Effects of Commercial *Fusarium* on Agarwood Formation in Cultivated *Aquilaria malaccensis* Lam. at Midhills, Nepal

A Dissertation Submitted for the Partial Fulfillment of the Requirement of a Master's Degree in Botany

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DECLARATION

The dissertation entitled **"Effects of Commercial *Fusarium* on Agarwood Formation in Cultivated *Aquilaria malaccensis* Lam. at Midhills, Nepal"** which is being submitted to the Amrit Campus, Institute of Science and Technology, Tribhuvan University, Nepal for the partial fulfilment of the requirement of Master's Degree in Botany has been carried out by me under the supervision of **Prof. Dr. Mukesh Kumar Chettri** and co-supervision of **Mr. Hari Sharan Adhikari**. This work has not been submitted to any other institution for any academic degree.

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RECOMMENDATION

This is to recommend that **Manoj Kafley** carry out the dissertation entitled **"Effects of Commercial *Fusarium* on Agarwood Formation in Cultivated *Aquilaria malaccensis* Lam. at Midhills, Nepal"** for the partial fulfilment of the requirement of a Master's Degree in Botany under our supervision. To our knowledge, this work has not been submitted to any other institution for any academic degree. He has fulfilled all the requirements laid down by the Amrit Campus, Institute of Science and Technology, Tribhuvan University, Lainchaur for the submission of the dissertation for the Master's Degree in Botany.

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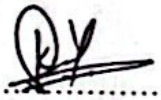

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Manoj Kafley

LIST OF ABBREVIATIONS AND ACRONYMS

$	US dollar
µm	micrometer
ANOVA	Analysis of variance
AR. 13	*Aspergilus* 13
C	column
Cm	Centimeter
DBH	Diameter at breast height
DPR	Department of plant resource
g	gram
GCMS	Gas chromatography-Mass spectrometry
i.e.	Id est (That is)
IUCN	International Union for Conservation of Nature
K.pa	kilopascal
L	liter
ll	length of the lower side
lu	length of the upper side
m	meter
Min	minute
ml	militer
mm	millimeter
m asl	meter above the sea level
NPr	Nepalese rupees
^{o}C	degree Celsius
Pvt	private
RFRI	Rain Forest Research Institute
s.n	serial number
Tl	total length

TABLE OF CONTENTS

DECLARATION ..... **Error! Bookmark not defined.**

RECOMMENDATION ..... **Error! Bookmark not defined.**

LETTER OF APPROVAL..... **Error! Bookmark not defined.**

ACKNOWLEDGEMENTS ..... **Error! Bookmark not defined.**

LIST OF ABBREVIATIONS AND ACRONYMS.....i

TABLE OF CONTENTS.....ii

LIST OF FIGURES v

LIST OF TABLES vi

ABSTRACT.....vii

सोध सार viii

CHAPTER 1 1

INTRODUCTION 1

1.1 Background 1

1.2 Justification 3

1.3 Research Questions 3

1.4 Objectives.....4

1.5 limitation 4

CHAPTER-2 5

LITERATURE REVIEW 5

CHAPTER-3 8

MATERIALS AND METHODS.....8

3.1 Study site.....8

3.2 Research design.....9

3.2.1 Arrangement of fungi.....9

3.2.2 Test plant.....9

3.2.3 Sampling 10
3.2.4 Inoculation methods 10
3.2.5 Curating 11
3.2.6 Harvesting 11
3.2.7 Measurement of agarwood 11
3.4 Cost analysis 13
3.5 Data analysis 13
CHAPTER-4 14
RESULT 14
4.1 Length of Discoloration 14
4.2 Relation of length of discoloration zone with diameter: 15
4.3 Agarwood percentage 17
4.4 Effect of inoculation on plant growth rate: 19
4.5 Effect of inoculated holes on the average agar wood per inoculation 20
4.6 Phytochemical compositions in Agarwood 21
4.7 Cost estimation 27
CHAPTER-5 28
DISCUSSION 28
5.1 Length of discoloration 28
5.2 Relation of discoloration zone and branch diameter 28
5.3 Agarwood percentage 29
5.4 Effect of inoculation on plant growth rate 30
5.5 Effect of numbers of holes in the mean weight of chips: 30
5.6 Chemical composition 30
5.7 Cost estimation 31
CHAPTER-6 32

CONCLUSION AND RECOMMENDATION.....32
6.1 Conclusion 32
6.2 Recommendation 32
REFERENCE:.....33
Annexes.....39
Photo plates:.....49

LIST OF FIGURES

Figure 1 Map of Nepal showing Study site. 8

Figure 2 Climate graph of the study area. A. Bharse, B. Damauli 9

Figure 3 Sampling technique 10

Figure 4 Researcher of this thesis inoculating the Fusarium 11

Figure 5 Boxplot, effect of treatment on Total length of discoloration zone. Same Lowercase letter above the boxplot area not significant at $p<0.05$. 15

Figure 6. Regression analysis between length of the discoloration zone (cm) and Diameter (cm). 16

Figure 7 Boxplot showing agarwood percentage 18

Figure 8 Agarwood growth rate. 20

Figure 9 Regression analysis 21

Figure 10 GCMS diagram of the agarwood oil. A. Bharse physical, B.Bharse fungus inoculation, C. Damauli physical, D. Damauli fungal inoculation 25

Figure 11 Heat map showing the percentage composition of compound in agarwood. (full name of compound is in annex) 26

Figure 12 Average oil extraction for each tree (ml) 27

LIST OF TABLES

Table 1 Length of discoloration (cm) 14

Table 2 Regression analysis between length of discoloration zone and Diameter 17

Table 3 Agarwood percentage in each treatment on both location 18

Table 4 ANOVA result of growth rate of agarwood plant across different treatment and location 19

Table 5 Growth rate of Agarwood 19

Table 6 ANOVA numbers of holes on the mean weight of agarwood 20

Table 7 Presence absence of the chemicals 21

ABSTRACT

Agarwood, a highly valuable fragrant wood, is obtained from the *Aquilaria malaccensis* tree. Natural formation of agarwood takes large amount of time so its formation can be accelerated by inducing with different methods, one of them is fungal inoculation. However, there is a lack of research on effective inoculation techniques in Nepal. Therefore, this study aims to investigate the influence of different treatment including research-based *Fusarium* on agarwood formation over a geographical area in central Nepal. The comparison among fungus inoculated, physical wounded and control treatment on the agarwood formation was done. This was Nepal's first artificial treatments inoculation of Fusarium in agarwood. The study was conducted on 30 selected trees and some branches of Aquilaria *malaccensis* in Gulmi, Satyawati Rular Municipality, Bharse and from Patan, Damauli. Purposive sampling was used to select the trees. The harvesting was done after 9 months of inoculation. The percentage yield of agarwood and essential oil content was analyzed and compared among the different geographical area using ANOVA. The relation between length of the discoloration zone with the different diameter of tree was analyzed using regression analysis . From the result it is found that the agarwood percentage is higher in fungal inoculation. The regression analysis revealed that the diameter of inoculated branch has significant positive effect on the length of discoloration. The chemical constituents of the essential oil were identified using the GCMS method. A total of 78 aromatic compounds were found. The findings will contribute to the growth of Nepal's agarwood sector by providing insight into the influence of *Fusarium* on agarwood formation. Also, the chemical examination of the essential oil will aid in understanding the composition and quality of agarwood, which may be advantageous for commercial use.

Key words: Discoloration zone, essential-oil, GC-MS, inoculation, resin formation

सोध सार

Aquilaria malaccensis एक सदाबाहार रुख हो । रुखहरु करिव ३० बर्षका भएपछि प्राकृतिक रुपमा ढुसीले संक्रमण गर्दछ र जसको बिरुद्दमा रुखले बिभिन्न secondary metabolites उत्पादन गर्दछ । उक्त बेलामा Heartwood Agarwood मा परिवर्तन हुन्छ , जुन अतिनै महंगो हुने गर्दछ तर बर्षौ पहिले बाट किसान देखि प्रयोगकर्ताहरुले छोटो समयमा Agar उत्पादन गर्न रुखमा काँटि ठोक्ने, छेडने तथा ढुसि प्रत्यागमन गर्ने चलन छ । यस अध्ययनले *Aquilaria malaccensis* रूखहरूमा अगरवुड निर्माणलाई गति दिन ढुसी इनोकुलेशन (विशेष गरी फ्युज्योरियम) प्रयोग गर्ने सम्भावनाको खोजी गरेको छ । यस अनुसन्धानमा पाटन (दमैली) र भार्से (गुल्मी) का ३० वटा खेति गरिएका रुखहरुमा ढुसी इनोकुलेशन, शारीरिक घाउ, र नियन्त्रण समूहहरूको अध्ययन गरीएको छ । नौ महिनापछि, ढुसी इनोकुलेशन समूहले Agarwood उत्पादन सबैभन्दा बढी देखायो , यसैगरि शारीरिक घाउले पनि Agarwood उत्पादन गर्न सफल भयो भने नियन्त्रण समूहमा Agarwood उत्पादन भएन । अध्ययनले रूखहरुको व्यास र discoloration क्षेत्रको लम्बाइ बीचको सकारात्मक सम्बन्ध पनि फेला पारेको छ । थप रूपमा, अध्ययनले Agarwood बाट निकालिएको आवश्यक तेलमा ७८ सुगन्धित यौगिकहरू फेला परेको छ । यस अनुसन्धानले नेपालमा दिगो अगरवुड उत्पादनको लागि मार्ग प्रदशन साथै देशको अगरवुड उद्योगलाई बढावा दिन सकिने सम्भावना देखाउँछ ।

CHAPTER 1

INTRODUCTION

1.1 Background

Aquilaria malaccensis is a tropical plant species widely known as agarwood-producing species from the family Thymelaeaceae. Agarwood is a resinous compound produced by plants as a response to physical wound as well as pathogen attacks (Karlinasari *et al*. 2015)**.** Furthermore, agarwood is a highly commercial non-timber forest product due to its important role in fragrances, aromatherapy, medicines, and religious activities (Chen *et al.* 2012). Over the past few decades, more than 150 compounds have been identified as constituents of agarwood, which comprise mixtures of chromones, volatile aromatic compounds and sesquiterpenoids (Naef , 2011). The value of agarwood has been estimated in the range of 9700 to 32000 USD per Kg, which is depends on quality grade of agarwood (Sen *et al.,* 2015; Buragohain and Dutta, 2024). One of its price considerations is the shape of chips, even same quality. In November 1994, all *Aquilaria* species have been listed in Appendix II of CITES (The Convention on the International Trade in Endangered Species of Wild Flora and Fauna) to prevent its excessive exploitation and to regulate its trade (CITES, 2004).

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. Naturally, production of agarwood in *Aquilaria* takes 10-20 years of time and it can develop only in 1-2% of *Aquilaria* trees. Thus, the supply of agarwood from wild sources is far less than the market demands (Zhao *et al*., 2024)

Agarwood farmers in different Asian countries have tried several wounding methods to produce agarwood, including chopping, nailing, holing and trunk breaking. These methods often take a long time, with generally inadequate and low quality in the agarwood production (Yagura *et al.,* 2005; Liu *et al*., 2013; Zhang *et al.,* 2014a; Li *et al.,* 2015).

Over the years, the practice has expanded to include the use of certain chemicals and microorganisms, and the creation of modern inducement kits. Several commercial inducement techniques are available in the market today including methods known as the CA Kit and the Taiwan and Pheerapan methods (Chen *et al.,* 2011). These approaches use microbes to accelerate the production of agarwood in standing trees. In addition, chemical inducers such as sodium chloride and hydrogen peroxide have also been applied in many countries (Chen *et al*., 2011; Zhang *et al.,* 2014b). However, this approach is becoming less preferable due to side effects of the chemicals which are harmful to the environment. More recently, using a chemical solution to induce agarwood in the whole tree (Agar-Wit), has been shown to yield high quality agarwood within 20 months after treatment (Zhang *et al.,* 2012). GC-MS profiles of essential oils from agarwood using this technique show high percentages of major sesquiterpene compounds, such as agarospirol and eudesmol. There are many inoculation techniques that employ various types of inducers for agarwood formation around the world. Different inducers can induce different kinds of agar wood formations even in the same species of *Aquilaria* trees.

The chemical components of agarwood are diverse and complex (Chen *et al*., 2012), contributing to the diversity of bioactivity and pharmacology, including neural activity, gastrointestinal regulation, antibacterial, anti-inflammation, and cytotoxicity (Wang et *al.,* 2018). A literature survey of agarwood plant materials showed that they contain sesquiterpenes, 2(-2-phenylethyl)-4H-chromen-4-one derivatives, genkwanins, mangiferins, iriflophenones, cucurbitacins, terpenoids and phenolic acids (Hashim *et al.,* 2016). Besides this wang *et al* (2018) has also summarized the new compound recorded in the agarwood after 2010 and found 154 new compounds identified from *Aquilaria* plants, 2-(2-phenylethyl)-4H-chromen-4-one derivatives and sesquiterpenes account for 57% and 35%, respectively.

