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# STUDY ON SENGON (*Falcataria moluccana)* RESISTANCE TO BOKTOR PEST (*Xystrocera festiva*) AND GALL RUST (*Uromycladium tepperianum*)

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# 1. INTRODUCTION

## 1.1. Background

Forest trees are a source of raw material for many of the essential needs of humans, including building material, paper products, firewood for heat and cooking, energy and many tree-crop foods. Forest trees also provide various ecological services, such as preservation of biodiversity, carbon sink, climate regulation and preservation of water quality; also represent our cultural and heritage.

Genomic research on forest trees is highly motivated by the need to support genetic improvement programs and develop diagnostic tools for the conservation, restoration and management of natural populations. Research progress in forest trees has been hindered by their long generation times and large genomes, the lack of well-characterized mutations for reverse genetic approaches, and limited funding. Nevertheless, over the past two decades progress has been made in forest tree genomics as a result of the efforts of a few dedicated researchers.

Genomics research in forest trees has unique advantages in that trees can be found in both the domesticated and non-domesticated condition. Research is needed in both areas: as trees are key drivers of terrestrial biodiversity and they are the largest producers of biomass, that research interests in genomics are focused on both ecologically and economically important species. Genome projects in most model and some non-model organisms begin with a reference genome sequence. This has not been true in forest trees owing to funding limitations and the large size of so many tree genomes. Forest tree genomes vary enormously in size and there is no clear model species for all forest trees, also no one species that is economically dominant over the others. This situation has surely hindered progress, but with the advent of next-generation sequencing (NGS) technologies, the large number of target species and the diversity among those species will become an asset for comparative genomic approaches.

Sengon is one of important fast growing legume species in Indonesia, grown either in monoculture or agroforestry system. Sengon is tropical multi-purpose trees, easily cultivated, and could produce nitrogen (Budelman, 2005). However, its main pest and disease, Boktor stem borer (*Xystrocera festiva*) and gall rust disease are capable of destroying sengon plantation (NFTA, 1989; Suharti *et al.*, 1998; Atmosuseno, 1999; Rimbawanto, 2006; Rahayu *et al.* 2009). Stem borer began attacking the 3-4 year old plants, and without adequate control of pests can ruin the entire crop. Meanwhile gall rust disease,

caused by the fungus *Uromycladium falcatarum*, attacks plants at all ages, from seedlings in the nursery until mature plants in the field, also potentially could damage the entire crop. Observations in the field showed that both the boktor pests and gall rust disease could attack plants at the same time causing enormous economic losses. The main concern is that the effective control method both for pests and diseases is not yet available.

Observations in the field showed that some individual trees show signs of resistance, because they can survive among severely affected populations. The existence of individuals who are resistant among the most vulnerable tress is highly expected, because the population of sengon in Indonesia generally, and Java particularly, are known to have a fairly high genetic diversity (Siregar *et al.*, 2005; Rahayu *et al.* 2009).

The results from Siregar et al. (2009) showed that sengon trees resistant to boktor pest have trypsin-inhibitor and alpha-amylase-inhibitor activities, which are significantly higher than the vulnerable ones. There is a possibility that the presence of inhibitors is a mechanism of resistance to these pests. For that, we need clarification by identifying the genes coding for these two inhibitors. Further research using microsatellite marker (Siregar 2016) showed that resistant sengon trees are different from susceptible ones, indicating a difference in the genetic background of the two accessions. Microsatellite markers thus could become guide for selection of resistant lines seedlings in a breeding and improvement program. Study by Shabrina et al. (2019) and Siregar (unpublished data) showed that there was different expression of certain genes in healthy sengon compared to gall-rust infected and boktor infested one. These different expressed candidate genes could be used further in the selection program for the resistant sengon.

Previous research results using scanned Electron Microscopy also showed that in some resistant lines of sengon against gall-rust disease the wood tissue could prevent the disease hyphae to enter the tissue and thus keep the tree healthy (Siregar 2016). Microsatellite marker utilized also able to differentiate resistant lines from susceptible ones, further indicating a genetic background for resistance to the disease. It was concluded that some gene(s) must be responsible for those resistance against those pest and disease.

Molecular biology has addressed functional questions, such as above mentioned problems, by studying individual genes, either independently or a few at a time. Although it constituted a reductionistic approach, it was extremely successful in assigning functional properties and biological roles to genes and gene products. The recent possibility of obtaining information on thousands of genes or proteins in one sole experiment, thanks to high-throughput methodologies (NGS, Next Generation Sequencing) for such as gene

expression (Holloway et al., 2002) or proteomics (MacBeath, 2002), has opened up new possibilities in querying living systems at the genome level that are beyond the old paradigm ‘one gene–one postdoc’. Relevant biological questions regarding gene or gene product interactions or biological processes played by networks of components, can now for the first time be addressed realistically. Nevertheless, genomic technologies are at the same time generating new challenges for data analysis and demand a drastic change in the habits of data management. Dealing with this overabundance of data must be approached cautiously.

### 1.2. Objectives

a. Analysis of gene expression related to boktor pest resistance and tumor rust using RT-PCR
b. Development of SNIP markers for Genome-wide association selection (GWAS) on the resistance properties of boktor pests and tumor rust
c. Identification of Open Reading Frames (ORFs) from transcriptomic sequence analysis and Blastx

### 1.3. Expected Output

a. Optimization of the protocol for the expression of multiple gene sequences using RT-PCR
b. New SNIP markers for selection of resistance properties related to boktor pests and tumor rust
c. The full length of the expressed genes, especially those related to the resistance of sengon to tumor rust and boktor pestsIdentification of Open Reading Frames (ORFs) from transcriptomic analyses and BLASTX

## 2. BENEFITS AND IMPORTANCE OF RESEARCH IMPLEMENTATION

Pest and disease attacks can occur when there is compatibility between the tree host and the pest and disease, and the interaction takes place at the optimum environmental conditions. Resistance or susceptibility to pests and diseases, is often associated with certain natural substances such as, proteins, enzymes, extractive, or secondary metabolites produced by individual trees. According to Finkeldey (2005) often, that natural material is a product(s) of one or few gene loci, which can be detected by molecular methods, or linked with several molecular genetic markers. Trees do not have immune system based on antibodies, such as found in animals, but they activate several defense mechanisms, such as the strengthening

of cell walls, and accumulation of proteins that are anti-microbial, and some form of bonded small substances (phytoalexins) (Lamb & Dixon, 1997). Therefore, the usual approach to investigate resistance is to compare the chemical content of resistant and susceptible individuals, or genetic profile of those individuals.

Research on defense mechanisms against pests and diseases of tropical tree species is rare. Such research on sengon in particular has never been done before, that this research would be the first and only one in the world. Previous investigation on the chemical content of two sengon accessions of resistant and susceptible ones resulted in discovery that the two different accessions had different activity levels of enzyme inhibitors, i.e. trypsin- and α-amylase inhibitors. Resistant accessions had significantly higher activity levels than susceptible ones (Siregar 2010). This result was in accordance with other phenomena on other agricultural plants from temperate regions, of which trypsin- and α-amylase inhibitors are responsible for insect pest resistance. Previous project report also found similar results on resistance against gall rust diseases. Sengon accessions resistant to the diseases have stronger activity of secondary metabolite, i.e. triterpenoid, saponins and steroids (Siregar 2016).

In further research, molecular markers can potentially differentiate genetic profile or background or gene expressions encoding the phenotypes, in this case resistance to pest and diseases. Siregar (2015, 2016) employed RAPD and microsatellite markers to differentiate genetic background of two sengon accessions, i.e. resistant and susceptible. Results showed that while RAPD failed to detect differences, microsatellite could separate the two accessions in the constructed dendrogram (Siregar *et al*. 2009; 2016). However, observation on microsatellite markers did not indicate direct link of the markers to the gene(s) responsible for resistance or susceptibility. These findings needs a follow up research on the differences related to genes responsible for those resistances. Such investigation requires detection of specific genes, cloning the fragment for isolation and identification of those genes.

Genomic and proteomic approaches using cDNA libraries are emerging that link the molecular and phenotypic bases of plant defenses and pathogen infection. Experiments to identify certain expressed gene started with isolating RNA which is a product molecule of an expressed gene. Due to unstable nature of RNA molecule, it is necessary to convert the RNA into cDNA. Massively parallel sequencing of cDNA using NGS is now an efficient route for generating enormous sequence collections that represent expressed genes. This approach provides a valuable starting point for characterizing functional genetic variation in

non-model organisms, especially where whole genome sequencing efforts are currently cost and time prohibitive.

