred. These incidences could be further explainable that the role and activities of fungi had no effect to form the agarwood because woundings and injuries in Kritsana trees could lead to form agar or oleoresin deposits. This experimental result was similar to that found in the studies of Rahman and Basak (1980), Rahman and Khisa (1984) and Gibson (1977). They were reported that there was no primary role of any specific fungus in the formation of agarwood as was previously believed.

Table 1. Experiment to determine the role of fungal infection and woundings in the formation of agarwood on Kritsana tree *(Aquilaria crassna)* treated by fungicidal pastes, surfaced oils paints and control

Treatments	Fungal infection in Kritsana trees				Conclusion of fungal infection	Occurence of agarwood
	Tree no. 1* (GBH 69 cm)	Tree no. 2* (GBH 100 cm)	Tree no. 3* (GBH 100 cm)	Tree no. 4* (DBH 108 cm)		
1. Control (openwounding alone)	+	+	+	+	+	Yes
2. Santar SM surfaced paint only	+	+	+	+	+	Yes
3. Santar SM + surfaced oil paint	–	–	–	–	–	Yes
4. Santar A surfaced paint only	+	+	+	+	+	Yes
5. Santar A + surfaced oil paint	–	–	–	–	–	Yes

Note + fungal infection
– No fungal infection
* results of 3 replicates.

Table 2. Fungi associated with agarwood test blocks from artificial wound of trees at Khao Yai National Park

Fungus species	Frequencies of associated fungi	
	Blotter Method (%)	PDA Method (%)
*1. *Fusarium oxysporum* Schlecht	10.0	11.6
*2. *Botryodiplodia theobromae* Pat.	8.3	8.3
3. Bacteria	6.7	5.0
*4. *Fusarium sp.*	3.3	–
5. *Mucor hiemalis* Wehmer	3.3	–
6. *Rhizopus sp.*	3.3	1.6
7. *Aspergillus niger* Gr.	3.3	1.6
8. *Penicillium sp.*	3.3	1.6
*9. *Fusarium stoveri* Booth	1.6	–
10. *Trichoderma viride* Pers.	1.6	1.6
11. *Chaetomium spirale* Zopf	1.6	1.6
12. *Cladosporium sp.*	1.6	1.6
13. *Curvularia lunata* (Wakk.) Boed	1.6	1.6
14. *Pithomyces sp.*	1.6	–
15. *Cephalosporium sp.*	1.6	1.6

* *known to be parasitic*

In Table 2, the fungi and bacteria recorded were listed with precentage occurrence by blotter and PDA methods. Fifteen species of fungi and bacteria were identified and several of these were saprophytic, but few were possibly parasitic (e.g. *Fusarium oxysporum* and *Botryodiplodia theobromae)*. The fungi found include 14 deuteromycetes (Fungi Imperfecti) and 1 bacterium. The species of fungi identified were similar to that found in other studies (Gibson, 1977; Rahman and Basak, 1980; Rahman and Khisa, 1984; and Subansenee *et al.*, 1984).

Infection of Fungi in Natural Agarwood and Kritsana Tree

As depicted in Table 2, most fungi detected were known to be saprophytic except only that *Fusarium oxysporum* was known to be parasitic in highest frequencies (10.0-11.6%). *Botryodiplodia theobromae* was found to be the second largest frequency (8.3%). This species was frequently known as facultative saprophyte which oftenly caused diseases in nursery soils, seeds, seedlings and many other tree species in forest plantations in the tropics. In natural agarwood fungi: *Mucor hiemalis, Rhizopus sp., Aspergillus niger, Penicillium sp. Fusarium spp., Trichoderma viride, Chaetomium spirale, Cladosporium sp., Curvularia lunata, Pithomyces sp.*, and *Cephalosporium sp.* were predominantly found. It was evident that agarwood decayed by various fungi would be lead to decrease in weight and degrade the quality of agarwood.

Table 3 showed 11 species of wood-rotting fungi decayed on Kritsana tree *(Aquilaria crassna)*. *Ganoderma applanctum*, a white rot, found to cause butt and heart rots in standing trees and dead trunks. *Ganoderma lucidum*, white rot, was also known to cause root and butt rots of standing Kritsana trees. *G. colossum*, also a white rot, was detected to decay on butt and heart rots on dead stumps. *Phellinus ferreus* and *Stereum ostrea*, unknown rot type, were found to decay on dead branches. *Porogramme calcicolor* (on brahches) and *Pleurocybella porrigens* (on dead trunks) caused white rot degradation. *Marasmius confluens, M. foetidus* and *Omphalina sp.* decayed on dead wood and bark of *A. crassana, Xylaria hypoxylon* (Ascomycotina : Sphaeriales) also caused white rot on bark. Most wood-rotting fungi listed in Table 3 demonstrated white rot types which they were capable of degrading hemicellulose, cellulose and lignin in agarwood. These fungi have played very important diseases on standing trees and dead agarwood; if *Aquilaria* species are raised in industrial plantations, those rotting-fungi should be avoided.

Seed-Borne and Seedling Diseases

Table 4 delineated 14 species of seed-borne fungi detected from 200 Kritsana seeds. Most prominent fungi isolated were *Aspergillus fravas* (48%), *Mucor sp.* (27%), *Botryodiplodia theobromae* (20%), *A. niger* (9%), *Rhizopus sp.* (9%), *Curvularia lunata* (5%), Cladosporium sp. (3%), and *Chaetomiun sp.* (2%). Very small frequencies were detected by *Trichoderma viride* (1%), *Verticillium sp.* (1%), *Fusarium oxysporum* (1%), *Peronophythora lichii* (1%) and bacteria (1%). The most important parasitic fungi were *Botryodiplodia theobromae, Fusarium sp. Verticillium sp., Fusarium oxysporum* and *Peronophythora lichii. They cause seed and seedling diseases in nurseries specially Peronophythora lichii* was

Table 3. Wood - rotting fungi from natural wounds on *Aquilaria crassna* at Khao Yai National Park and at Khao soi Dao Wildlife Sanctuary.