Fusarium is a large genus of imperfect fungus that is of great importance because many species are major plant diseases, generate a diverse variety of secondary metabolites, and/or cause opportunistic mycoses in humans (Nelson *et al.,* 1981). Although *Fusarium* research has expanded our understanding of this essential group of fungus over the last century, many elements of its biology remain unexplored (Austwick, 1982; Michniewicz, 1989; Vesonder, 1989). *Fusarium* species cause a wide variety of plant diseases. Most *Fusarium* illnesses are soil-borne, and unlike pathogens that infect

plant aerial parts, the methods by which *Fusarium* infects its hosts are poorly known. Although *F. oxysporum* may exhibit small morphological changes during infection, structures unique to infection, such as appressoria or infection pegs, have not been found (Mendgen *et al.*, 1996; Lagopodi *et al*., 2002). *Fusarium* also lacks host-specific poisons such as those generated by *Alternaria* and *Cochliobolus* species. Nonetheless, prior to the introduction of powerful molecular tools, insights into *Fusarium* pathogenicity were achieved viz. *Fusarium culmorum, F. graminearum and F. temperatum.* After the entry of fungus, a mold infection may then occur, and in response, the tree produces a salutary self-defense material to conceal damages or infections. While the unaffected wood of the tree is relatively light in color, the resin dramatically increases the mass and density of the affected wood, changing its color from a pale beige to yellow, orange, red, dark brown or black (Zhao et al., 2024)

1.2 Justification

From previous study, It is found that most of the research are only focused on the scientific content only, nonetheless are not useful for farmers. Sustainable commercial harvesting of agar wood will benefit the farmers. This will also cause the economic enhancement, quality agarwood formation. From the literature review it is found that different inoculation methods have been proposed by different scientist (Shivanand *et al*., 2022) to enhance the agarwood formation, but none of the scientific test has been done in Nepal. So effective inoculation technique to speed the agarwood formation is need for Nepal should be studied, which in turns possibly will upgrade the economy of the related farmers. So, I wanted to do this research that could be very beneficial for the ground levels farmers.

1.3 Research Questions

The study was based on the following research questions.

1. How different treatments (physical wounding and *Fusarium* inoculation) impact on agarwood formation?
2. Does different treatment affect the growth rate of the plants?
3. What role does diameter of plants plays in agarwood formation?

4. Does there any difference on the chemical constituents of agarwood oil formed by different treatment?
5. Does the ecological zone play any role in the variation of agar formation?

1.4 Objectives

The general objective of the study is to anlyze the effects of commercial *Fusarium* on Agarwood Formation in Cultivated Aquilaria malaccensis Lam. at Midhills Nepal.

The specific objectives are:

1. To analyze the impact of different treatments on agarwood formation.
2. To measure the effects of different treatments on the growth rate of the plants.
3. To analyze the relation between the host tree diameter and agarwood formation.
4. To analyze the chemical constituents with agarwood oil of different treatment.

1.5 limitation

- There was time constrains. This study would have give best result if we had 5/6 years of times, to analyze the total effect of the treatments, as this stydy was done for the sake of thesis, and thesis has time limit, so I did this study for 9 months.
- This experimental setup is too expensive. The trees used in this research are valuable and expensive, so this study was done in few plants.

CHAPTER-2

LITERATURE REVIEW

Aquilaria malaccensis (family Thymelaeaceae) is a prominent agarwood producer. The high market demand for agarwood has seriously affected natural sources of *A. malaccensis*. Appendix II of the Convention on International Trade in Endangered Species currently lists the species as endangered Flora and Fauna in the Wild (CITES 2010). To address the need for environmentally friendly agarwood manufacturing, *Aquilaria* trees are presently being planted on a massive basis, and efforts are being undertaken to develop efficient artificial methods for inoculating young plantation trees with agarwood. Existing artificial agarwood inducement methods include bark removal, axe and nail wounding, burning, and fungus infection (CITES, 2004; CITES, 2005a, 2005b; Pojanagaroon & Kaewrak, 2006; Barden *et al*. 2007; IUCN, 2009). These methods take a long time to make agarwood and give a poor agarwood yield. To date, some comparatively new and efficient methods have been developed, such as Blanchette's cultivated agarwood kits (CA-Kits) from the University of Minnesota (Blanchette & Heuveling, 2009) and the Chinese Academy of Medical Sciences and Peking Union Medical College's whole-tree agarwood-inducing technique (Agar-Wit) (Liu *et al.*, 2013). Agarwood induction with a chemical solution has been demonstrated to generate high-quality agarwood within 20 months of treatment (Zhang *et al.*2012).

Han and Win *et al* (2016) investigated how the same inducer with different inoculation procedures influences agar wood chip production and discovered that agar wood formation is controlled by numerous parameters such as inoculated hole size, inoculation techniques, time, and amount of inducer. Mohamed *et al.* (2014) investigated the influence of different fungi on agarwood production in young *Aquilaria malaccensis* (Lam.) trees throughout time. Their study detected the typical alternation in the length and light intensity of the ensuing discoloration after 3 and 6 months. When they measure the severity of the discoloration, a positive connection with time was seen. So they concluded that time, not the species of any of the examined fungus, had a significant influence on the length and degree of staining.

Faizal *et al* (2020) induced agarwood in *Gyrinops versteegii*, one of Indonesia's most prevalent agarwood-producing trees. In this work, they employed 12 trees and used an injection method to wound four branches on each tree. They employed two strains of

the endophytic fungus *Fusarium solani* recovered from the provinces of Gorontalo and Jambi. When compared to injured control wood, the infected wood exhibited a large resinous zone after 3 months. The presence of various sesquiterpenes typical of agarwood was found by gas chromatographic-mass spectrometric analysis of the infected samples. These included the chromone derivatives 2-(2-phenylethyl) chromen-4-one, 6-methoxy-2-(2-phenylethyl) chromen-4-one, and 6,7-dimethoxy-2-(2-phenylethyl) chromen-4-one, as well as alloaromadendrene, -eudesmol, and -selinene. They conclude that this strategy successfully promoted agarwood formation in a few of months and may be utilized to improve agarwood cultivation success.

Faizal *et al.* 2017 also artificially induced the formation of agarwood by injection and inoculation of cultivated *Aquilaria malaccensis* with four strains of *Fusarium solani* isolated from different places in Indonesia. Their results showed that *A. malaccensis* responded differently upon wounding and fungal inoculations compared to healthy trees. All wounded and inoculated samples resulted in the formation of typical discoloration zone surrounding injection sites. Hasibuan *et al* (2013) has studied the effect of inoculated *Fusarium* in agarwood and the results revealed that the features of inoculated and uninoculated *Aquilaria microcarpa* wood were partially different. There were variances in the color of the wood, the odor, the deposit inside the lumen vessel, and the frequency of included phloem. Elemol, baimuxinal, 3-phenyl-2-butanone, and chromen-4-one were found in *Fusarium*-inoculated wood.

The agarwood was naturally infected with *Aspergillus, Lasiodiploidia, Chaetomium, Fusarium,* and *Penicillium* species, according to the study. Further research into enzyme activities implicated in disease revealed that *Aspergillus* isolate AR13 had stronger cellulase, ligninolytic, and laccase activities than other isolates. The current work has provided a potential chance to further research into the production of microbial strains for artificial inoculation in agar trees to promote agarwood formation. (Sangareswari *et al.* 2016)

Thapa *et al.* 2020 investigated the agarwood growth rate of Nepal in the Gulmi district and discovered that the mean annual diameter increment was 0.582, 0.36, and 0.31 cm. The average yearly height increase was 0.86, 0.69, and 0.50 m. At ages 6, 9, and 12, the mean annual volume increment was 0.0000456, 0.000282, and 0.000308 m3, respectively. According to Adhikari *et al.* 2021, planting *Aquilaria* in Nepal's hill agro-ecosystems and cultivating agarwood as a crop utilizing modern technologies

might generate a new income for the region. So, the best inoculants and inoculation technique should be studied.

From the literature review it is found that most of the work were focused on artificial inoculation in different parts of the world but this practice has not been scientifically conducted in Nepal. Therefore this study attempts for the scientific inoculation practice to form agarwood in Nepal.

CHAPTER-3

MATERIALS AND METHODS

3.1 Study site

The inoculation was done in Satyawati Gaupalika, Bharse, Gulmi . Where Mr. Dhan Bahadur Pun planted agarwood at 4.4 hector of land in 2004. The latitude and longitude of the study site was 28º 03' 12"N and 83º 27' 51" E and the altitude of the site was 1895 m asl (Figure 1). Then another study was done in the Himalayan Herbal Pvt., Damauli, where Mr. Krishna Bahadurr Gurung has cultivated agarwood in 70 ropani in 2005. The latitude and longitude was 27s 58' 11" N and 84d 15' 24" E and the altitude was 403 m asl.

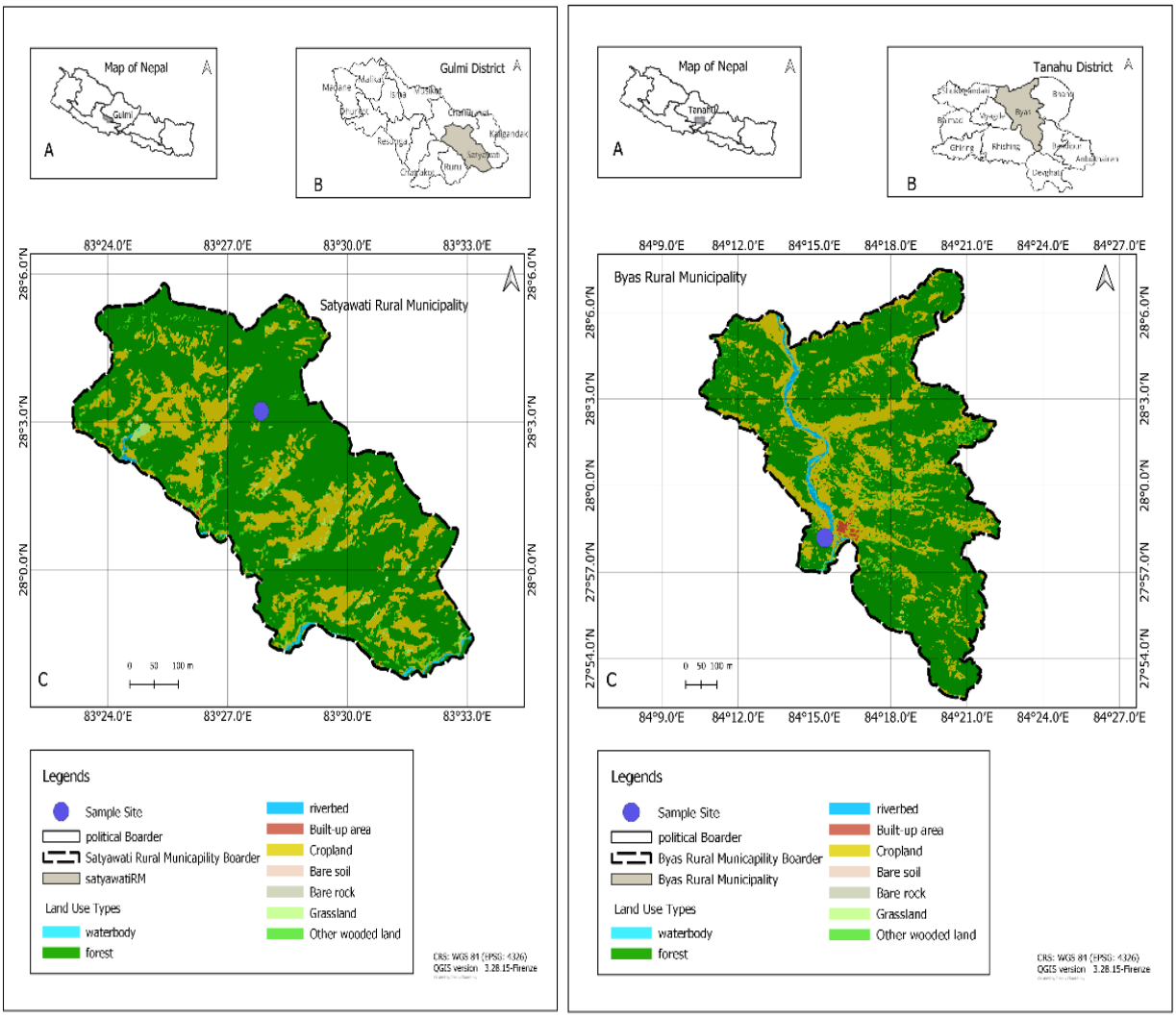

Figure 1 Map of Nepal showing Study site.

The climatic data was obtained from climatechart.net developed by Zepner et al. (2020). The mean annual temperature in the study sites of the Bharse is 15.9 ^{o} C and the annual precipation was 1507 mm. Similarly at Damauli the average temperature is 23.3 ^{o}C and the precipation is 2388.9 mm (Figure 2).

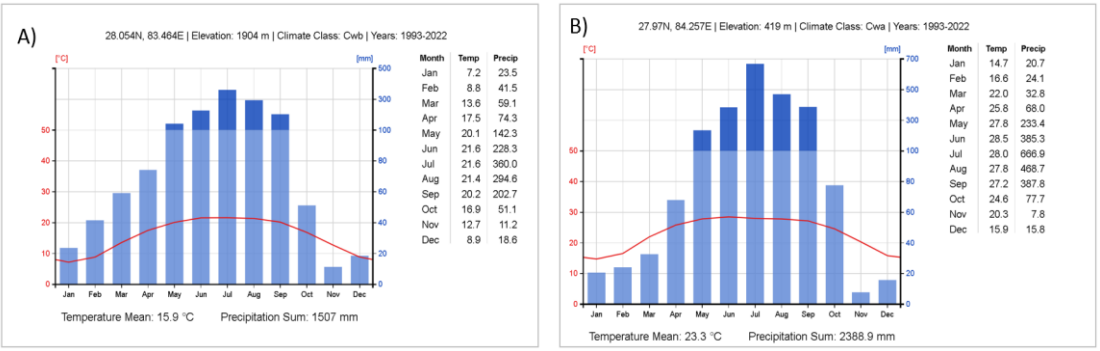

Figure 2 Climate graph of the study area. A. Bharse, B. Damauli

3.2 Research design

Five replicates for each fungus inoculation, physical wounding and control treatment in the agarwood tree were selected on the both sites.

3.2.1 Arrangement of fungi

The fungus used in the inoculation was research-based *Fusarium* spp. developed by Rain Forest Research Institute (RFRI) India.