Expressed Sequence Tags (EST) sequencing is an attractive alternative to whole genome sequencing because the majority of most eukaryotic genomes is non-coding DNA, and EST sequences lack introns and intragenic regions that render analysis and interpretation of data more difficult (Bouck and Vision 2007). ESTs thus have a high functional information content, and often correspond to genes with known or predicted functions (Andersen and Lubberstedt 2003). Large collections of EST sequences have proven invaluable for gene annotation and discovery (Emrick *et al*. 2007) comparative genomics (Vera *et al*. 2008), development of molecular markers (Barbazuk *et al*. 2007; Novaes *et al*. 2008) and for population genomic studies of genetic variation associated with adaptive traits (Namroud *et al*. 2008).

One example, genomic tools are now being developed to accelerate the identification of resistance genes and the development of blight resistant American chestnut. In this context, a central objective of The Fagaceae Genomic Tools Project (http://www.fagaceae.org) is the sequencing of the transcriptomes of chestnut, oak and beech species with the long-term goal of isolating genes underlying resistance to the chestnut blight. To identify genes involved in resistance to *C. parasitica*, Barakat *et al*. (2009) have sequenced the transcriptome from fungal infected and healthy stem tissues collected from blight-sensitive American chestnut and blight-resistant Chinese chestnut (*Castanea mollissima*) trees using ultra high throughput pyrosequencing. They identified many defense-related genes differentially expressed in canker vs. healthy stem in chestnuts, which provide many new candidate genes for developing resistance to the chestnut blight and for studying pathways involved in responses of trees to necrotrophic pathogens.

Large collections of EST sequences from transcriptomic NGS is comparable to a more conventional cDNA library constructed by cloning, however such library still need expression studies to confirm that the gene(s) in question are properly expressed in the organism studied (Mizrachi *et al*. 2010; Sun *et al*. 2010). In such cases expression studies of expressed gene(s) often employed RT-PCR analysis.

## 3. METHOD

### 3.1. Plant Materials

Plants materials are sengon leaves which would be taken from tree accessions resistant and susceptible to either/or both boktor pest and gall rust, which belonged to sengon plantation of National Forest Estate (Perum Perhutani) in Kediri, East Java, as well as community plantations in West Jawa.

### 3.2. Isolation of Genomic DNA and Total RNA

DNA genome isolation procedure used protocols from *Qiagen DNeasy plant mini kit* from Qiagen (2012). The first step was washing Sengon (*F. moluccana*) leaf samples thoroughly under running water and weighed for 100 mg. Using mortar and pestle leaf sample was ground with liquid nitrogen until powdered. Powdered sample was put into a 1.5 ml tube filled with 500 µl AP1 buffer, 4 µl RNAse A stock (100 mg/ml) and PVP 26%. The powdered sample was put into 1,5 µl tube that contained 500 µl buffer AP1, 4µl RNAse A stock (100 mg/ml) and PVP 26%, it was mixed to be homogeneous. Then the solution was incubated at 600C for 20 minutes at a speed of 250 rpm. The next step was the separation of DNA with its impurities such as proteins, polysaccharides and polyphenols by adding 130µl of P3 buffer, then it was centrifuged at a speed of 11 000 rpm for 5 minutes. The supernatant was moved to QIAshredder and centrifuged at a speed of 11 000 rpm for 2 minutes. The liquid which passed through the QIAshredder membrane was moved into a new tube and added 1.5x V AW1 buffer then it was homogenized with pipetting technique, then incubated at room temperature for 2 minutes. The solution was then transferred to DNeasy mini spin column and re-incubated at room temperature for 5 minutes. It was centrifuged at a speed of 10 000 rpm for 1 minute. The liquid passed through the mini spin column membrane was discarded and centrifuged again if there was still solution left. 500 µl buffer AW2 was added into mini spin column and centrifuged at a speed of 10 000 rpm for 1 minute. The liquid that passed through the mini spin column membrane was discarded and as much as 500 µl of AW2 buffer was re-added into the mini spin column and centrifuged at a speed of 10 000 rpm for 1 minute. Then it was replaced into a new 1.5 ml micro tube and 100 µl of AE buffer was added to the mini spin column for DNA elution and incubated at room temperature for 5 minutes as well as centrifuged at a speed of 11 000 rpm for 2 minutes. The isolated DNA was electrophoresed at 1% agarose at a voltage of 75 v for 45 minutes, then visualized with Kodak Gel Logic 200.

Total RNA Mini Kit from Geneaid was used to isolate total RNA. As much as 0.1 g of sengon stem cambium crushed with liquid nitrogen to become a powder. Then, it was put into a tube containing 500 µL of DRR buffer and 5 µl of β-mercaptoetanol and mixed to be homogenous. For the cambium sample of susceptible sengon, it was incubated at 60 °C for 2 minutes using a heatblock. The solution was transfered into Filter Column then centrifuged at a speed of 5000 rpm with a temperature of 4 °C for 10 minutes. The filtrated solution was transferred to a 1.5 ml tube and mixed with half volume of absolute ethanol then mixed with pipetting technique. The solution was transfered into RB Column and centrifuged at a speed of 10 000 rpm at 4 °C for 2 minutes. The liquid was discarded, as much as 400 µl of W1 buffer was added into RB Column and centrifugated at a speed of 10 000 rpm at 4 ° C for 1 minute. The liquid was discarded again and as much as 600 µl of Wash Buffer was added. The addition of Wash Buffer was carried out 2 times with a speed of 10 000 rpm centrifugation at 4 °C for 1 minute for the first rinsing and 2 minutes for the second rinsing with the same centrifugation speed and temperature. The liquid that washed was discarded and it was centrifuged again at a speed of 10 000 rpm at 4 °C for 3 minutes to dry the RB Column. RB Column was moved to the new 1.5 ml tube and 12.5 µl of RNAse free water was added. The tube was incubated in the room temperature for 2 minutes. Then, it was centrifuged at a speed of 10 000 rpm at 4 °C for 2 minutes. The elution was done twice so that a total RNA of 25 µl was produced.

The results of total RNA isolation were tested by quantitative and qualitative techniques. Quantitative test is the measurement of total RNA concentration (ng/µL) by measuring absorbance at 230, 260, and 280 nm. As many as 1 µL of RNA sample was measured using a Implen Nanophotometer NP80 (Implen GmbH, Munich, Germany). The absorbance ratio values A260/280 and A260/230 were calculated to determine the total RNA purity that has been isolated. While the qualitative test of total RNA sample was analyzed by agarose gel electrophoresis technique, as much as 5 µL of RNA sample was mixed with 1 µL of loading buffer using micropipette on parafilm paper. The electrophoresis was conducted at a voltage of 100 V for 45 minutes, the bands formed on the electrophoregram results were visualized with Kodak Gel Logic 200.

### 3.3. cDNA Synthesis

Rever Tra Ace® qPCR RT Master Mix kit and gDNA Remover (Toyobo) was used in cDNA synthesis from sengon cambium. There were two stages contained on this kit, it were degradation of DNA impurity and reverse transcription reaction. DNA degradation

solution was made by mixing 4X DN Master Mix and gDNA remover on RNA template with a ratio of 50: 1. The composition of the material for cDNA synthesis is presented in Table 1. The RNA template, 4x DN MM and NFW were mixed and incubated at 37 °C for 5 minutes using a PCR machine. After that 5x RT Mmix II was added into the solution, then it continued with the second incubation process on incubation stages of 37 °C for 15 minutes, 50 °C for 5 minutes, and 98 °C for 5 minutes. The mixture was then stored at -20 °C. 5X Reverse Transcription Master Mix II is a solution that contains oligodT primers and random primers. The primary used is the AAI primer that has been published by Barbosa *et al.* (2010) and designed primers.

Table 1 The composition for cDNA synthesis

| Material | Volume |
|---|---|
| RNA | 5 µl |
| 4x DN MM | 3 µl |
| NFW (*Nuclease Free Water*) | 2 µl |
| 5x RT Mmix buffer | 2 µl |
| Total | 10 l |

### 3.4. Designing of Specific Primer for PCR and RT-PCR

The genes obtained from the DEG results were used to design primers for verification of gene expression using RT-PCR. Sequence-based primer design was performed with Primer 3 web-based software (https://bioinfo.ut.ee/primer3-0.4.0/). The criteria used to design the primer include: target gene length 80-150 bp, percentage of GC content between 45-55%, length of primer base 17-25 bases, at the last base at the 3 'end preferably base G or C, melting temperature ranges from 60-65 °C (Takara 2016). The genes used for the primer design were genes that are expressed differently (logFC> 2) and have been confirmed to be associated with pests or diseases in other plants based on the annotation results.