Fungus species	Type of rot	Injuries
1. *Ganoderma applanatum* (pers. ex Wallr.) Pat.	White rot	Butt & heart rot on standing tree
2. *G.lucidum* (Fr.) Karst.	White rot	Root & butt rot on living tree
3. *G.colossum* (Fr.) Karst.	White rot	Butt & heart rot on dead tree
4. *Phellinus ferreus* (Pers.) B. et G.	Unknown	Decayed on branches
5. *Stereum ostrea* (Fr.) Fr.	Unknown	Decayed on branches
6. *Porogramme calcicolor* (Sacc.) Lowe	White rot	Decayed on branches
7. *Marasmius confluens* (Fr.) Pers.	Unknown	Decayed on dead wood
8. *M.foetidus* (Sow.) Fr.	Unknown	Decayed on dead wood
9. *Omphalina sp.*	*Unknown*	*Decayed on bark*
10. *Pleurocybella porrigens* (Pers. ex Fr.) Sing.	White rot	Decayed on dead trunk
11. *Xylaria hypoxylon* (L.) Grev. (Ascomycotina)	White rot	Decayed on bark

known to cause severe damage as downy mildew and damping-off diseases on seedlings in nursery within a few weeks after germination from seeds.

Mycorrhizal Association

Mycorrhizal fungi associated with Kritsana tree *(Aquilaria crrassna)* grown in natural habitat were studied at Khao Yai National Park. Azygospores and chlamydospores of vesicular-arbuscular mycorrhizal (VAM) or endomycorrhizal fungi were extracted from 10-20 cm soil depth along the tree roots and rhizosphere of natural Kritsana trees using wet sieving and decanting techniques as described by Gerdemann and Nicolson (1963). The most commonly observed VAM fungus was identified as *Glomus sp.* This VAM species was believed to promote the tree growth and supported the nutritional uptake and nutrient recycling of Kritsana trees in nature. If Kritsana trees is raised into a large scale plantations in future, the VAM fungus *Glomus sp.* should be propagated and developed in the seedbeds before transplanting into field sites.

CONCLUSIONS

The role and activities of fungi affecting in artificial woundings and natural association with Kritsana or agarwood trees were studied. The results could be concluded as the followings:-

1. As exhibited in Table 1, it was concluded that there was no specific role and activities of fungi in the formation of agarwood or oleoresin deposits in Kritsana trees but lead to degrading and discolorating in the living trees. All fungi imperfecti such as *Fusarium oxyspoum, Botryodiplodia theobromae, Trichoderma viride, Cladosporium sp., Curvularia*

Table 4. Seed – borne diseases investigated from the 200 seeds of *Aquilaria crassna* at Khao Yai National Park.

Fungus species	Frequency by Blotter Method (%)
1. *Aspergillus flavus* Gr.	48
2. *Mucor sp.*	27
3. *Botryodiplodia theobromae* Pat.	20
4. *Aspergillus niger* Gr.	9
5. *Rhizopus sp.*	9
6. *Curvularia lunata* (Wakk.) Boed.	5
7. *Cladosporium sp.*	3
8. *Chaetomium sp.*	2
9. *Fusarium sp.*	1
10. *Trichoderma viride* Pars.	1
*11. *Verticillium sp.*	1
*12. *Fusarium oxysporium* Schlecht	1
**13. *Peronophythora lichii* Chen ex Ko et al.	1
14. Bacteria	1

* Parasitic fungi

** No. 13 found to cause downy mildew and damping – off disease on seedlings of *Aquilaria crassna* within a few weeks after germination.

lunatu, Aspergillus spp., Penicillium sp. and other bacteria (Table 2) had been recorded as pioneer invaders.

2. Many wood-rotting fungi, as indicated in Table 3, were usually known as white rot types of decay and caused root rot, butt and heart rots, decayed bark, broken branches and sap rot through open woundings of Kritsana trees. This group of fungi normally caused damage and weakened the growth of Kritsana trees.

3. From the results of this experiment it might be concluded that under natural condition agar or Kritsana formation is initiated through the interaction caused by woundings or injuries (physical, mechanical, chemical and biological actions) followed by invasion of pioneer fungi and bacteria which entered primarily through the openings created by broken branch stubs, insects, birds or animals.

4. As provided in Table 4, there were many seed-borne fungi and bacteria colonize on Kritsana seeds. These microorganisms damaged to the quality and viability of the seeds and could be transmitted to cause severe damage of Kritsana seedlings in nurseries and younger trees transplanted.

5. The VA mycorrhizal *Glomus sp.* was identified from extracted soils along the tree roots of Kritsana trees.

6. Further research should be made to elucidate the synthetic mechanism of physio-biochemical processes in the formation of oleoresin deposits of agarwood within Kritsana trees.

ACKNOWLEDGMENT

The authors were greatly thankful to the International Foundation for Science (IFS) of of the Swedish Government for providing research grant to support this research project (1985-1990).

Special gratitued was conveyed to the Royal Forest Department, Kasetsart University-Faculty of Forestry and the National Research Council of Thailand for permission to continue the research studies in the field.

All thanks were also due to many authorities for entirely providing facilities, assistance and cooperation.

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