3.2.2 Test plant

The targeted plant for the inoculation is *Aqualaria malencesis*. The plant with different diameter and height were used for the inoculation. Agarwood plant is evergreen tree reaching 20 meters in height. It is native to Bengal and northeastern India. Its fragrant wood is used commercially and medicinally. The tree produces white flowers and ovoid, tailed seeds within 3-5 cm long capsules. It grows in humid and high-rainfall regions with temperatures ranging from 20 to 28°C and prefers sandy loam soils. Seeds are readily sown within a week of collection for achieving a high germination rate (Shivanand *et al*., 2022)

Scientific Classification of *Aquilaria malaccensis.*

Kingdom: Plantae

Clade: Tracheophytes

Clade: Angiosperms

Order: Malvales

Family: Thymelaeaceae

Genus: *Aquilaria*

Species: *A. malaccensis* Lam.

3.2.3 Sampling

Purposive sampling was done. Mostly multi-stemmed trees were selected for treatment. From each study area 15 trees were selected i.e total 30 plants were selected (Figure 3, 4). And their height and diameter at DBH were measured. Small marks were made at DBH so that DBH can be measured at the same height after 9 months during yielding.

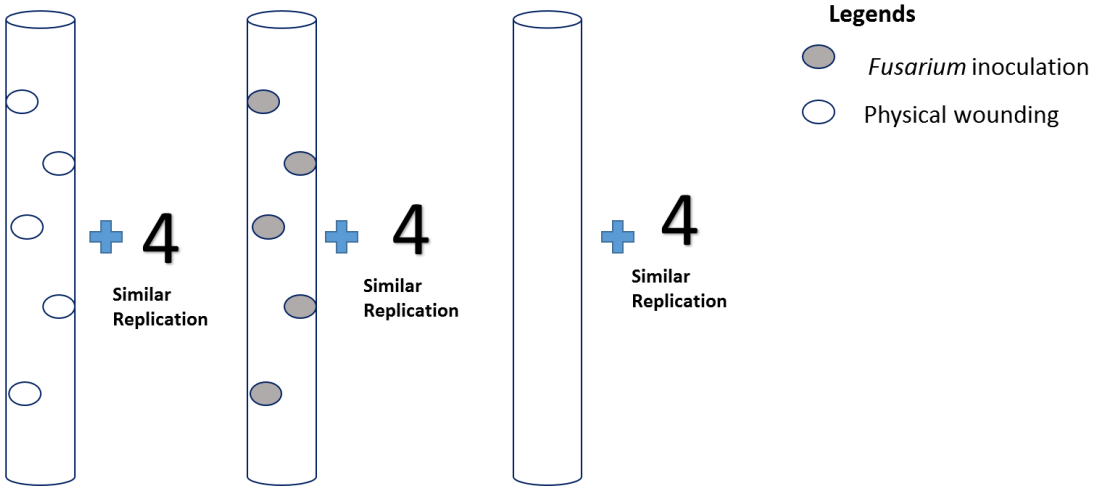

Figure 3 Sampling technique

3.2.4 Inoculation methods

The inoculation was done by-syringe method, deign was based on the Han *et al.*, (2012), Holes were drilled using a 10 mm driller in spiral manner. The inducer (fungus)

Figure 4 Researcher of this thesis inoculating the *Fusarium* by using syringe method

was added directly into open holes until full (Figure 4). At last, cotton was inserted to plug the holes.

3.2.5 Curating

In each three months gap, 10 ml molasses solution (1 molasses: 2 water) was added in the holes for two times in fungal inoculated trunks with the help of syringe. This helps in the proper growth of the inoculated fungi.

3.2.6 Harvesting

Harvesting was done after 9 months of inoculation. The plants and/or branches were cut down and the agarwood chips were harvested.

3.2.7 Measurement of agarwood

Fresh weight of each plant was measured using digital beam balance after cutting down of the tree for yielding. Height and diameter at the DBH were measured using a tape. Unnecessary branches as well as bark were removed using a sickle. Then, white part of the wood was separated with the adze, sickle and carving equipment. As agarwood

is formed inside the whole branch, to accurately measure the agarwood yield per tree, we had separate resign completely from the white wood.

The length of discoloration zone on the upper side (Lu) and lower side (Ll) of the drill hole was measured and noted down. Then each chip was separated with the help of hand saw, adze, chisels and carving equipment. The length of chips was measured by vernier caliper. They were air dried for 14 days and then agarwood yield per tree were calculated. The percentage weight of agar in the tree was calculated by using formulae.

$$\text{Agarwood percentage yield} = \frac{Weight\ of\ chips}{fresh\ weight\ of\ \ tree}\ X\ 100\ \%$$

3.3 Chemical analysis

The agarwood is collected from stem of *Aqualaria malasensis*. The samples were coded according treatments. The residual wood other than agar resin were removed and shed dried for few days. Agar-reign samples were cleaned, dried and cut into thin slices, and mechanical grinding was done. Using the hydro distillation method, the essential oils for the resinous samples from artificially induced agarwood were extracted. Then for distillation 100 g of ground agarwood were soaked in 600 mL of distilled water for the entire night then the wood was transferred to a 1 L round-bottomed flask. The flask was placed in front of a Clevenger type apparatus with running tap water in order to cool it. The distillation process continued for five hours. The extracted essential oil was gathered, separated with n-hexane, and dried at 45^0C in a vacuum rotary evaporator. Glass vial containing the essential oil were stored at 4 ^{0}C until further examination.

Further chemical analysis of the essential oil was done at department of plant resources (DPR), Thapathali. By using GC-MS, the essential oils were examined. A Shimadzu GC-MS-QP2010 Plus that was available from the Department of Plant Resources was used for the GC-MS analysis. The analysis was conducted using a capillary column, SH-RTX-5MS (60 m × 0.32 mm × 0.25 µm), where the stationary phase was a cross bond of 5% diphenyl/95% dimethyl polysiloxane. The following parameters were used for the GC analysis: 50 C for the column oven; 250 C for the injection; 250 C for the ion source; 200 C for the interface; 80 splits ratios for split injection mode; 53.8 kPa for helium pressure; 112.3 mL/min for total gas flow; and 1.35 mL/min for the column. The GC-MS system begins by heating the oven to 50 °C for one minute, then raises it to 23 at a rate of 3 ◦C for 9 min. Electron ionization mode was used for mass spectral

detection, with scanning occurring between 40 and 350 m/z. It took seventy minutes in total to analyse one sample.

3.4 Cost analysis

Cost analysis was done using the cost value as suggested by Sen *et al.*, 2015.

3.5 Data analysis

Primary data was entered into Excels and ANOVA was done to compare the length of the discoloration zone above the wounding, below the wounding, total length of the discoloration zone, agarwood percentage, plant growth rate. Regression analysis was done for the length of the discoloration zone affected by the diameter of the inoculated tree, and the relation between mean weight of the chips per hole and the number of holes in the trees.

CHAPTER-4

RESULT

4.1 Length of Discoloration

No discoloration was observed in the control group. Among the fungus and physical treatments, the fungus treatment exhibited a significantly higher length of discoloration (Table 1).

When comparing the ecological zones of Damauli and Gulmi, there was no significant difference in the total length of the discoloration zone. However, in the fungus treatment, the length of the discoloration zone in Gulmi was numerically higher than in Damauli but this difference is not significant(Table 1; Figure 5).

Table 1 Length of discoloration zone (cm) in the wood with different treatments.

	Damauli			Gulmi		
Treatment	Control	Fungus	Physical	Control	Fungus	Physical
Lu	0.00 ± 0.00	2.56 ± 0.09	0.77 ± 0.03	0.00 ± 0.00	4.30 ± 0.46	0.86 ± 0.03
Ll	0.00 ± 0.00	3.22 ± 0.14	0.85 ± 0.03	0.00 ± 0.00	5.57 ± 0.42	1.10 ± 0.05
Tl	0.00 ± 0.00	5.78 ± 0.21	1.62 ± 0.05	0.00 ± 0.00	9.88 ± 0.80	1.96 ± 0.07

Lu: length of discoloration zone on the upperside of drill hole, Ll: length of discoloration zone on the lower side of the drill hole, Tl: Total length of discoloration.

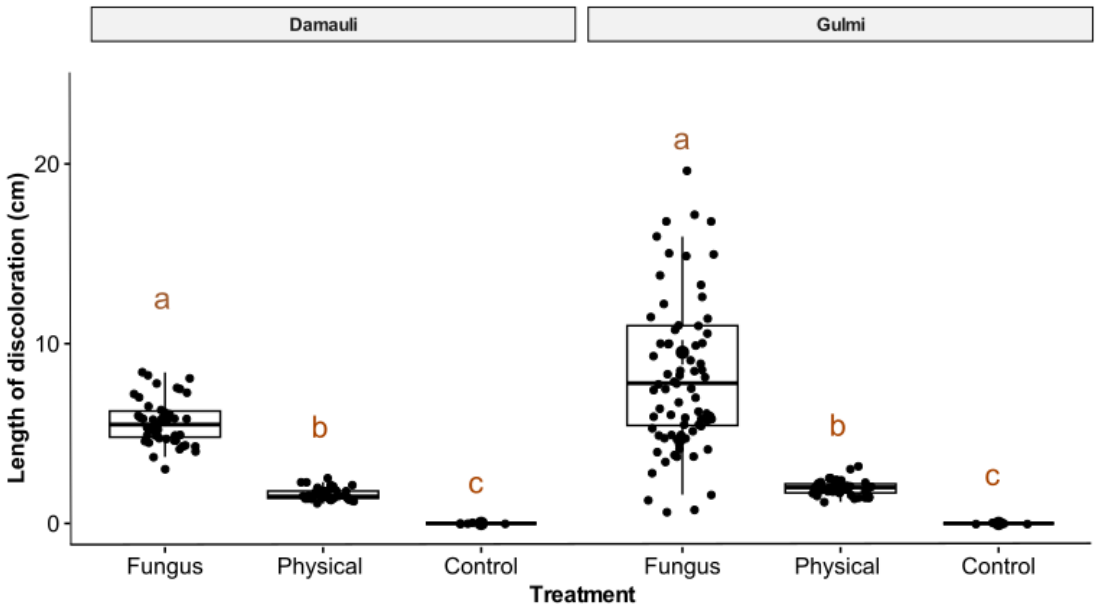

Figure 5 Boxplot, effect of treatment on total length of discoloration zone. (Same Lowercase letter above the boxplot area not significant at $p<0.05$.)

4.2 Relation of length of discoloration zone with diameter:

The overall regression analysis shows that length of the discoloration zone is significantly influenced by the diameter of the trunk as indicated by the linear regression analysis (Figure 6). The model explains 35% of the variability in the length of the discoloration zone, suggesting that a substantial portion of the changes in length of discoloration zone can be attributed to changes in the diameter of the trunk.

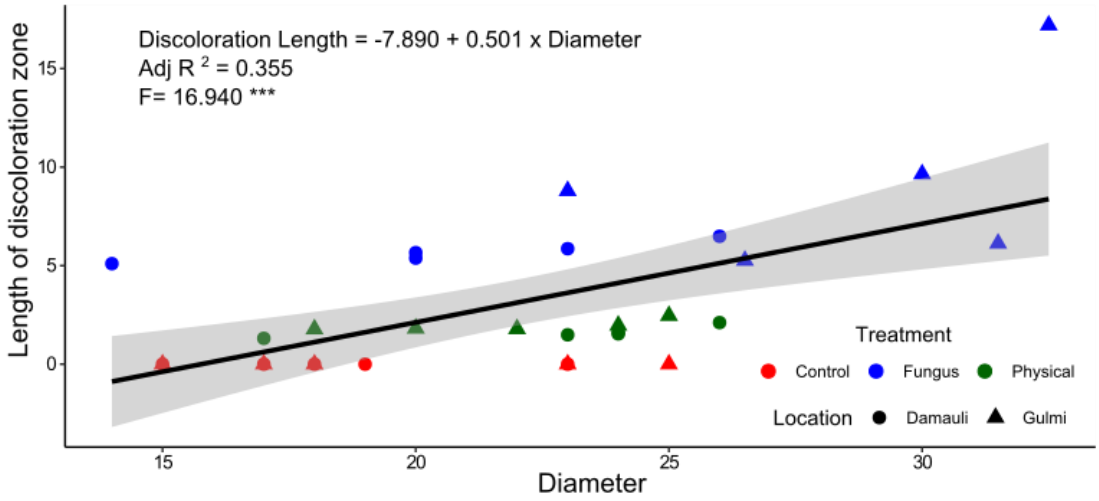

Figure 6. Regression analysis between length of the discoloration zone (cm) and Diameter (cm).

The regression results across all treatments between Damauli and Gulmi was also done. The result shows that the Fungus treatment in Gulmi is particularly noteworthy in trunk with higher diameter, exhibiting a substantial positive relationship beween the length of discoloration zone with trunk diameter, with a coefficient of 1.043 and the R-squared value of 0.67 ($P<0.1$). This substantial R-squared value underscores the treatment's significant explanatory power, indicating that approximately 67% of the variability in the length of the discoloration zone in Gulmi's Fungus treatment can be elucidated by changes in trunk diameter. Similarly, the Fungus treatment in Damauli also demonstrates a significant positive relationship, with a coefficient of 0.111 ($p < 0.05$). In contrast, the Control treatments in both Damauli and Gulmi exhibit no significant correlation with trunk diameter, as indicated by coefficients of 0.000 and p-values exceeding 0.1. This suggests that variations in trunk diameter do not exert a significant impact on the length of the discoloration zone under Control conditions.

Furthermore, the Physical treatment in Damauli reveals a noteworthy positive association (coefficient = 0.069) (Table 2), indicating that changes in trunk diameter contribute to variations in the length of the discoloration zone in this context. Similarly, the Physical treatment in Gulmi demonstrates a moderate positive relationship (coefficient = 0.077), suggesting a significant effect of trunk diameter on the observed discoloration.The results shows the treatment-specific nature of the relationship between trunk diameter and discoloration length.