### 3.5. PCR and RT-PCR

Polymerase Chain Reaction (PCR) is an enzymatic method for DNA amplification by in vitro technique (Yusuf 2010). Each cycle consists of three stages, there are denaturation, annealing and elongation stages. On the denaturation stage, the template DNA, which is double strands is made into single strands by an increase in temperature (94-96°C). The next stage is the primer attachment stage or annealing, i.e. with a lower temperature depending on the primer used. Primer attaches to the complementary DNA template of the base sequence. This attachment is specific to the target sequence. The sequence of nucleotide

primer will be the determinant on which part of the primer will attach (anneal) to the genome. Incorrect temperature causes no attachment or primer attach in misplace. The next stage is the elongation stage. The temperature used is in accordance with the optimal temperature of the DNA polymerase activity. Generally, temperature used is between 65-72°C. The PCR reaction composition presented in Table 2.

Table 2 PCR composition of specific gene

| Material | Volume |
|---|---|
| Template (50 ng/µl) | 2 µl |
| Primer F 10µM | 0.5 µl |
| Primer R 10µM | 0.5 µl |
| Green Go Taq Mix | 5 µl |
| NFW | 2 µl |
| Total | 10 µl |

All materials were mixed on the vortex, then it was reacted into the PCR machine with the reaction as presented in Table 3.

Table 3 Stage of specific gene PCR reaction

| Stage | Temperature ($^0$C) | Time (minute) | ∑ cycle |
|---|---|---|---|
| Pre-denaturation | 95 | 2 | 1 |
| Denaturation | 95 | 0.5 | 35 |
| Annealing | * | 1 | 35 |
| Elongation | 72 | 2 | 35 |
| Post-elongation | 72 | 5 | 1 |

*annealing temperature depends on primer used

Annealing temperature was optimized. PCR products were electrophoresed in agarose 2% with 50bp marker. The cDNA products were diluted 50-fold with deionized water before use as a template in real-time PCR. The quantitative reaction was performed on an IQ5 Multicolor Real-Time PCR Detection System (Bio-Rad, USA) using the Power SYBR Green PCR Master Mix (Applied Biosystems, CA, USA). The reaction mixture (20 µL) contained 2× Power SYBR Green PCR Master mix, 0.9 µM each of the forward and reverse primers, and 1 µL of template cDNA. PCR amplification was performed under the following conditions: 50°C for 2 min and 95°C for 30 s, followed by 40 cycles of 95°C for 15 s and 62°C for 1 min.

The cDNA product was then eluted to a concentration of 10-25 ng / µ before being used as a template in real-time PCR. The RT-PCR process was carried out using the StepOneTM Plus System Real-Time PCR from Applied Biosystem. The recorder used was Power SYBR Green PCR Master Mix (Applied Biosystems, CA, USA). The reaction

mixture (10 µL) contained 2 × Power SYBR Green PCR Master mix, 0. µM of forward and revers primers each, and 1 µL of cDNA prints. RT-PCR amplification was carried out in pre-denaturation stages of 95 ° C for 3 minutes, followed by 40 cycles consisting of denaturation of 95 ° C for 10 seconds, annealing for 10 seconds, extension for 20 seconds.

Analysis of the relative expression of target genes using the relative quantification method with a comparison of the value of Ct ($2^{-\Delta\Delta CT}$) (Schmittgen & Livak 2008) with the following formula formula:

$\Delta Ct\ P = Ct_{GT} - Ct_{HG}$;

$\Delta Ct\ K = Ct_{GT} - Ct_{HG}$;

$\Delta\Delta Ct = \Delta Ct_P - \Delta Ct_K$;

Expression= $2^{-\Delta\Delta Ct}$

Note: $Ct_{GT}$=target gene expression, $Ct_{HG}$=housekeeping gene expression, ΔCt P: Ct treated sample; ΔCt K: Ct control; ΔΔCt: difference in Ct values between treated sample and control.

### 3.6. Compilation of the sengon reference genome (*Falcataria moluccana* miq.) de novo

**Sequencing DNA** DNA isolated from samples of boktor-resistant and puru rust-resistant sengon leaves sequencing using Illumina NovaSeq 6000 by Novogen AIT, Singapore. Raw data results sequencing 72.6 GB in size with FASTQ file format.

**QC read data (fastq)** Result data sequencing which was uploaded on platform Masers are checked for quality using FASTQC software (Trivedi *et al.* 2014). Data that has good quality (Q>30) can be processed directly assembly and if the data has poor quality (Q<30) it is done filtering data first using the FILTERQC software.

**Genome Assembly: De Novo Assembly** Assembly reads data that has gone through quality checks is carried out using three software, namely Platanus (Kajitani *et al.* 2019), Ray (Biosvert *et al.* 2010), and SOAP denovo (Li *et al.* 2010). Results assembly then checked for completeness of the genome data using the BUSCO software (Simão *et al.* 2015).

**Genome Annotation** Results assembly from Ray software then annotated to find out the functional genes. FASTA data results assembly sequence was also extracted using Samtools v1.10. The TI and AAI sequences were then aligned (alignment) with TI and AAI sequence analysis results transcriptomics (Shabrina 2020) on the Geneious Prime®2021.1 program. The previously used TI and AAI sequences were aligned using CAP3 software (Huang and Madan 1999) and clustered using CD-HIT software (Li and Godzik 2006) withidentity threshold by 95%.

## 4. RESULTS AND DISCUSSION

### 4.1. Total RNA Isolation

RNA isolation is the basis of molecular biology to analyze gene expression in living things. Total RNA isolation is the first step in the detection of -amylase inhibitor, trypsin inhibitor and actin genes. The isolation results are the template material in the cDNA synthesis process for gene detection using Real Time PCR. It plays an important role in gene expression and protein biosynthesis. The expression of these genes will control all components at the cellular level to the individual.

L S1 S2 S3 B1 B2 B3

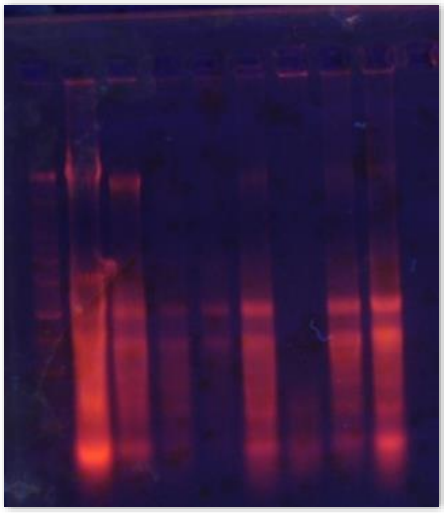


Figure 1 Results of total RNA isolation. L = Ladder; S1 = total RNA isolation of healthy samples 1; S2 = total RNA isolation result of healthy sample 2; S3 = total RNA isolation of healthy samples 3; B1 = total isolation of RNA samples attacked by boktor 1; B2 = total isolation of RNA samples attacked by boktor 2; B3 = total isolation of RNA samples attacked by boktor 3

Based on Figure 1, RNA isolation was successfully carried out which was indicated by the appearance of double bands on the 1% agarose electrophoresis visualization. The quality of the RNA isolation results can be seen from two aspects, namely qualitative and quantitative aspects. The qualitative aspect shows the appearance of two bands in samples S1, S2, S3, B2 and B3 with the same fluorescence thickness (intensity), thick, and quite sharp. While in sample B1 has less clear RNA band fluorescence and there issmear in each sample that shows contamination by substances that come from inside or outside the sample

in the RNA. Extraction factor is a factor that can 15affect the quality of an RNA. According to Amanda and Cartealy (2015), high RNAse activity is found in several types of tissues and cells. In addition, contamination can occur during the isolation of RNA from human skin, so it is necessary to wear gloves during the isolation process. The presence of carbohydrate compounds, anthocyanins, phenolics, or other metabolites can reduce the quality and quantity of RNA produced.

Based on the results of Figure 1 of the six samples used, the RNA quality of the healthy samples formed was better than the samples that were attacked by the boktor pest. It is in the samples attacked by boktor pests that have a reddish color and are not fresh and the boctor pests cause wound tissue and powder that sticks under the bark which changes the color of the bark of sengon (Puspitarini 2006). Another cause is caused by the production of endogenous ribonuclease (RNAse) which increases due to the attack or injury of the pathogen (Hartati 2002).

The quality of total RNA from the quantitative aspect was seen in terms of the concentration and ratio of the total RNA purity. The results of the Nanodrop spectrophotometer measurement show the size of the total RNA concentration and the ratio of purity A280/A260 and A260/230 for each sample are presented in Table 4.