Table 2 Regression analysis between length of discoloration zone and Diameter

	Damauli			Gulmi		
	Control	**Physical**	**Fungus**	**Control**	**Physical**	**Fungus**
Diameter	0	0.069	0.111**	0	0.077	1.043*
	0	-0.031	-0.023	0	-0.037	-0.423
Constant	0	0.037	3.402***	0	0.286	-18.134
	0	-0.716	-0.491	0	-0.818	-12.23
Observations	5	5	5	5	5	5
R2		0.621	0.883		0.587	0.67
Adjusted R2		0.495	0.844		0.449	0.56
Residual Std. Error (df = 3)	0	0.213	0.208	0	0.213	3.312
F Statistic (df = 1; 3)		4.921	22.677**		4.263	6.083*

Note: *p<0.1; **p<0.05; ***p<0.01

4.3 Agarwood percentage

The summary of agarwood percentages across various treatments and locations is presented in Table 3, along with the results of ANOVA. The analysis reveals a significant impact of both location and treatment, as well as their interaction, on agarwood formation (Table 3). According to the analysis of variance, the highest percentage of agarwood is observed in the fungal treatment, followed by the physical treatment, while the control treatment shows no agarwood formation (Table 3). Although the numerical value of agarwood is higher in the fungal treatment in Gulmi ($0.96 \pm 0.04\%$) compared to Damauli ($0.54 \pm 0.02\%$), the difference is not statistically significant (Figure 7). However, in the physical treatment, the agarwood percentage in Gulmi is significantly higher than in Damauli. No agarwood formation is noted in the control treatment.

Table 3 Agarwood percentage in each treatment on both location

Location	Treatment	TW	WC	AP%
Damauli	Control	6036.80 ± 397.60 b	0.00 ± 0.00 b	0.00 ± 0.00 e
	Physical	10096.60 ± 1693.14 ab	20.20 ± 2.82 b	0.22 ± 0.03 d
	Fungus	12426.20 ± 2018.34 ab	66.40 ± 9.80 b	0.54 ± 0.02 b
Gulmi	Control	6160.00 ± 405.71 b	0.00 ± 0.00 b	0.00 ± 0.00 e
	Physical	9812.80 ± 513.21 b	37.40 ± 3.03 b	0.38 ± 0.02 c
	Fungus	17756.20 ± 3373.45 a	174.00 ± 36.88 a	0.96 ± 0.04 a
Two-way ANOVA across different location and treatment		L=df(1), F: 1.4 T=df(2), F:12.91 *** L x T= df(2), 1.55	L=df(1), F: 10.57** T=df(2), F:32.08 *** L x T= df(2), 6.08 **	L=df(1), F: 87.38*** T=df(2), F:452.3 *** L x T= df(2), 34.52 ***

Note: Significance codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 ;TW: Total weight of wood (g), WC: weight of chip (g) , AP%: agarwood percentage; Different letters indicates significant differences agarwood percentage within each column.

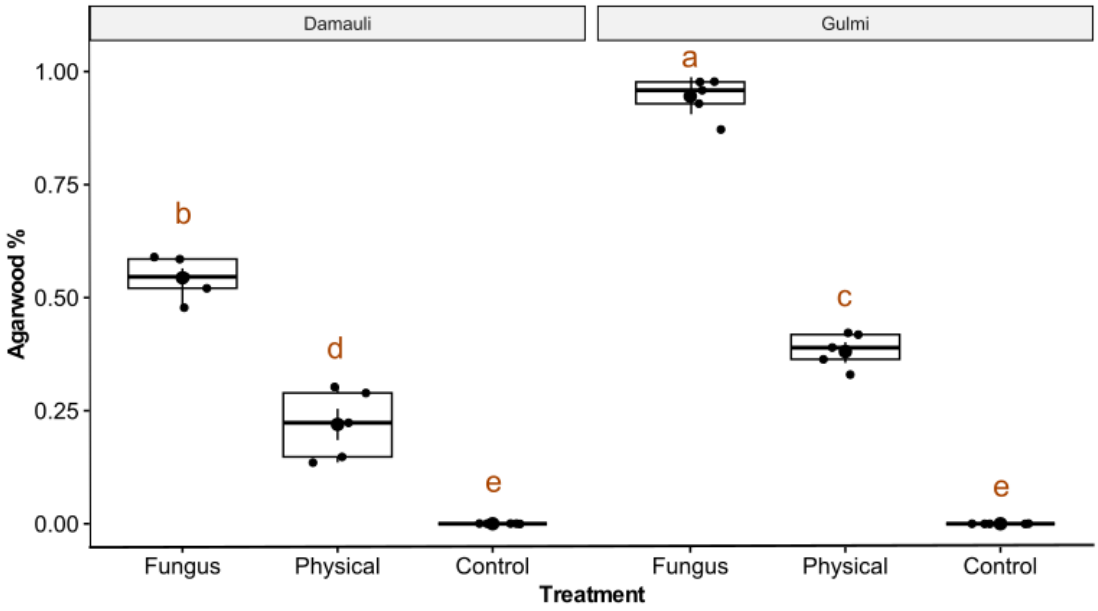

Figure 7 Boxplot showing agarwood percentage for each treatment and ecological zone

4.4 Effect of inoculation on plant growth rate:

The analysis of variance (ANOVA) conducted on plant growth rates reveals that inoculation does not exert a significant effect on the growth rate of the plants (Table 4).

Table 4 ANOVA result of growth rate of agarwood plant across different treatment and location

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Location	1	0.93	0.927	0.277	0.603
Treatment	2	4.24	2.12	0.634	0.539
Location:Treatment	2	6.86	3.431	1.026	0.374
Residuals	24	80.24	3.343		

The average plant growth rate ranges from 9.7 to 10 cm per month across the treatments. Furthermore, the ANOVA results indicate that there is no discernible impact of inoculation on plant growth rates, and the differences between ecological zones also do not significantly affect plant growth. Specifically, when considering the plant growth rates in Damauli and Gulmi under different treatments, it becomes apparent that there are no significant differences attributable to either the treatment or the location. For instance, in Damauli, the control, fungus, and physical treatments exhibit growth rates of 8.9±0.8, 8.9±0.5, and 9.3±0.5 cm per month, respectively. Similarly, in Gulmi, the control, physical, and fungus treatments show growth rates of 10.0±0.6, 8.8±1.4, and 8.7±0.5 cm per month, respectively (Figure 8; Table 5).

Table 5 Growth rate of Agarwood

Location	Treatment	IL	FL	GR
Damauli	Control	216.6±10.0	235.8±10.3	8.9±0.8
	Fungus	239±14.9	260.2±15.7	8.9±0.5
	Physical	244.6±11.1	267.6±13.0	9.3±0.5
Gulmi	Control	297.4±17.2	328.8±18.4	10.±0.6
	Physical	326.4±24.9	354.2±23.9	8.8±1.4
	Fungus	477.8±60.2	518.4±63.4	8.7±0.5

IL: Initial length of branch in cm, FL: length of branch after 9 months, GR: Growth rate in cm per month.

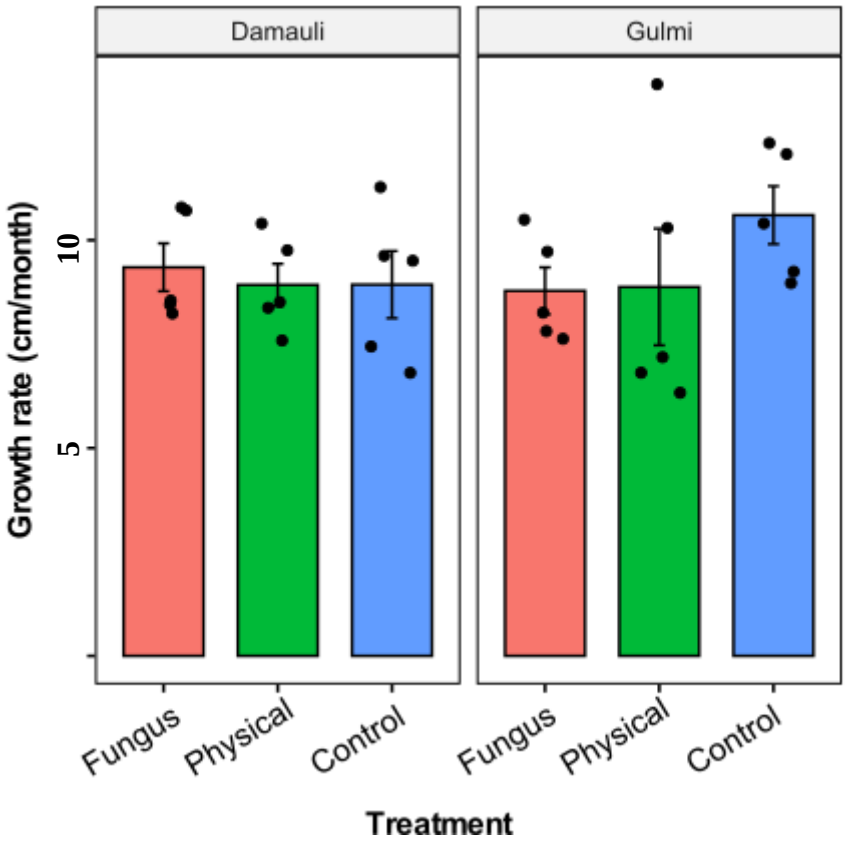

Figure 8 Agarwood growth rate.

4.5 Effect of inoculated holes on the average agar wood per inoculation

The number of inoculations on the same tree does not affect the mean weight of agar wood on per inoculation (Table 6, Figure 9).

Table 6 ANOVA numbers of holes on the mean weight of agarwood

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
WC	1	0.09	0.09	0.021	0.885
Residuals	40	167.91	4.198		

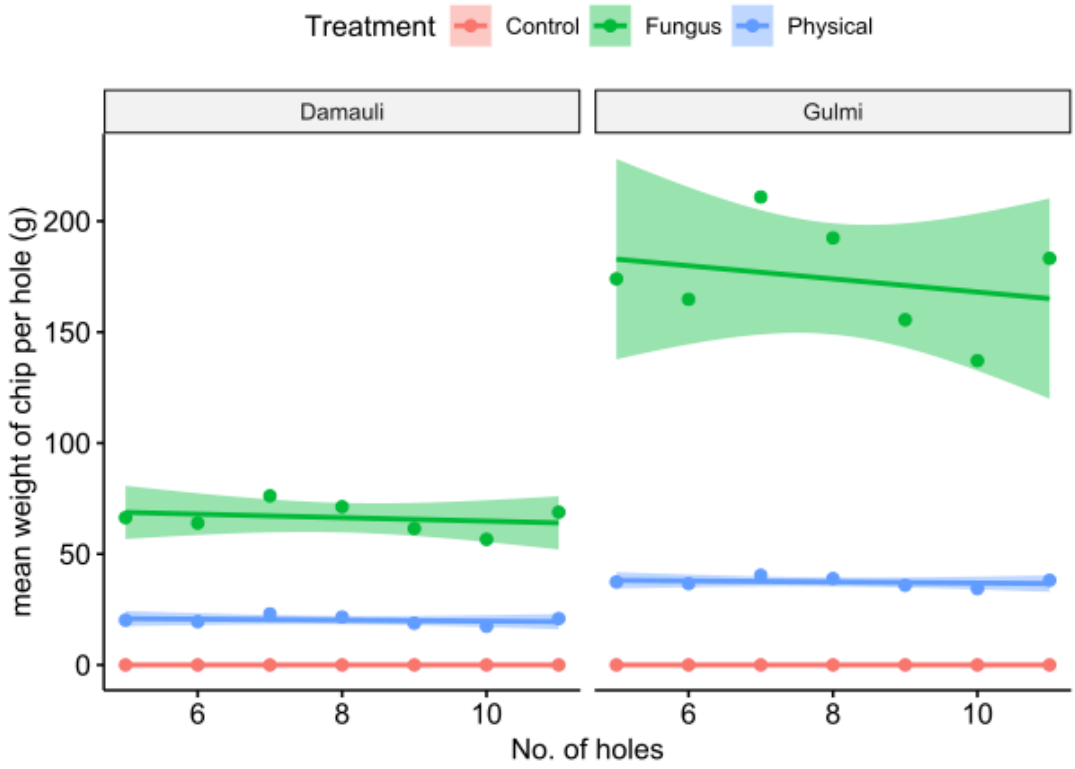

Figure 9 Regression analysis of mean weight of chips per hole and number of holes

4.6 Phytochemical compositions in Agarwood

From the GCMS result it is found that there are 78 phytochemical compounds in the agarwood (Table 7, Figure 10, 11). The numbers of phytochemicals in fungi inoculated sample is greater than that of physical wound in both ecological site. Notably, sesquiterpenes such as "Agarofuran <alpha->," Hinesol, Cubenol, Selinene <beta->, Gurjunene <alpha->, Muurolene <gamma->, Vetispirene <beta->, Aromadendrene epoxide, Aristolone, α-caryophyllene, Nootkatane, and Gurjunene <gamma-> were found abundantly. Among the major compounds identified, 4-phenyl-2-butanone constituted 32.1% of the total, followed by jinkoh-eremol at 6.5% and α-guaiene at 5.8% (Figure 11, Annex 7).

Table 7 Presence absence of the chemicals

Name of chemicals	Bharse		Damauli	
	Fungi	Physical	Fungi	Physical
14,15-Bisnorlabdane <8,13:13,20-diepoxy->	+	-	-	-
Acalea <trans->	+	+	-	+
Acoradiene <beta->	-	-	+	-

Agarofuran <alpha->	+	+	+	+
Amorpha-4,9-dien-2-ol	+	+	+	-
Amorpha-4,9-diene <7,14-anhydro->	-	-	+	+
Amorphene <alpha->	-	-	+	-
Amorphene <delta->	+	-	-	+
Aristola-1(10),8-diene	+	+	+	+
Aristolone	+	+	+	+
Aromandendrene epoxide <allo->	-	+	-	+
Atlantol <beta->	+	-	+	+
Bicyclogermacrene	-	-	-	+
Cadalene <8,9-epoxide->	+	+	+	+
Cadin-4-en-10-ol	+	+	+	+
Cadina-1(6),4-diene	-	-	+	+
Cadinene <delta->	+	+	+	-
Cadinene <gamma->	+	+	-	-
Calamenen-10-one <10-nor->	+	+	+	+
Calamenene <cis->	+	+	+	-
Caryophyllene <14-hydroxy-(Z)->	+	-	-	-
Cembrene	+	+	-	+
Chamigrene <beta->	-	-	+	-
Cinnamaldehyde <(2E), -hexyl->	-	-	+	-
Copaen-11-ol <alpha->	+	+	+	-
Copaen-4-alfa-ol <beta->	-	+	-	-
Costol <alpha->	+	+	+	+
Costol <beta->	+	+	+	+
Costol <gamma->	+	+	-	-
Cubebol <epi->	-	+	-	+
Cubenol <1-,10-di-epi->	+	+	+	+
Cyclocolorenone	+	+	-	-

Cyclocolorenone <epi->	+	+	+	+
Cyperotundone	-	-	-	+
Dauca-5,8-diene	-	-	+	-
Dihydromayurone	-	-	-	+
Dodecanenitrile	-	-	+	-
Epiligulyl oxide	-	+	-	-
Eremophilone	-	+	+	+
Eudesma-6,11-diene <cis->	-	-	+	-
Eudesmol <10-epi-gamma->	+	+	+	+
Eudesmol <gamma->	+	-	+	-
Farnesyl acetate <2E,6E>-	+	-	-	-
Germacra-4(15),5,10(14)-trien-1-alpha-ol	-	-	+	-
Guaia-3,9-dien-11-ol <cis->	+	+	-	+
Guaiac acetate	+	+	-	-
Guaiazulene	+	+	+	+
Gurjunene <alpha->	-	-	-	+
Gurjunene <gamma->	+	-	-	-
Himachalene oxide <beta->	+	+	+	+
Hinesol	+	+	+	+
Humulene <alpha->	+	+	-	-
Isoprecyclemone E	+	-	-	+
Isovalencenol <(E)->	+	-	+	-
Lanceol <cis->	+	+	+	+
Longifolene <iso->	+	+	+	+
Methanonaphthalene-8-ethanol <1,3,4,5,6,7-hexahydro-, ß,1,1,5,5-pentamethyl-, 2H-2,4a-> isomer II	-	-	+	-
Modhephen-8-beta-ol	+	-	+	-
Mustakone	-	-	-	+

Muurola-4,10(14)-dien-1-beta-ol	+	+	+	+
Muurolene <15-oxy-alpha->	+	+	+	+
Muurolene <gamma->	-	-	+	-
Muurolol <alpha-, epi->	+	+	+	-
Naphth-1-ol <1,2,3,4,4a,7,8,8a-octahydro-, 4-isopropyl-, 1,6-dimethyl->	-	-	-	+
Nootkatol	+	+	+	+
Nootkatone	+	+	-	+
Phenethyl ketone <methyl->	+	+	-	-
Presilphiperfol-7-ene	-	-	+	-
Presilphiperfolan-8-ol	-	-	-	+
Quinoline <2-isobutyl->	+	+	+	+
Rosifoliol	-	-	-	+
Selinene <beta->	+	-	+	-
Thujopsadiene <cis->	-	+	-	+
Thujopsenal	-	-	+	-
Valencene <13-hydroxy->	+	+	+	+
Valerianol	+	+	-	-
Vetispirene <beta->	+	+	-	-
Vetivone <beta->	+	+	-	-
Zierone	+	+	+	+

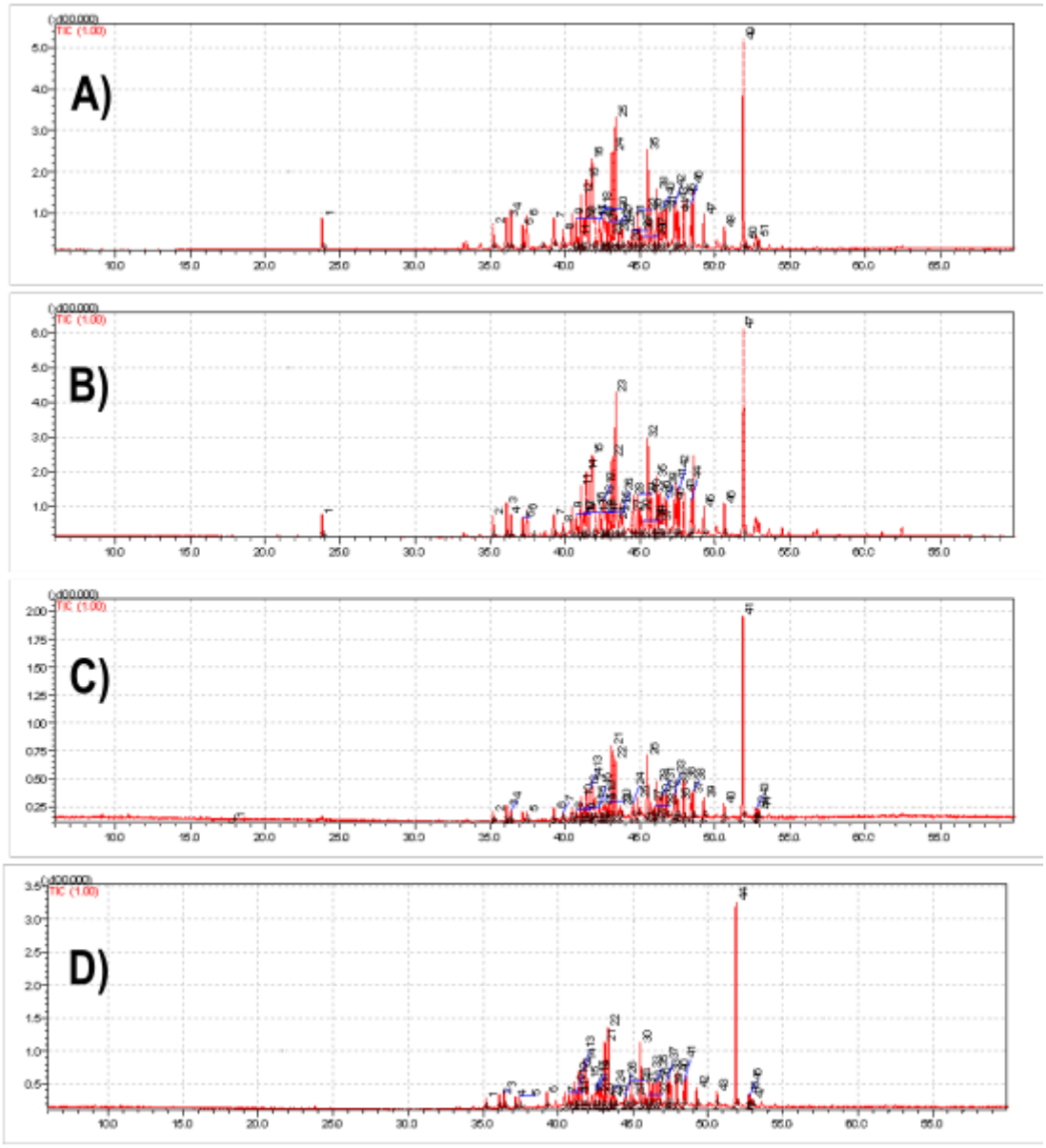

Figure 10 GCMS diagram of the agarwood oil. A. Bharse physical, B.Bharse fungus inoculation, C. Damauli physical, D. Damauli fungal inoculation

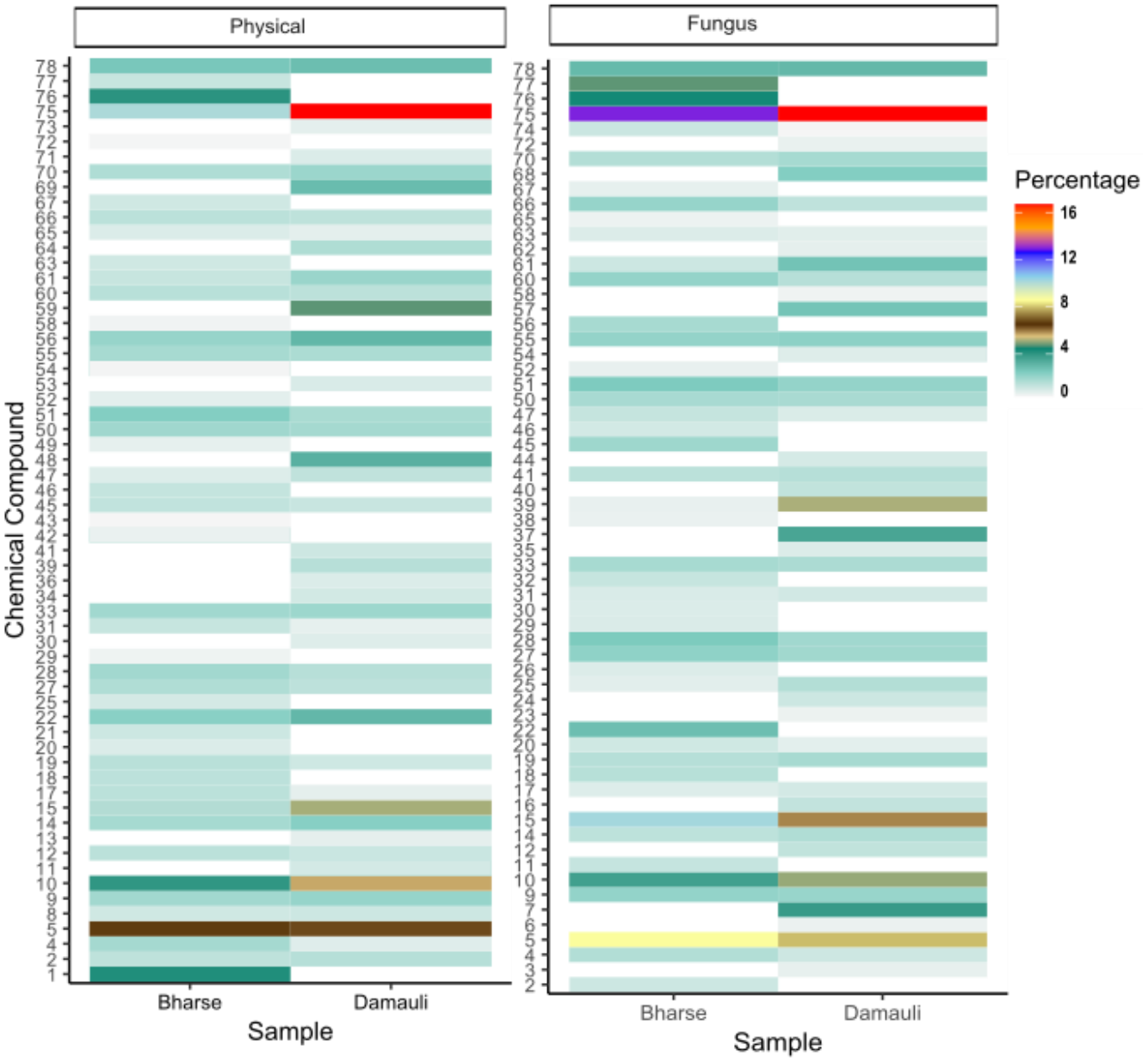

Figure 11 Heat map showing the percentage composition of compound in agarwood. (Full name of compound is in annex 7)

4.7 Cost estimation

Agarwood chips yield was calculate for each treatment group (physical wounding, and *Fusarium* inoculation) based on the average agarwood chips from tree sample. The control group exhibited no chips as well as oil production. The physical yield 135 gm in Damauli whereas 250 g in the Ghulmi form each five trees. Similarly for the *Fusarium* inoculation the chips yields was 730 g and 460 g in Gulmi and Damauli respectively (Figure 12). The value of agarwood has been estimated in the range of 9700 to 32000 USD per Kg, which is depends on quality grade of agarwood (Sen *et al.*, 2015; Buragohain and Dutta 2024). Assuming the lowest quality of our chips also, the price is US $ 9700 per kg. The average price per tree for the physical wounding treatment was calculated to be US $ 485 in Gulmi and US $ 262 per tree in Damauli , while *Fusarium* inoculation yielded an average price of US $ 1416 and 893 per tree in Gulmi and Damauli respectively.

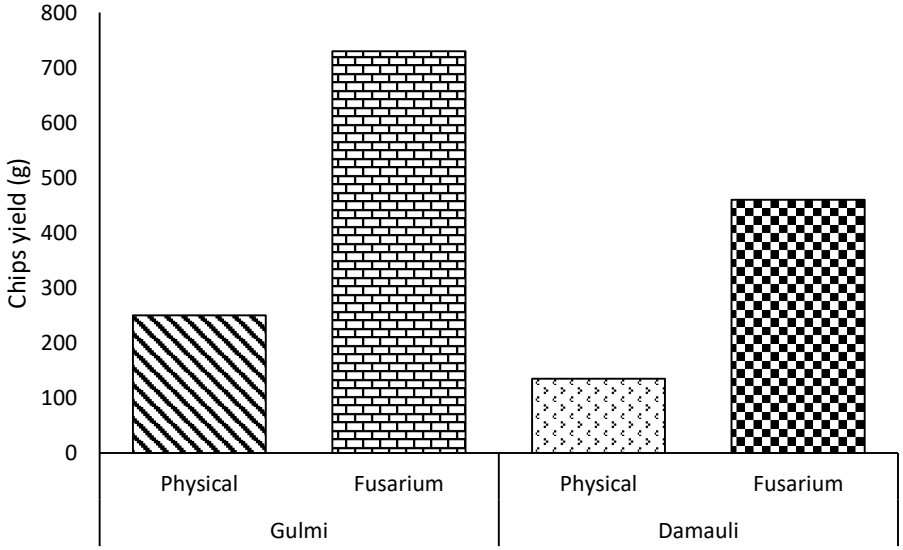

Figure 12 Chips yield (g) from five trees for each treatments.