Table 4 Nanodrop spectrophotometer results of total RNA concentration and purity ratio

| Sample | RNA consentration (ng/µl) | Purity ratio to protein (A280/A260) | Purity ratio against polysaccharides (A260/A230) |
|---|---|---|---|
| S1 | 614.60 | 1.985 | 0.687 |
| S2 | 597.60 | 2.080 | 1.154 |
| S3 | 542.60 | 2.114 | 1.640 |
| B1 | 1194.2 | 2.008 | 0.867 |
| B2 | 737.40 | 1.994 | 0.946 |
| B3 | 301.36 | 2.072 | 1.045 |

The concentration and quality of the cDNA used is one of the determinants of the success of the process real time PCR. The concentration and quality of this cDNA was measured using a machine NanoDrop spectrophotometer. The concentration of RNA can be seen from the results of the absorbance value (light absorption) of ultraviolet (UV) light on the sample at a wavelength of 260 nm (A260). The value of total RNA concentration in Table 4 was obtained from the conversion of the A260 value of total RNA isolates to the value of total RNA concentration. A value of 1 in A260 of single-stranded RNA (ss-RNA)

represents 40 ng/µl (Amanda and Cartealy 2015) so that the value of 16The total RNA concentration obtained was in the range of 301.36–1194.2 ng/µl. The value of the total RNA concentration was positively correlated with the quality of the RNA band formed as seen from the comparison between the two aspects. The greater the total RNA concentration, the better the intensity and sharpness of the fluorescence of the total RNA band formed, while the smaller the total RNA concentration, the more faint the intensity and sharpness of the fluorescence of the total RNA band formed. The value of the total RNA concentration of the four samples indicated that the total RNA could be used as a template for cDNA synthesis. The value of the total RNA concentration affects the success of the cDNA synthesis process.

The purity of RNA can be seen from the absorbance of the sample at wavelengths of 230 nm, 260 nm and 280 nm and the ratio of A280 to A260 and A260 to A230. According to Sambrook and Russell (2001), the purity ratio value of A280/ A260 should range from 1.8–2.0. A purity ratio of A280/A260 below this range indicates that there is protein contamination. The value of the purity ratio A280/A260 of the four samples was in the proper range and some did not. This shows that all samples have a fairly good purity of protein contaminants, although there are still smear. Meanwhile, based on the A260/230 purity ratio, a sample has good RNA purity if it has an A260/230 purity ratio value ranging from 2.0–2.4 (Farrell 2005). The purity ratio of A260/A230 in the four samples has a lower ratio value compared to the provisions. This could be due to the presence of contaminants in the sample in the form of polysaccharides (Rapley and Heptinstall 1998). RNA purification efforts are needed, one of which is RNA resuspension (1 g/µL) with DNAse RNAsefree Thermo Scientific Kits (Chandrawijaya *et al.* 2013). However, in this study, RNA purification was not carried out. Based on the results of RNA quality both in terms of qualitative or quantitative terms, the four samples can be used for the next process, namely the optimization of primers for genes encoding -amylase inhibitors, trypsin inhibitors and actin and optimization using RT-PCR for gene expression analysis.

The quality of cDNA can be seen from the absorbance ratio values of A260/A280 and A260/A280. According to Okanti *et al.* (2020) a good A260/A280 ratio ranges from 1.8-2.0. The ratio value indicates that the cDNA is free of contaminants such as protein. Furthermore, the good A260/A230 ratio has a 2.0-2.4 ratio. Ratio values below or exceeding this number can be caused by polysaccharide contamination. The concentration of cDNA used can be seen in appendix 1.

Based on the data obtained, the concentration of cDNA used for the TI4 gene ranged from 29.05-65.90 ng/µl. All samples have an A260/A280 ratio value between 1.8-2.0 except

for the S3 sample which has a ratio of 1.76, while the A260/A230 ratio above 2.0 is found in two samples (S1 and B3), sample B2 has a ratio of 1,579 and the other three samples (S2, S3, B1) have a ratio value between 1.8 to 1.9. This shows that the cDNA used for the TI4 gene still contains contaminants in the form of proteins and polysaccharides. The concentrations used for the Actin 1 gene ranged from 20.75-58.00 ng/µl. The value of the A260/A280 ratio has exceeded 1.8 in all samples, which means that the cDNA is quite clean from protein contamination. In addition, there are two samples (B1 and B2) with a ratio value of A260/A230 below 2.0, which means that there are still polysaccharide contaminants in both samples.

The next step after obtaining RNA isolates with good quality is cDNA synthesis through a reverse transcription process (reverse transcription). The quality test of the cDNA formed was verified using PCR with the primers used, namely from the gene housekeeping (actin). Research on gene primers housekeeping on sengon has not been done before, therefore cDNA quality testing is carried out in conjunction with the optimization of the primary gene encoding the actin gene as a housekeeping gene.

### 4.2. Sengon inhibitor trypsin gene expression test (*Falcataria moluccana* miq.) against boktor pest attack

The final result of the RT-PCR process, one of which is the formation of several curves, namely the amplification curve (amplification curve) and the melting curve or melting curve (melting curve). Melting Curve Analysis (MCA) was used to determine the specificity of the amplicon. The specific amplicon is characterized by the appearance of a peak melting temperature on the MCA curve and no increase in fluorescence on the amplification curve. To strengthen the analytical data, it is necessary to determine the sensitivity, detection limit, and efficiency of the method. The optimization of the primer temperature by RT-PCR has been carried out several times and from the 20 experiments the two best results were obtained. Primer TI4 at 61.7 °C and Actin 1 primer at 61.7 °C. The TI4 gene is the target gene to be quantified while the Actin 1 gene is the housekeeping gene.