CHAPTER-5

DISCUSSION

5.1 Length of discoloration

The formation of agarwood is generally associated with the wounding and fungal infection of the *Aquilaria* trees (Liu Y. *et al.* 2013; Mohamed *et al.* 2014). This study highlighted how different treatments affect the formation of agarwood in *Aquilaria malaccensis*. As expected, the fungus treatment showed a much bigger area of discoloration, confirming what other studies have found that agarwood can be triggered by the presence of fungi (Mahamad *et al.* 2014; Azren *et al.* 2019). Similarly, the physical treatment caused agarwood to form, possibly because the tree reacts to wounds. On the other hand, the control group didn't show any agarwood formation, suggesting that the studied agarwood trees might be too young or didn't have the natural triggers for agarwood development (Shivanand et al., 2022). It's interesting to that in *Aquilaria malaccensis*, agarwood usually forms in response to natural events like lightning, wind breaking branches, or attacks by pests and diseases. These events create wounds that make the tree's defense system activate (Mohamed *et al*. 2010). Present findings support the idea that agarwood forms as a biochemical defense response to stress and intruders. While physical wounds can cause agarwood, our study shows that *Fusarium* fungi induce a higher level of agarwood formation, pointing out the different impacts of stressors on the process (Mohamed *et al*. 2010;Rasool and Mohamed, 2016).

Furthermore we used physical drilling in Damauli and electric borer in Gulmi, that may be the reason for more agarwood formation in Gulmi as compared to Damauli. So, we recommend to use effective drilling and making deep holes while making holes for agarwood formation.

5.2 Relation of discoloration zone and branch diameter

In the present study, the overall regression analysis revealed a significant influence of trunk diameter on the length of the discoloration zone, aligning with previous findings suggesting a positive association between agarwood yield and larger stem diameters (Surtharti *et al.* 2011). The treatment-specific analysis uncovered further relationships,

with Gulmi's Fungus treatment exhibiting a notably strong positive correlation between the length of the discoloration zone and trunk diameter. This finding underscores the treatment-specific nature of agarwood formation, suggesting that the choice of inoculation method can significantly impact the outcome. These insights contribute to the ongoing refinement of agarwood cultivation techniques, emphasizing the importance of considering treatment type, geographical location, and tree age for optimal results in different ecological contexts as suggested by Rasool & Mohamed (2016).

This investigation highlight the importance of considering the diameter of plants during the inoculation process. The positive correlation observed between trunk diameter and the length of discoloration zone highlights that plants with larger diameters exhibit more extensive agarwood.

5.3 Agarwood percentage

The examination of agarwood percentages under different treatments and locations, as outlined in Table 3 and analyzed through ANOVA, underscores the significant impact of both location and treatment, including their interaction, on agarwood formation. Fungal treatment displayed the highest agarwood percentage, followed by the physical treatment, while the control treatment exhibited no agarwood formation. The association between agarwood formation and the self-defense mechanism of *Aquilaria* trees in response to various stresses, such as wounds and fungal infections, is well-established in literature (Gao *et al.* 2012; Singh and Sharma, 2015). The resin secreted by the trees serves as a defense reaction, accumulating around wounds over time to form agarwood (Subasinghe and Hettiarachchi, 2013). The evolving understanding of *Aquilaria*-fungal interactions has resulted the adoption of deliberate wounding coupled with biological inoculation for agarwood induction, surpassing the efficacy of sole mechanical wounding (Jong *et al.* 2014). Our results align with these findings, highlighting that using fungal inoculation for agarwood production works better. Our study also supports the what Jong *et al.* (2014) discovered. That means although the traditional method of physically wounding the trees is cheaper it doesn't give good-quality agarwood and has uncertain results (Azren *et al.* 2019). So, our study emphasizes that using *Fusarium* fungi to induce agarwood is a powerful method, and

it provides important ideas for improving how we grow agarwood in the commercial scale.

5.4 Effect of inoculation on plant growth rate

There is not significant impact of inoculation in the growth rate of the plant. This means that the inoculation does not hamper the plant growth. The inoculation of fungus i.e. *Fusarium* or just a physical wound also does not have any significance effect on the plant's growth. This means that the inoculation in the agarwood can be done anytime without affecting its growth.

5.5 Effect of numbers of holes in the mean weight of chips:

From the result of two way-ANOVA it is found that the total numbers of holes in the plant does not affect mean weight of chips. Rather they are affected by the types of inoculation. So high number of inoculations can yield the higher chips weight but the mean weight is not affected by the number of the holes.

5.6 Chemical composition

This study represents the first comprehensive examination of agarwood treatment and chemical analysis in Nepal. Agarwood cultivation is relatively recent in Nepal, but it is widely found in Southeast Asia and parts of India like Assam. Our findings reveal a diverse array of volatile substances in agarwood, particularly aromatic and sesquiterpene compounds. Interestingly, healthy trees lacked sesquiterpenes but contained significant organic acids. Additionally, the inoculation method used to induce agarwood production significantly affected oil composition in healthy trees.

Altogethr 78 different chemicals were isolated from the agarwood oil in the present sudy. Similar chemicals also reported by others in different parts of the world. Oil of *Aquilaria malaccensis* contains aromatic chemicals, chromone and sesquiterpenes. In the Malaysian agarwood oil compounds like β-agarofuran, nor-ketoagarofuran, and α-eudesmol were identified (Tajuddin *et al.* 2013, Ahmad and Kulkarni, 2017). Additionally, research by Mohamad *et al.* (2014) also reported sesquiterpenes such as β-agarofuran and α-agarofuran. Another study in Indonesia, conducted by Nakanishi et al. (1983), identified jinkohol, agarospirol, and nootkatane in *A. Malaccensis*.

Furthermore, Pripdeevech *et al.* (2011) reported the presence of β-dihydroagarofuran and cyclocolorenone in Thai agarwood.

The study reveals that agarwood comprises diverse volatile substances, particularly from aromatic and sesquiterpene groups. As the numbers of chemicals in fungal inoculated agarwood oil is more than the physical wounding, we can infer that the inoculation method also significantly influences the oil composition. Our treatment method specially *Fusarium* effectively stimulates resin synthesis in *A. malaccensis*, resulting in high levels of aromatics and sesquiterpenes. Additionally, a small number of volatiles primarily contribute to agarwood's characteristic odor, indicating the success of our project.

5.7 Cost estimation

The price of agarwood can vary depending on the quality and source of the chips. Agarwood, derived from Aquilaria trees, is highly valued for its unique scent and medicinal properties (Ali et al., 2016). The quantity and quality of agarwood can be classified into different groups based on resin accumulation levels, with certain branches yielding higher quantities of oil with specific compositions of sesquiterpenes and fatty acids (Huy et al., 2022). Agarwood oil has been used in various products, including skin care items, due to its beneficial properties (Malik et al., 2023). In our study *Fusarium* inoculation generated significantly higher average prices per tree compared to physical wounding for agarwood production, with values ranging from US $ 262 to US $ 1416 . *Fusarium* inoculation could lead to increased revenue for producers and a more sustainable agarwood industry.

CHAPTER-6

CONCLUSION AND RECOMMENDATION

6.1 Conclusion

In conclusion, this study demonstrates that both physical wounding and fungal inoculation can successfully induce agarwood chip formation in *Aquilaria* trees. However, fungal inoculation (*Fusarium)* exhibits a more pronounced positive impact on agarwood formation compared to physical wounding methods. Importantly, neither treatment method appeared to hinder the growth rate of the trees.

Furthermore, a positive relation was observed between the diameter of the inoculated tree and the length of the resulting discoloration zone, indicating that larger trees possess a higher potential for producing greater quantities of agarwood. Interestingly, the two sites Gulmi and Damauli did not appear to significantly influence agarwood formation. However, differences were observed in the chemical composition of the agarwood produced through each treatment i.e by *Fusarium* inoculation and physical wounding.

6.2 Recommendation

Based on present study, following is recommended,

- Our study recommends the use of artificial inoculation, particularly emphasizing the efficacy of suitable fungal inoculants like *Fusarium* over the comparatively slower physical wounding method.
- We recommend that practitioners consider their inoculation strategies based on plant diameter to maximize agarwood yield.
- Practitioners should account for ecological variations when cultivating agarwood and implementing fungal inoculation techniques for optimal results.
- We recommend to use effective drilling and making deep holes while making holes for agarwood formation.
- Other species of fungus should be explored for agarwood formation.
- Fungus inoculation should be done in the other native species to check the resign formation.

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ANNEXES

Annex 1: Growth rate calculation

S.N	Location	Sample	Treatment	Height (cm) (initial)	Height (cm)(final (after 9 month)	Growth rate
1	Gulmi	C1	Control	250	276	10.4
2	Gulmi	C2	Control	290	325	12.06897
3	Gulmi	C3	Control	300	337	12.33333
4	Gulmi	C4	Control	290	316	8.965517
5	Gulmi	C5	Control	357	390	9.243697
6	Gulmi	F2	Fungus	305	337	10.4918
7	Gulmi	F4	Fungus	589	635	7.809847
8	Gulmi	F5	Fungus	590	635	7.627119
9	Gulmi	F6	Fungus	360	395	9.722222
10	Gulmi	F8	Fungus	545	590	8.256881
11	Gulmi	P10	Physical	395	420	6.329114
12	Gulmi	P2	Physical	240	273	13.75
13	Gulmi	P3	Physical	340	375	10.29412
14	Gulmi	P4	Physical	334	358	7.185629
15	Gulmi	P7	Physical	323	345	6.811146
16	Damauli	C1	Control	235	251	6.808511
17	Damauli	C2	Control	204	227	11.27451
18	Damauli	C3	Control	215	231	7.44186
19	Damauli	C4	Control	187	205	9.625668
20	Damauli	C5	Control	242	265	9.504132
21	Damauli	F1	Fungus	241	267	10.78838
22	Damauli	F2	Fungus	255	276	8.235294
23	Damauli	F4	Fungus	213	231	8.450704
24	Damauli	F5	Fungus	280	310	10.71429
25	Damauli	F7	Fungus	234	254	8.547009
26	Damauli	P1	Physical	205	225	9.756098
27	Damauli	P3	Physical	235	255	8.510638
28	Damauli	P4	Physical	290	312	7.586207
29	Damauli	P5	Physical	215	233	8.372093
30	Damauli	P7	Physical	250	276	10.4

Annex 2: Diameter (cm) and discoloration length (cm)

S.N.	Location	Treatment	Dimater	Discoloration_length
1	Gulmi	Control	17	0
2	Gulmi	Fungus	32.5	19.2
3	Gulmi	Fungus	31.5	14.65
4	Gulmi	Fungus	30	8.8
5	Gulmi	Fungus	26.5	9.13
6	Gulmi	Control	25	0
7	Gulmi	Physical	25	2.46
8	Gulmi	Physical	24	1.97
9	Gulmi	Control	23	0
10	Gulmi	Fungus	23	7.26
11	Gulmi	Physical	22	1.79
12	Gulmi	Physical	20	1.82
13	Gulmi	Control	18	0
14	Gulmi	Physical	18	1.78
15	Gulmi	Control	15	0
16	Damauli	Control	17	0
17	Damauli	Fungus	26	6.49
18	Damauli	Physical	26	2.11
19	Damauli	Physical	24	1.61
20	Damauli	Physical	24	1.54
21	Damauli	Control	23	0
22	Damauli	Fungus	23	5.86
23	Damauli	Physical	23	1.49
24	Damauli	Fungus	20	5.38
25	Damauli	Fungus	20	5.66
26	Damauli	Control	19	0
27	Damauli	Control	18	0
28	Damauli	Physical	17	1.31
29	Damauli	Control	15	0
30	Damauli	Fungus	14	5.1

Annex 3: Agarwood percentage calculation

S.N	Location	Treatment	Tretment	Weight of chip (g)	total weight (g)	Agarwood percentage
1	Damauli	C1	Control	0	4900	0
2	Damauli	C2	Control	0	5390	0
3	Damauli	C3	Control	0	6174	0
4	Damauli	C4	Control	0	6664	0
5	Damauli	C5	Control	0	7056	0
6	Damauli	F1	Fungus	61	10345	0.589657
7	Damauli	F2	Fungus	69	14450	0.477509
8	Damauli	F4	Fungus	83	15935	0.520866
9	Damauli	F5	Fungus	87	15934	0.546002
10	Damauli	F7	Fungus	32	5467	0.58533
11	Damauli	P1	Physical	29	10023	0.289335
12	Damauli	P3	Physical	12	3967	0.302496
13	Damauli	P4	Physical	23	10300	0.223301
14	Damauli	P5	Physical	18	12170	0.147905
15	Damauli	P7	Physical	19	14023	0.135492
16	Gulmi	C1	Control	0	5000	0
17	Gulmi	C2	Control	0	5500	0
18	Gulmi	C3	Control	0	6300	0
19	Gulmi	C4	Control	0	6800	0
20	Gulmi	C5	Control	0	7200	0
21	Gulmi	F2	Fungus	155	15250	1.016393
22	Gulmi	F4	Fungus	165	16552	0.996858
23	Gulmi	F5	Fungus	178	18404	0.967181
24	Gulmi	F6	Fungus	71	8925	0.795518
25	Gulmi	F8	Fungus	301	29650	1.015177
26	Gulmi	P10	Physical	40	9560	0.41841
27	Gulmi	P2	Physical	48	11360	0.422535
28	Gulmi	P3	Physical	32	10300	0.31068
29	Gulmi	P4	Physical	32	8220	0.389294
30	Gulmi	P7	Physical	35	9624	0.363674

Annex 4: length of discoloration zone calculation (cm)