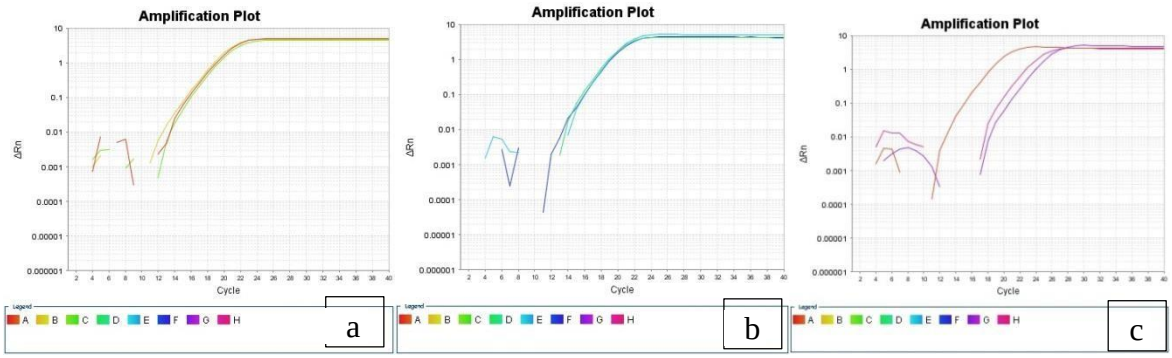



Figure 2 The sample TI4 gene amplification curve a) S1, b) S2 and c) S3

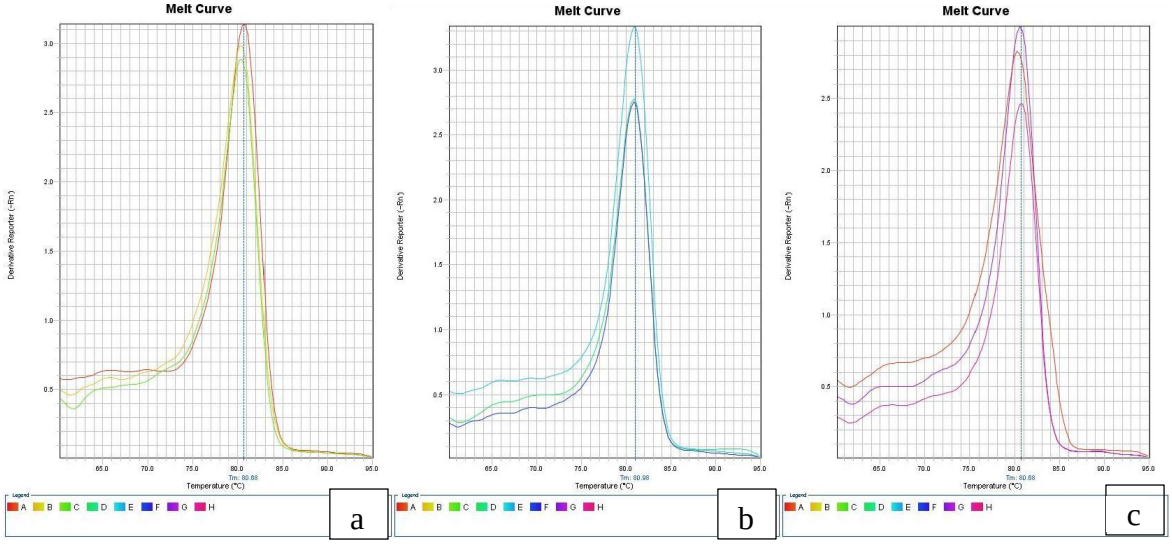



Figure 3. Melting curve of TI4 gene samples a) S1, b) S2 and c) S3

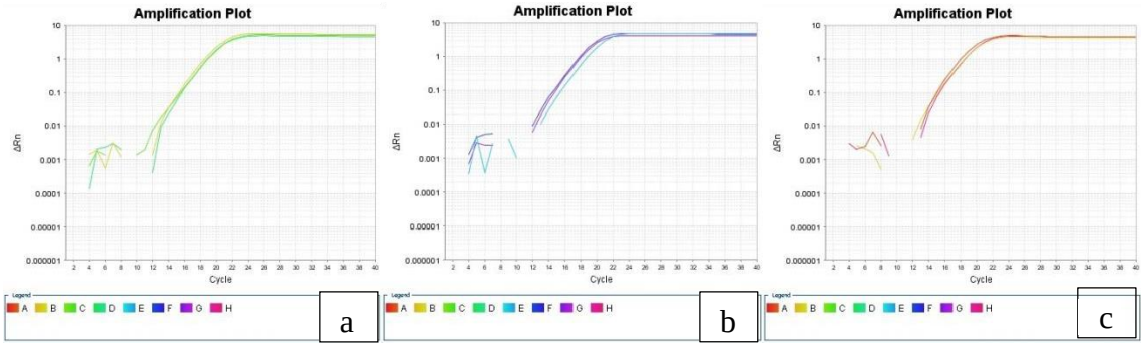



Figure 4 Sample TI4 gene amplification curve a) B1, b) B2 and c) B3

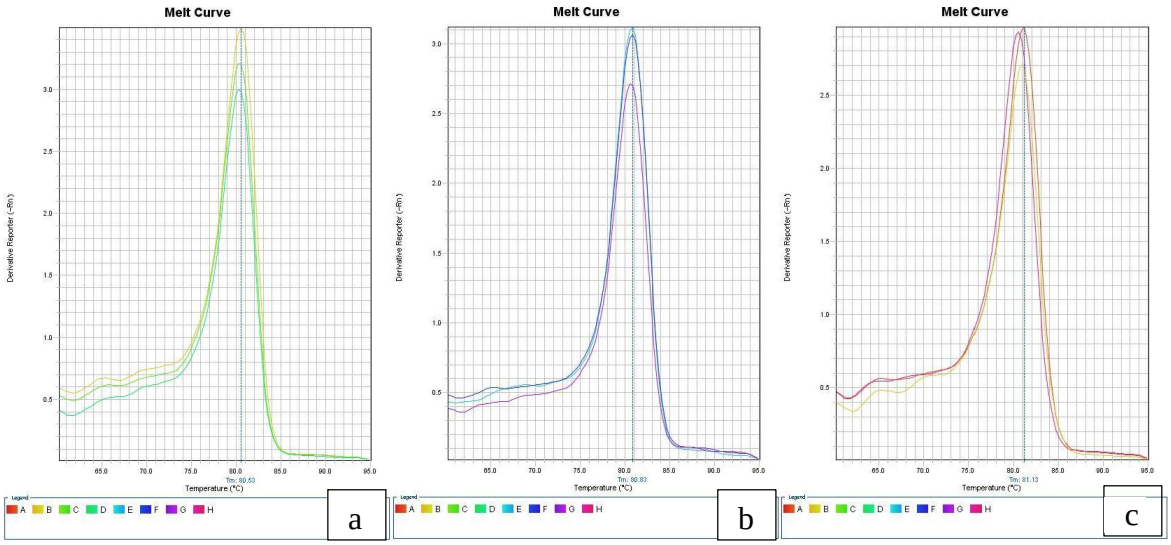



Figure 5 Melting curve of TI4 gene samples a) B1, b) B2 and c) B3

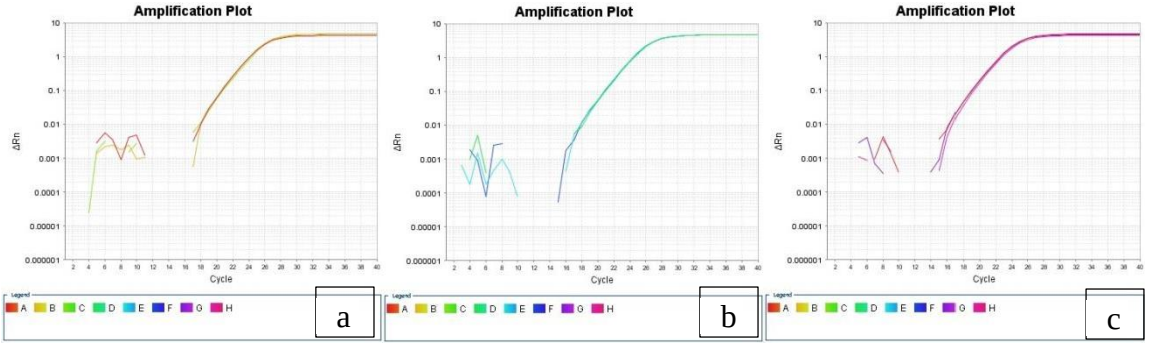



Figure 6 Actin gene amplification curve 1 sample a) S1, b) S2 and c) S3

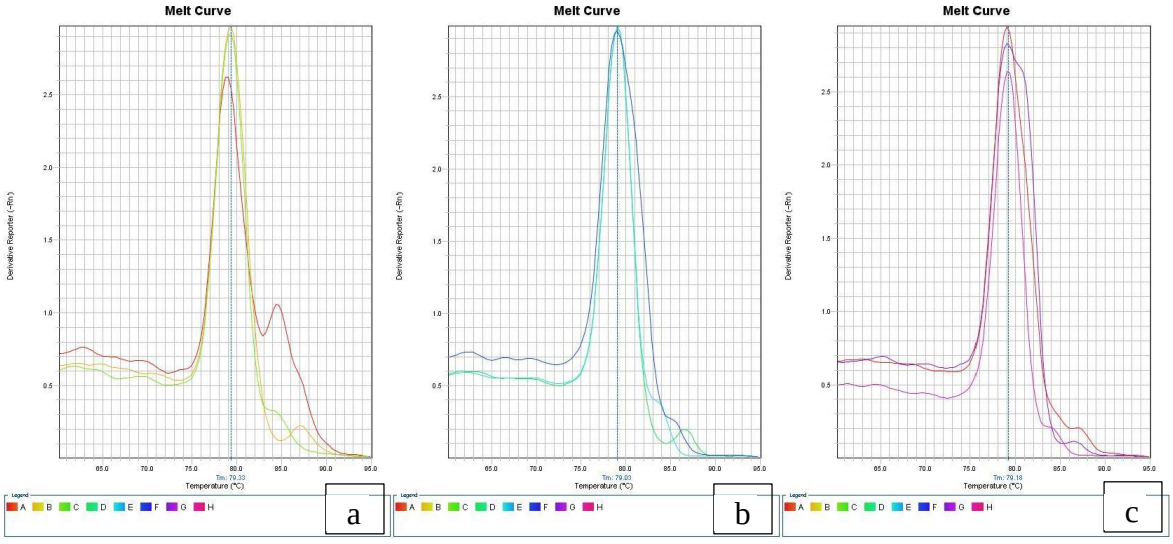



Figure 7 Actin gene melting curve 1 sample a) S1, b) S2 and c) S3

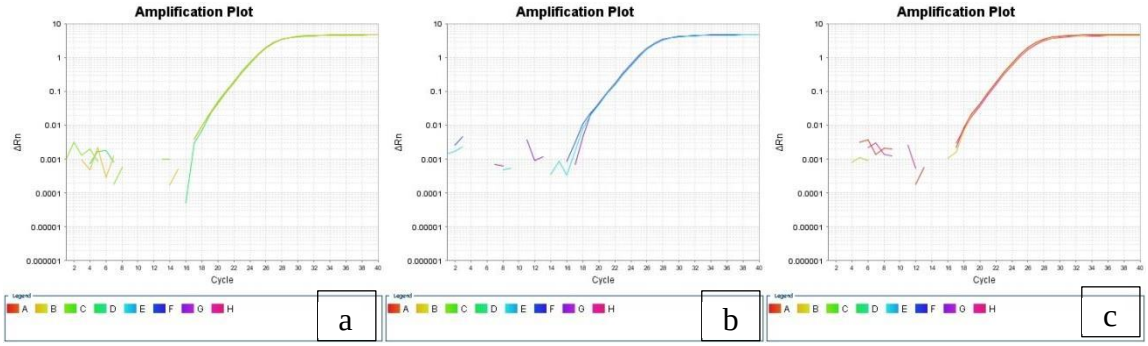



Figure 8 Actin gene amplification curve 1 sample a) B1, b) B2 and c) B3

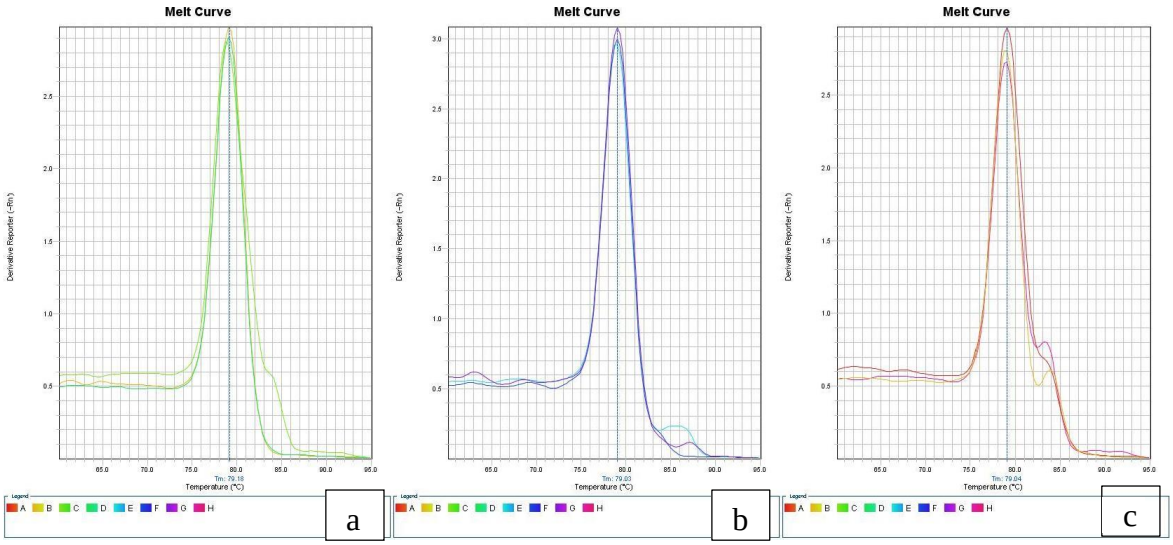



Figure 9 Melting curve of Actin 1 sample a) B1, b) B2 and c) B3

Optimization of the primer temperature by RT-PCR has been carried out several times to obtain the two best results. Primer TI4 at 61.7 ℃ and Actin 1 primer at 57 ℃. The TI4 gene is the target gene to be quantified while the Actin 1 gene is the housekeeping gene.

The results of the curves in Figures 2 and 4 show that the TI4 gene has an amplification curve that is quite good in all sample replications, both in samples of resistant sengon (S1, S2, S3) and in samples of sengon attacked by boktor pests (B1, B2, B3). A good amplification curve is indicated by the formation of a neat curve to phase plateau and looks tight on all replicates of the same sample. The results of the amplification curve that are not good are only shown by sample S3 (sengon resistance 3rd replication) where one curve is not close to the other two curves. The amplification curve for the Actin 1 gene is shown in Figure 6 and Figure 8. Based on the results obtained, the amplification curve for the Actin 1 gene has looked good in all sample replications.

According to Tooy *et al.* (2016) melting curve analysis is required to ensure the specificity of the amplification real-time PCR. Therefore the two yield curvesreal time PCR

is required for experimental validation real time PCR. The melting curve of the TI4 gene is shown in Figure 3 and Figure 5, the TI4 gene has a fairly good qPCR result in all sample replications.

The melting curve (melting curve) can be said to be good if it only has one peak and the curve looks neat. The melting curve of the Actin 1 gene is shown in Figure 6 and Figure 8. Based on the two figures, the average curve produced is quite good. This can be seen from the uniformity of the curve that is formed and only produces one peak. The unfavorable results are only shown by the 1st sample S1 in Figure 7(a). The figure shows that there is one curve that forms two peaks. In addition, in Figures 7(b) and 7(c) it can be seen that the curve is not completely tight but only has one peak. According to Polosoro and Enggarini (2016), if the peak of the dissociation curve is more than one, this means that the PCR reaction produces more than one product, whereas if the peak of the dissociation curve shows different values, it means that the PCR products produced are different from each other melting with two peaks can also be caused by contaminants carried during the transfer of reagents into the tube strips. In this study, the amplification curve and curve good melting, so it can be said that the Actin 1 gene marker can amplify specific PCR products because the received signal is purely from Actin 1.

$C_T$ Value obtained from the RT-PCR experiment can then be analyzed using the 2 analysis method-ΔΔC T. $C_T$ Value related to the number of amplicons produced during the reaction. The lower $C_T$ value the more amplicon produced (Schmidgen and Livak 2008). $C_T$ Value the results of the RT-PCR experiment can be seen on the appendix page. $C_T$ Value From the TI4 gene sample it can be seen that $C_T$ value of all samples have close values, which range from 17.62 to 23.6456, while $C_T$ value for the Actin 1 gene sample with a $C_T$ value, The smallest value is owned by sample S31, which is 16.2608 and the largest value is owned by sample B11, which is 19.0701 (Appendix 4 and 5). The first table shows $C_T$ value from samples of the TI4 gene. Based on the table it can be seen that $C_T$ value of