S.N.	Sample no		Location	Treatment	Lu	Ll	Total
1	F2	H1	Gulmi	Fungus	2.1	7.9	10
2	F2	H2	Gulmi	Fungus	3.2	5.7	8.9
3	F2	H3	Gulmi	Fungus	3	4.8	7.8
4	F2	H4	Gulmi	Fungus	2.5	3.6	6.1
5	F2	H5	Gulmi	Fungus	2.8	3.2	6

6	F2	H6	Gulmi	Fungus	3.5	3.9	7.4
7	F2	H7	Gulmi	Fungus	2.7	2.8	5.5
8	F2	H8	Gulmi	Fungus	2.6	3.1	5.7
9	F2	H9	Gulmi	Fungus	2.1	2.6	4.7
10	F2	H10	Gulmi	Fungus	4.8	3.4	8.2
11	F2	H11	Gulmi	Fungus	0.7	0.9	1.6
12	F2	H12	Gulmi	Fungus	2	2.1	4.1
13	F2	H13	Gulmi	Fungus	1.4	2.3	3.7
14	F4	H1	Gulmi	Fungus	9.5	10.1	19.6
15	F4	H2	Gulmi	Fungus	1.9	2.1	4
16	F4	H3	Gulmi	Fungus	5.7	5.7	11.4
17	F4	H4	Gulmi	Fungus	8.8	8	16.8
18	F4	H5	Gulmi	Fungus	4.5	4.8	9.3
19	F4	H6	Gulmi	Fungus	2	2.9	4.9
20	F4	H7	Gulmi	Fungus	11	12.5	23.5
21	F4	H8	Gulmi	Fungus	12.3	11	23.3
22	F4	H9	Gulmi	Fungus	11.2	7.9	19.1
23	F4	H10	Gulmi	Fungus	3	4.5	7.5
24	F4	H11	Gulmi	Fungus	20.7	21.3	42
25	F4	H12	Gulmi	Fungus	12	13.5	25.5
26	F4	H13	Gulmi	Fungus	2.5	4.5	7
27	F4	H14	Gulmi	Fungus	3	2.9	5.9
28	F4	H15	Gulmi	Fungus	13	9.5	22.5
29	F4	H16	Gulmi	Fungus	15	10	25
30	F4	H17	Gulmi	Fungus	18.5	6.5	25
31	F5	H1	Gulmi	Fungus	3	2.8	5.8
32	F5	H2	Gulmi	Fungus	2.1	2	4.1
33	F5	H3	Gulmi	Fungus	3.5	5	8.5
34	F5	H4	Gulmi	Fungus	1.9	3.5	5.4
35	F5	H5	Gulmi	Fungus	2.3	3	5.3
36	F5	H6	Gulmi	Fungus	1.8	3.1	4.9
37	F5	H7	Gulmi	Fungus	3	2.5	5.5
38	F5	H8	Gulmi	Fungus	1.7	3	4.7
39	F5	H9	Gulmi	Fungus	1.6	4.4	6
40	F5	H10	Gulmi	Fungus	1.4	2	3.4
41	F5	H11	Gulmi	Fungus	3	3.4	6.4
42	F5	H12	Gulmi	Fungus	1.8	2.9	4.7
43	F5	H13	Gulmi	Fungus	1.7	2	3.7
44	F6	H1	Gulmi	Fungus	2.6	5.3	7.9
45	F6	H2	Gulmi	Fungus	3	7	10
46	F6	H3	Gulmi	Fungus	2.8	16.5	19.3
47	F6	H4	Gulmi	Fungus	2.5	5	7.5
48	F6	H5	Gulmi	Fungus	2.5	6	8.5
49	F6	H6	Gulmi	Fungus	2.8	5.5	8.3
50	F6	H7	Gulmi	Fungus	3.1	5	8.1
51	F6	H8	Gulmi	Fungus	2.2	3.7	5.9
52	F6	H9	Gulmi	Fungus	2.4	4.3	6.7
53	F6	H10	Gulmi	Fungus	2.3	3.5	5.8

54	F8	H1	Gulmi	Fungus	2.1	5.6	7.7
55	F8	H2	Gulmi	Fungus	8.3	10.9	19.2
56	F8	H3	Gulmi	Fungus	3.7	8.5	12.2
57	F8	H9	Gulmi	Fungus	2	2.9	4.9
58	F8	H10	Gulmi	Fungus	4.9	6.1	11
59	F8	H1	Gulmi	Fungus	3.6	7	10.6
60	F8	H2	Gulmi	Fungus	2.1	1.7	3.8
61	F8	H3	Gulmi	Fungus	2.2	2.9	5.1
62	F8	H9	Gulmi	Fungus	6.6	9.4	16
63	F8	H10	Gulmi	Fungus	2.8	8	10.8
64	F8	H1	Gulmi	Fungus	4.8	5.2	10
65	F8	H2	Gulmi	Fungus	3.8	6.2	10
66	F8	H3	Gulmi	Fungus	1.9	2.5	4.4
67	F8	H9	Gulmi	Fungus	5.7	2.8	8.5
68	F8	H10	Gulmi	Fungus	3.2	9.4	12.6
69	F8	H1	Gulmi	Fungus	3.2	10.1	13.3
70	F8	H2	Gulmi	Fungus	3	8	11
71	F8	H3	Gulmi	Fungus	2.1	4.1	6.2
72	F8	H9	Gulmi	Fungus	2.5	6.6	9.1
73	F8	H10	Gulmi	Fungus	2.6	7.3	9.9
74	F8	H11	Gulmi	Fungus	7	4.5	11.5
75	F8	H12	Gulmi	Fungus	1.7	2.9	4.6
76	P7	H1	Gulmi	Physiscal	1	1	2
77	P7	H2	Gulmi	Physiscal	0.9	0.9	1.8
78	P7	H3	Gulmi	Physiscal	0.9	1	1.9
79	P7	H4	Gulmi	Physiscal	0.9	1.1	2
80	P7	H5	Gulmi	Physiscal	0.9	1	1.9
81	P7	H6	Gulmi	Physiscal	0.5	0.7	1.2
82	P7	H7	Gulmi	Physiscal	0.7	0.9	1.6
83	P7	H8	Gulmi	Physiscal	0.8	1.4	2.2
84	P7	H9	Gulmi	Physiscal	0.6	0.8	1.4
85	P4	H1	Gulmi	Physiscal	0.8	1.2	2
86	P4	H2	Gulmi	Physiscal	0.8	1.6	2.4
87	P4	H3	Gulmi	Physiscal	1	1	2
88	P4	H4	Gulmi	Physiscal	0.9	1.4	2.3
89	P4	H5	Gulmi	Physiscal	0.8	1	1.8
90	P4	H6	Gulmi	Physiscal	0.9	0.9	1.8
91	P4	H7	Gulmi	Physiscal	0.7	0.8	1.5
92	P10	H1	Gulmi	Physiscal	1.1	1	2.1
93	P10	H2	Gulmi	Physiscal	1	1.5	2.5
94	P10	H3	Gulmi	Physiscal	1.4	1.6	3
95	P10	H4	Gulmi	Physiscal	0.9	1.6	2.5
96	P10	H5	Gulmi	Physiscal	1	1.3	2.3
97	P10	H6	Gulmi	Physiscal	1	2.2	3.2
98	P10	H7	Gulmi	Physiscal	0.6	1.4	2
99	P10	H8	Gulmi	Physiscal	1	1.1	2.1
100	P2	H1	Gulmi	Physiscal	1.1	1.3	2.4
101	P2	H2	Gulmi	Physiscal	1	1.1	2.1

102	P2	H3	Gulmi	Physiscal	1	1	2
103	P2	H4	Gulmi	Physiscal	0.8	0.9	1.7
104	P2	H5	Gulmi	Physiscal	0.6	0.9	1.5
105	P2	H6	Gulmi	Physiscal	0.8	0.9	1.7
106	P2	H7	Gulmi	Physiscal	0.7	0.7	1.4
107	P2	H8	Gulmi	Physiscal	0.7	0.8	1.5
108	P3	H1	Gulmi	Physiscal	0.9	1.1	2
109	P3	H2	Gulmi	Physiscal	0.9	1.3	2.2
110	P3	H3	Gulmi	Physiscal	1	1.2	2.2
111	P3	H4	Gulmi	Physiscal	0.9	1	1.9
112	P3	H5	Gulmi	Physiscal	0.9	0.9	1.8
113	P3	H6	Gulmi	Physiscal	0.8	0.9	1.7
114	P3	H7	Gulmi	Physiscal	0.7	0.7	1.4
115	P3	H8	Gulmi	Physiscal	0.6	0.8	1.4
116	C1	H1	Gulmi	Control	0	0	0
117	C2	H2	Gulmi	Control	0	0	0
118	C3	H3	Gulmi	Control	0	0	0
119	C4	H4	Gulmi	Control	0	0	0
120	C5	H5	Gulmi	Control	0	0	0

Annex 5: Certification of participation in the training programme

वर्षा वन अनुसंधान संस्थान
RAIN FOREST RESEARCH INSTITUTE
भारतीय वानिकी अनुसंधान एवं शिक्षा परिषद्
Indian Council of Forestry Research & Education
(पर्यावरण, वन एवं जलवायु परिवर्तन मंत्रालय, भारत सरकार के अधीन)
(Under Ministry of Environment, Forest and Climate Change, Govt. of India)
देववन, जोरहाट - ७८५०१०, असम
Deovan, Jorhat - 785010, Assam

TRAINING COMPLETION CERTIFICATE

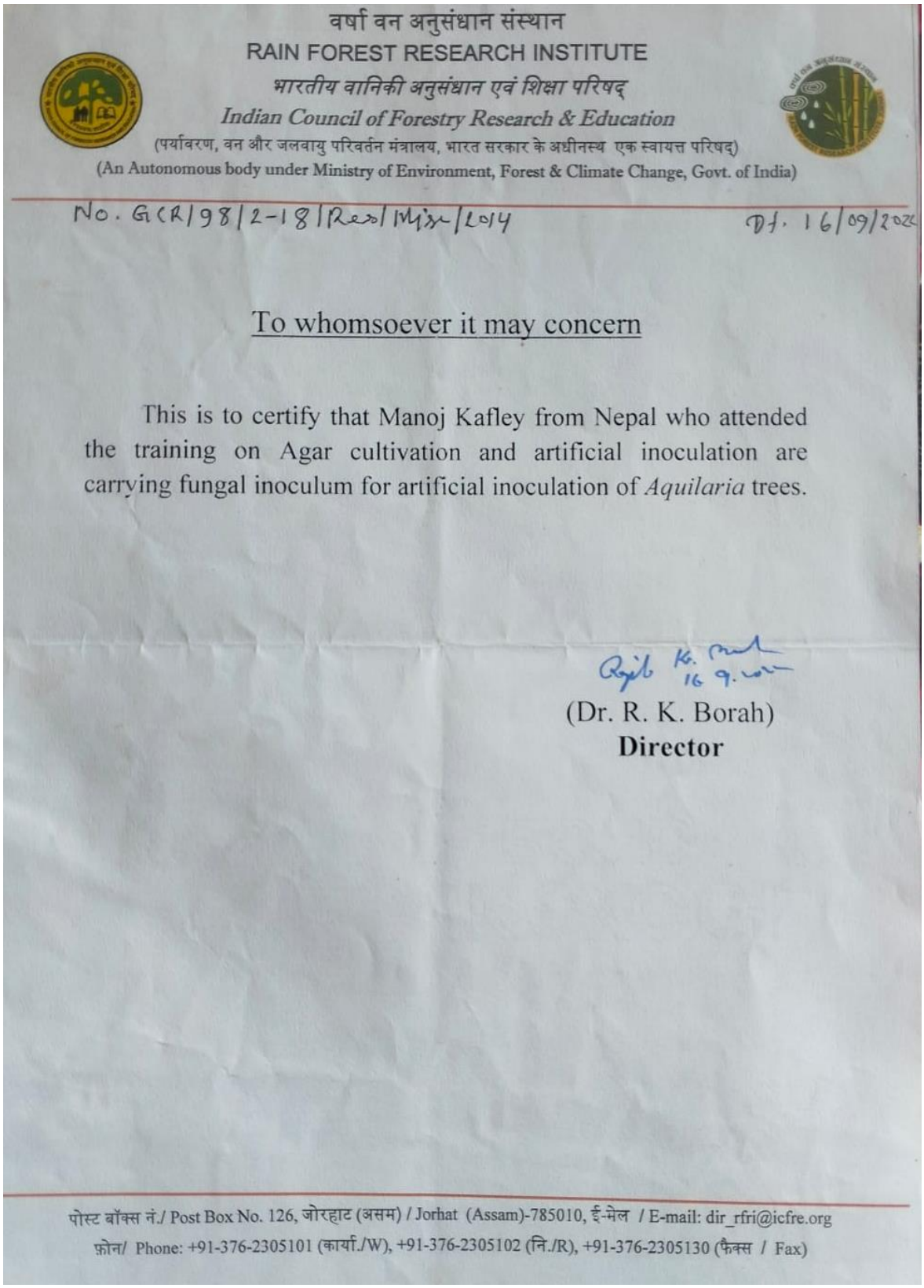
This is to certify that **MANOJ KAFLEY, Kathmandu, Nepal** has successfully completed **SKILL DEVELOPMENT TRAINING ON AGARWOOD CULTIVATION AND ARTIFICIAL INOCULATION** organized by RFRI, Jorhat (Assam) during 14-16 September, 2022.

RFRI, Jorhat
16-09-2022

(R. K. Kalita)
Course Coordinator

(Dr. R. K. Borah)
Director

Annex 6: letter for carrying fungal inoculum.



वर्षा वन अनुसंधान संस्थान
RAIN FOREST RESEARCH INSTITUTE
भारतीय वानिकी अनुसंधान एवं शिक्षा परिषद्
Indian Council of Forestry Research & Education
(पर्यावरण, वन और जलवायु परिवर्तन मंत्रालय, भारत सरकार के अधीनस्थ एक स्वायत्त परिषद्)
(An Autonomous body under Ministry of Environment, Forest & Climate Change, Govt. of India)

No. GCR/98/2-18/Res/Misc/2014 Dt. 16/09/2024

To whomsoever it may concern

This is to certify that Manoj Kafley from Nepal who attended the training on Agar cultivation and artificial inoculation are carrying fungal inoculum for artificial inoculation of *Aquilaria* trees.

Rajib K. Borah 16.9.24

(Dr. R. K. Borah)
Director

पोस्ट बॉक्स नं./ Post Box No. 126, जोरहाट (असम) / Jorhat (Assam)-785010, ई-मेल / E-mail: dir_rfri@icfre.org
फ़ोन/ Phone: +91-376-2305101 (कार्या./W), +91-376-2305102 (नि./R), +91-376-2305130 (फैक्स / Fax)

Annex 7: Name of compound found in agarwood:

[1] "Phenethyl ketone <methyl->"
[2] "Amorphene <delta->"
[3] "Cadinene <gamma->"
[4] "Cadinene <delta->"
[5] "Copaen-11-ol <alpha->"
[6] "Agarofuran <alpha->"
[7] "Atlantol <beta->"
[8] "Nootkatol"
[9] "Eudesmol <gamma->"
[10] "Cubenol <1-,10-di-epi->"
[11] "Hinesol"
[12] "Guaiac acetate"
[13] "Muurolol <alpha-, epi->"
[14] "Valerianol"
[15] "Cadin-4-en-10-ol"
[16] "Cyclocolorenone"
[17] "Isovalencenol <(E)->"
[18] "Guaia-3,9-dien-11-ol <cis->"
[19] "Himachalene oxide <beta->"
[20] "Vetivone <beta->"
[21] "Muurolene <15-oxy-alpha->"
[22] "Selinene <beta->"
[23] "Aristolone"
[24] "Amorpha-4,9-dien-2-ol"
[25] "Gurjunene <gamma->"
[26] "Calamenen-10-one <10-nor->"
[27] "Caryophyllene <14-hydroxy-(Z)->"
[28] "Farnesyl acetate <2E,6E>-"
[29] "Vetispirene <beta->"
[30] "Muurola-4,10(14)-dien-1-beta-ol"
[31] "Lanceol <cis->"
[32] "Calamenene <cis->"
[33] "Costol <gamma->"
[34] "14,15-Bisnorlabdane <8,13:13,20-diepoxy->"
[35] "Costol <beta->"
[36] "Humulene <alpha->"
[37] "Zierone"
[38] "Costol <alpha->"
[39] "Aristola-1(10),8-diene"
[40] "Quinoline <2-isobutyl->"
[41] "Cyclocolorenone <epi->"
[42] "Cembrene"
[43] "Nootkatone"
[44] "Longifolene <iso->"
[45] "Cadalene <8,9-epoxide->"
[46] "Acalea <trans->"
[47] "Guaiazulene"
[48] "Valencene <13-hydroxy->"
[49] "Modhephen-8-beta-ol"
[50] "Cubebol <epi->"
[51] "Copaen-4-alfa-ol <beta->"
[52] "Eudesmol <10-epi-gamma->"
[53] "Thujopsenal"
[54] "Eremophilone"
[55] "Aromandendrene epoxide <allo->"
[56] "Epiligulyl oxide"
[57] "Muurolene <gamma->"
[58] "Dauca-5,8-diene"
[59] "Cadina-1(6),4-diene"
[60] "Amorphene <alpha->"
[61] "Dodecanenitrile"
[62] "Eudesma-6,11-diene <cis->"
[63] "Chamigrene <beta->"
[64] "Germacra-4(15),5,10(14)-trien-1-alpha-ol"
[65] "Amorpha-4,9-diene <7,14-anhydro->"
[66] "Acoradiene <beta->"
[67] "Methanonaphthalene-8-ethanol <1,3,4,5,6,7-hexahydro-, ß,1,1,5,5-pentamethyl-, 2H-2,4a-> isomer II"
[68] "Presilphiperfol-7-ene"
[69] "Cinnamaldehyde <(2E), -hexyl->"
[70] "Thujopsadiene <cis->"
[71] "Naphth-1-ol <1,2,3,4,4a,7,8,8a-octahydro-, 4-isopropyl-, 1,6-dimethyl->"
[72] "Gurjunene <alpha->"
[73] "Presilphiperfolan-8-ol"
[74] "Isoprecyclemone E"
[75] "Cyperotundone"
[76] "Mustakone"
[77] "Bicyclogermacrene"
[78] "Dihydromayurone"

Annex: 8 Report of GCMS analysis

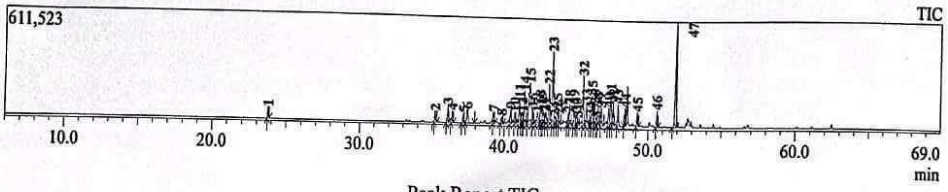
Department of Plant Resources
Thapathali, Kathmandu
Instrument Section
Test Report

Issued To: Manoj Kafley
Sample Name: Agarwood-Bharse F2
Sampled by: Customer
Received Date: 2023-10-08
Sample Amount: 5 mL
Report No.: 121
Detector Type: MS
Column Type: RTX-5MS

Sample Type: Essential Oil
Sample ID: IG-65
Analyzed Date: 2023-10-10
Report Issue Date: 2023-12-18
Injection Volume: 1.00 uL
Packing of Sample: Glass Vial
Library Used: FFNSC 4.0
Dimension of Column: 60mX0.32mmX0.25um

GCMS Profiling

Chromatogram Agarwood-Bharse F2



Peak Report TIC

Peak#	R.Time	Area	Area%	Name
1	23.785	232655	0.80	Phenethyl ketone <methyl->
2	35.202	280746	0.97	Cubebol <epi->
3	36.090	439990	1.52	Cadinene <gamma->
4	36.385	273198	0.94	Cadinene <delta->
5	37.159	250117	0.86	Copaen-11-ol <alpha->
6	37.439	463680	1.60	Agarofuran <alpha->
7	39.245	277866	0.96	Copaen-4-alfa-ol <beta->
8	39.847	201554	0.70	Nootkatol
9	40.446	424544	1.46	Eudesmol <10-epi-gamma->
10	40.738	289012	1.00	Cubenol <1-,10-di-epi->
11	41.061	683514	2.36	Hinesol
12	41.236	321853	1.11	Guaiac acetate
13	41.280	274521	0.95	Muurolol <alpha-, epi->
14	41.422	1152650	3.98	Valerianol
15	41.794	2625255	9.05	Cadin-4-en-10-ol
16	41.977	375348	1.29	Cyclocolorenone
17	42.315	573401	1.98	Cembrene
18	42.525	550185	1.90	Guaia-3,9-dien-11-ol <cis->
19	42.627	507421	1.75	Himachalene oxide <beta->
20	42.725	360949	1.24	Thujopsenal
21	42.890	351973	1.21	Muurolene <15-oxy-alpha->
22	43.121	990226	3.42	Aristolone
23	43.372	2226330	7.68	Amorpha-4,9-dien-2-ol
24	43.498	222969	0.77	Eremophilone
25	43.704	447036	1.54	Calamenen-10-one <10-nor->
26	43.812	380571	1.31	Aromandendrene epoxide <allo->
27	44.471	309178	1.07	Vetispirene <beta->
28	44.551	593346	2.05	Muurola-4,10(14)-dien-1-beta-ol

1 / 2

Peak#	R.Time	Area	Area%	Name
29	44.190	104538	0.42	Farnesyl acetate <2E,6E>-
30	44.461	176108	0.70	Vetispirene <beta->
31	44.544	381374	1.52	Muurola-4,10(14)-dien-1-beta-ol
32	44.856	457763	1.82	Lanceol <cis->
33	45.024	226217	0.90	Calamenene <cis->
34	45.115	135897	0.54	Costol <gamma->
35	45.494	1063978	4.23	14,15-Bisnorlabdane <8,13:13,20-diepoxy->
36	45.723	477569	1.90	Costol <beta->
37	45.998	185083	0.74	Humulene <alpha->
38	46.101	656994	2.61	Zierone
39	46.289	418795	1.66	Costol <alpha->
40	46.530	478779	1.90	Aristola-1(10),8-diene
41	46.749	421448	1.67	Quinoline <2-isobutyl->
42	47.270	481722	1.91	Cyclocolorenone <epi->
43	47.422	582177	2.31	Cembrene
44	47.573	369741	1.47	Nootkatone
45	47.922	525395	2.09	Longifolene <iso->
46	48.408	460794	1.83	Cadalene <8,9-epoxide->
47	49.251	357668	1.42	Acalea <trans->
48	50.608	216110	0.86	Guaiazulene
49	51.887	2542144	10.10	Valencene <13-hydroxy->
50	52.009	114998	0.46	Isovalencenol <(E)->
51	52.777	127875	0.51	Modhephen-8-beta-ol
		25168349	100.00	

Analyzed By

Checked By

1. The above results refer only to the submitted sample and test performed.
2. This report cannot be used for any publicity or advertisement without the written consent of Department of Plant Resources, Kathmandu.
3. Test report shall not be reproduced, except in full, without the written consent of the laboratory.
4. The name of the sample was claimed by the customer.

Photo plates:

Photo plate 1 Preliminary field visit (A), Researcher participating in training in India (B,C,D).

Photo plate 2 Innoculation at Damauli

Photo plate 3 Innoiculation at Bharse (A-C); Weight measurement of Branches (D)

Photo plate 4 yielding and carving

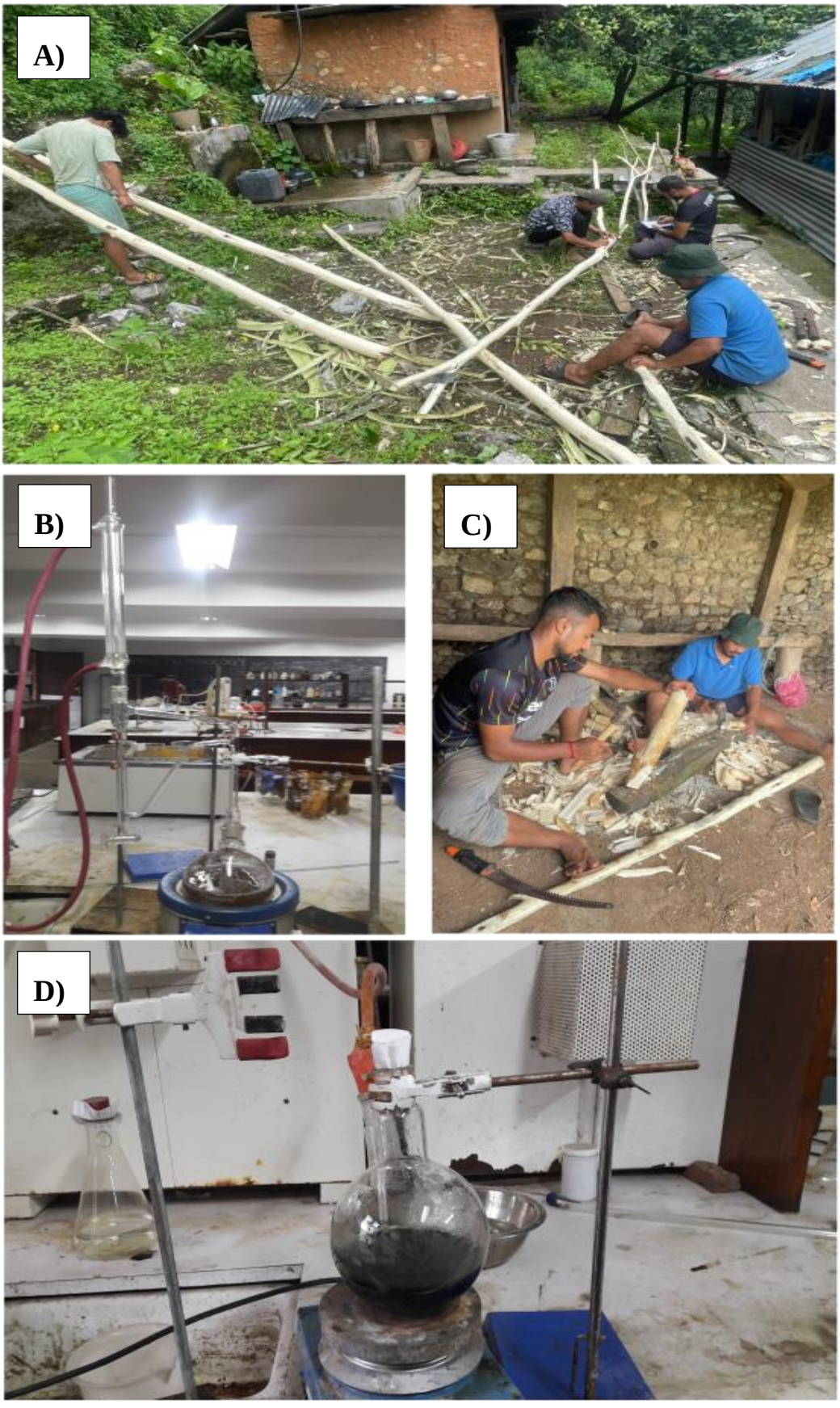


Photo plate 5 yielding and carving (A, C); laboratory work (B, D)

Photo plate 6 Researcher and Agarwood farm owner of Gulmi (from left to right 1.Manoj Kafley (Researcher), 2.Mr. Dhan Bahadur Pun and 3.Bhuvan Pun and his daughter. 4. Mr Assish Adhakari friend.