all samples have close values, which range from 24.33 to 26.99. The second table shows $C_T$ value for the Actin 1 gene sample. $C_T$ value, the smallest value is owned by sample S31, which is 22.36 and the largest value is owned by sample B31, which is 24.69. $C_T$ value nearby can show that quantitatively the concentration of the sample used is relatively the same (Polosoro and Enggarini 2016), in this experiment it can be seen that the concentration of cDNA used for the two types of markers has different values because of the difference in $C_T$ value which is quite large. According to Yahya (2019), the lower the cDNA concentration used, the higher $C_T$ value formed will be smaller. This statement is in line with the cDNA

concentration used for the TI4 gene which has a larger range so that $C_T$ value resulting gene is also higher than the Actin 1 gene.

Gene expression calculations generally use the $2^{-\Delta\Delta C_T}$ method between the target gene and normalizer (housekeeping gene) while $C_T$ compare $\Delta\Delta C_T$ of the target gene with $\Delta C_T$ calibrator and $\Delta C_T$ normalizer. Difference in value (Ratnasari 2015). Quantification results using the $2^{-\Delta\Delta C_T}$ method found in appendix 5. The results showed that the expression level of the TI4 gene in samples of sengon attacked by boktor was 0.91 times higher than the gene expression in healthy sengon trees.

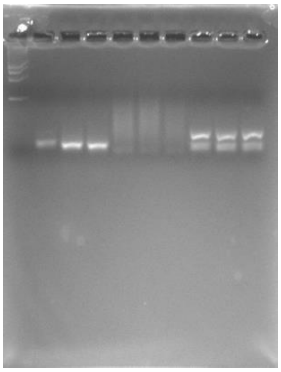


Figure 10 Results of electrophoresis visualization TI4 gene.

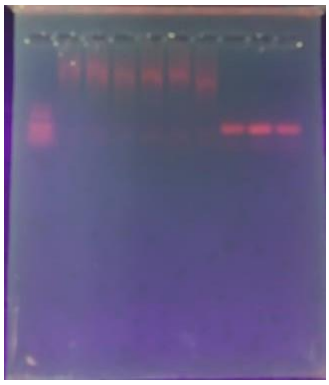


Figure 11 Electrophoresis visualization results Actin 1 gene.

The RT-PCR results were then tested by electrophoresis. Figure 10 shows the results of the visualization of the TI4 gene electrophoresis gel, while Figure 11 is the result of visualization of the Actin 1 gene. From the two images, it can be seen that the TI4 and Actin 1 genes have a clean single band. A gene that has good quality can be seen from the formation of intact DNA bands on gel electrophoresis (Miftakhurohmah *et al.* 2016). This indicates that the TI4 and Actin 1 genes were successfully obtained in the observed sengon cambium.

### 4.3. Sengon Inhibitor α-Amylase Gene Expression Test (*Falcataria moluccana* Miq) attacked by Pest Boktor

Real Time PCR is a modification of the conventional PCR method that can quantify and observe directly the amplification of a DNA with the help of fluorescent reporter. fluorescent reporter is a substance that can bind to the target DNA and emit a fluorescence signal that describes the amount of product produced. fluorescent reporter consists of two types namely fluorescent dye andprobe. fluorescent dye is a substance that can fluoresce

when it binds to the target DNA, whereas probe is a primer that is labeled with a radioactive substance and can fluoresce with the same technicality as fluorescent dye (Cubist 2006). fluorescent reporter used in this research is SYBR Green (SYBR Select Master Mix). The results obtained from RT-PCR in the form of amplification curves melt curve and melt peak curve. Optimization of various aspects needs to be done in the process Real Time PCR in order to obtain an amplification curve that passes through threshold (threshold line describing the fluorescence produced by DNA amplification) and the curve meltpeak curve which has a peak that is formed in the form of single peak and the results of electrophoresis that form a band according to the target size. In addition, optimization is processed Real Time PCR This is done in order to remove the dimers formed in the previous conventional PCR process. One aspect that can be optimized is the concentration of cDNA samples because DNA samples with high concentrations have the potential to have high contaminants so that the concentration of mixed samples is recommended to range from 1-100 ng/µl (Applied Biosystem 2010). The treatment concentration of cDNA samples used for the optimization process was 10 ng/µl for the six cDNA samples (S1, S2, S3, B1, B2 and B3) with three replications carried out on both primers, namely primers AAI 2 and Actin 1 using the annealing temperature of the previous optimization results (61°C and 61.7 °C). The results of the cDNA sample amplification curves on AAI 2 and Actin primers are shown in Figure 12 and Figure 13.

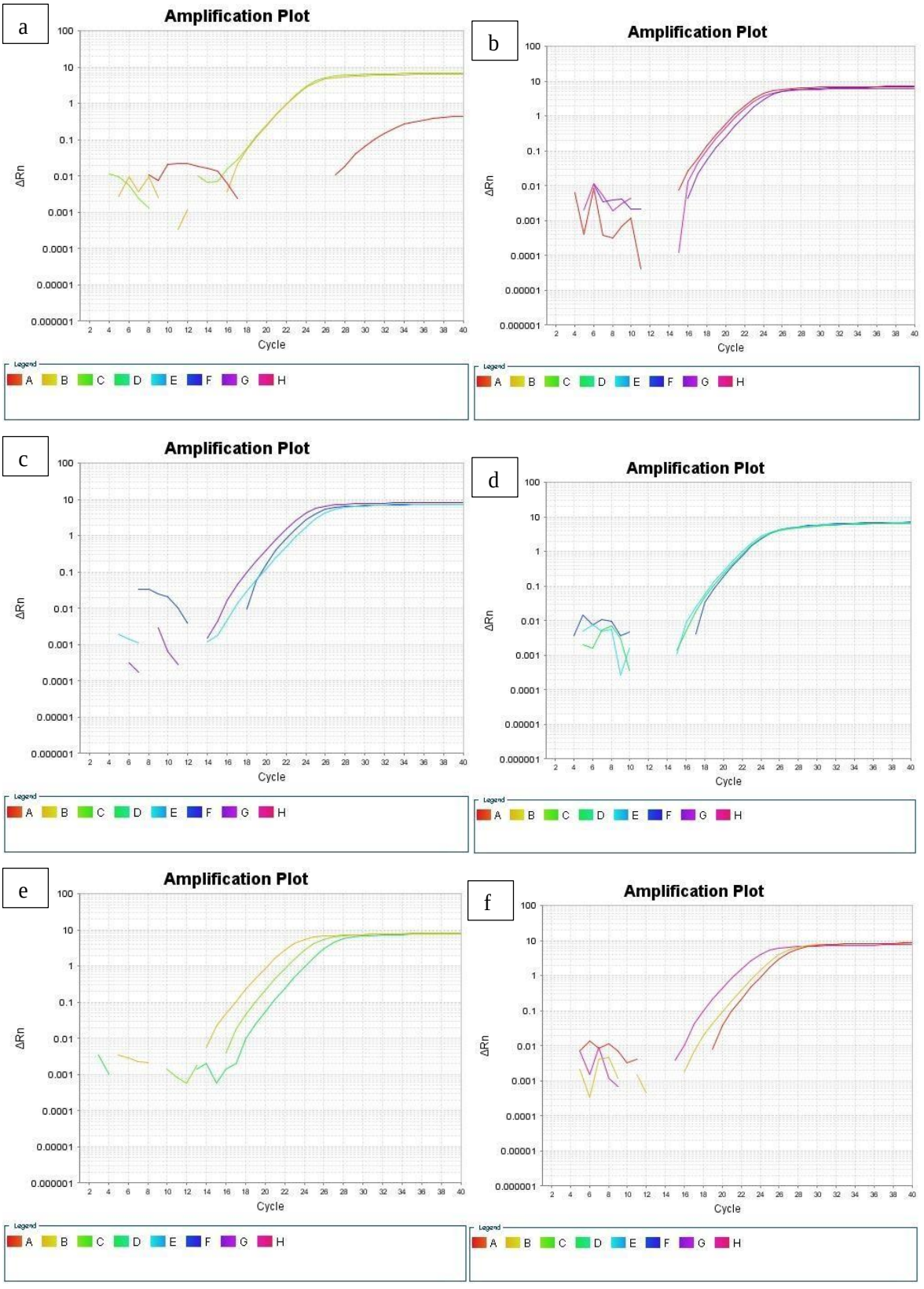



Figure 12 AAI 2 amplification curve. A = S1; B = S2; C = S3; D = B1; E = B2; F = B3.

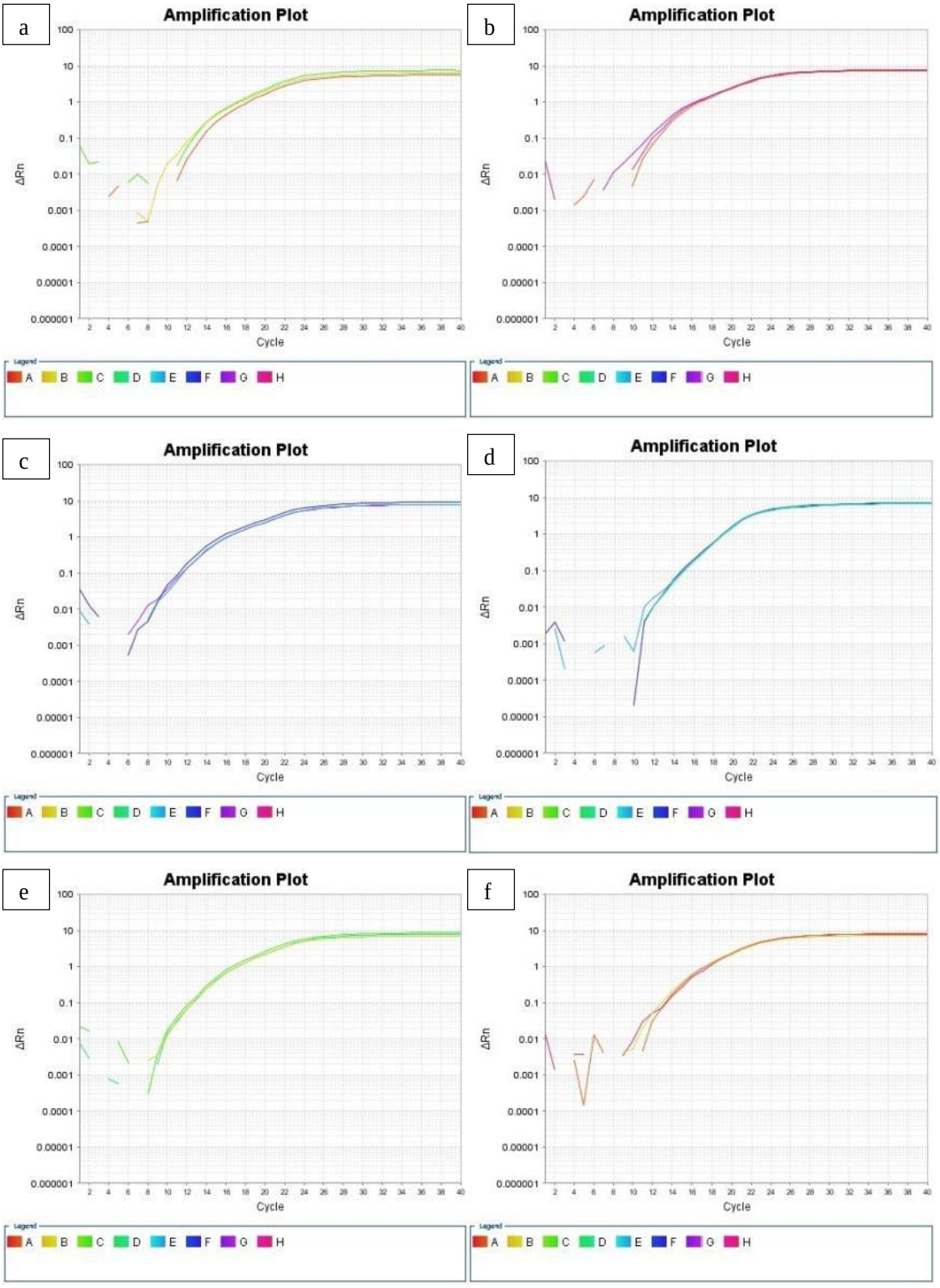



Figure 13 Actin amplification curve 1. A = S1; B = S2; C = S3; D = B1; E = B2; F = B3

The results in Figure 12 and Figure 13 show that the amplification curve is formed quite well from all samples on AAI 2 and Actin 1 primers as seen from peak 26 which was constant and parallel in all three replicates. The S1 sample in the AAI 2 primer showed that there was 1 replication which had a curve that was not constant so it looked not tight in one of the replicates.

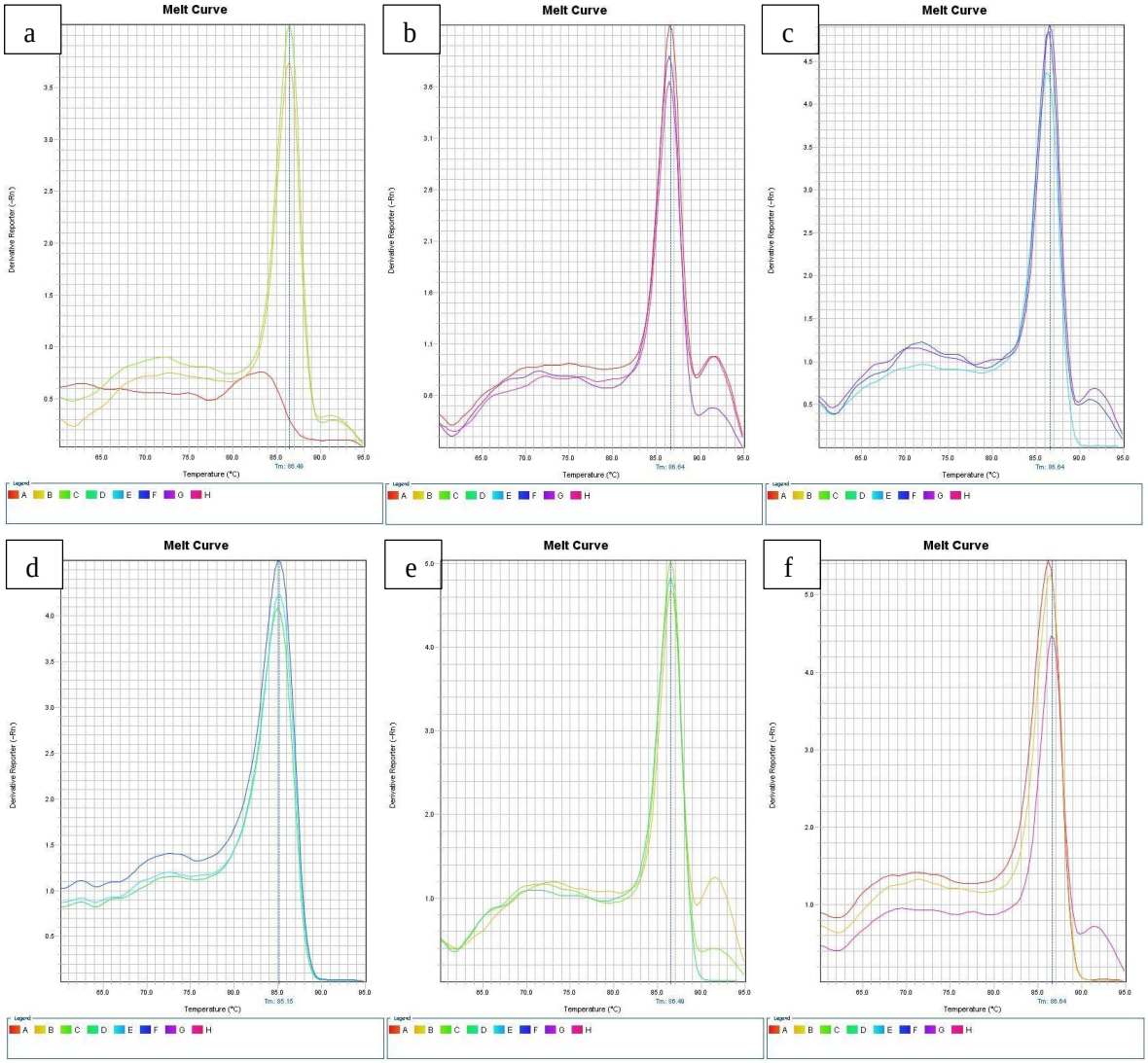



Figure 14 Meltpeak curve AAI 2. A = S1; B = S2; C = S3; D = B1; E = B2; F = B3.

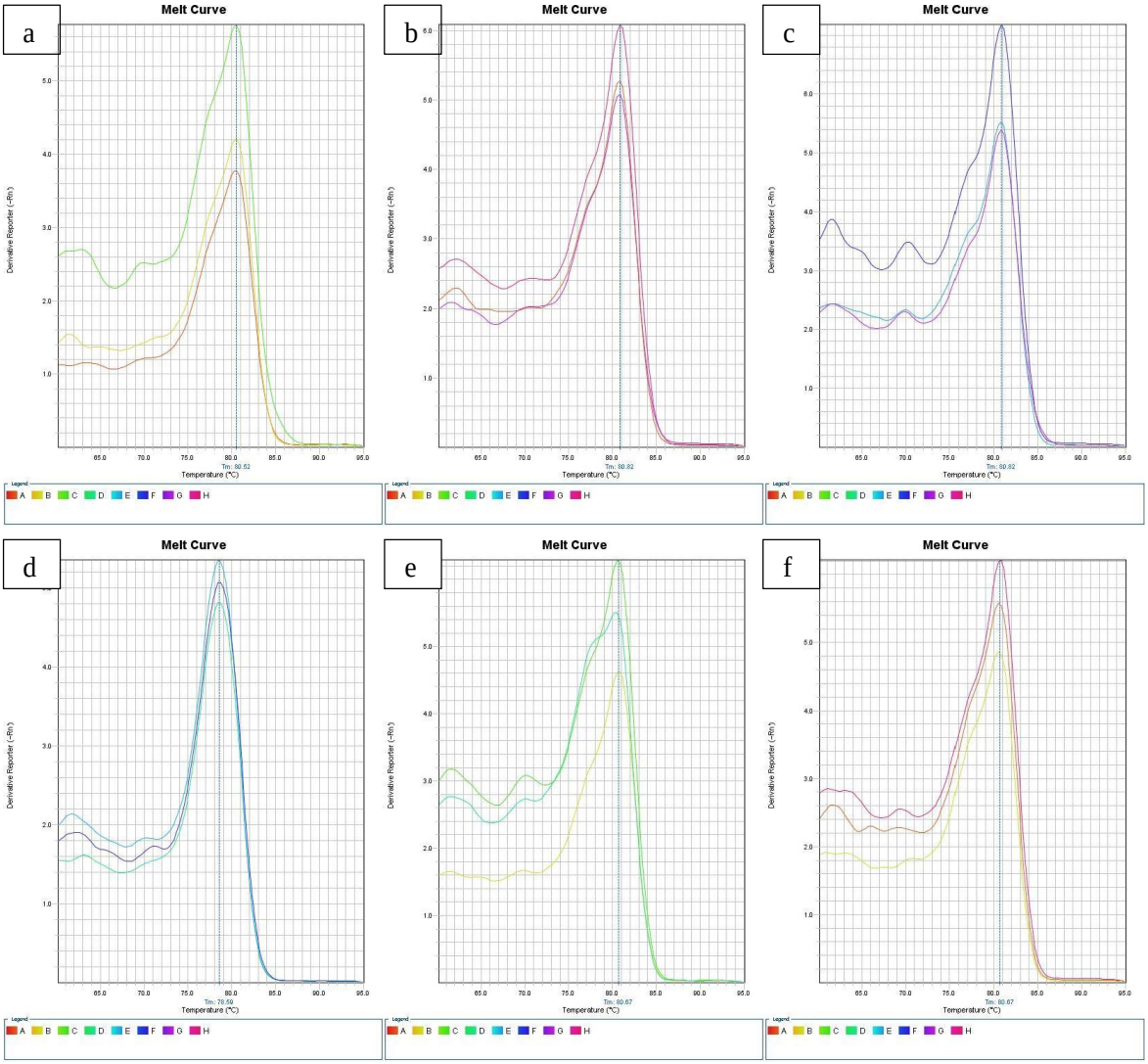



Figure 15 Meltpeak curve Actin 1. A = S1; B = S2; C = S3; D = B1; E = B2; F = B3

Meltpeak curve is the peak melt curve which can indicate the specificity of the product formed. The results in Figure 14 and Figure 15 show that meltpeak that formed in all samples and replicates on primers AAI 2 and Actin 1 was good enough because it formed one peak indicating the specificity of the primer. Results melt curve Actin 1 primer shows curves that are still not very tight and some are still bulging but still have one peak. Optimization of cDNA concentration was then carried out on November 5, 2021 for actin 1 and November 10, 2021 for primer AAI 2. The treatment of concentration of cDNA samples used for the optimization process was 10 ng/µl for the six cDNA samples (S1, S2, S3, B1, B2 and B3) with three replications performed on both primers, namely primers AAI 2 and Actin 1 using the annealing temperature of the previous optimization results °C for Actin 1

and 61 primers °C for primer AAI 2). Curve result amplification of cDNA samples on AAI 2 and Actin primers are shown in Figure 16 and Figure 17.

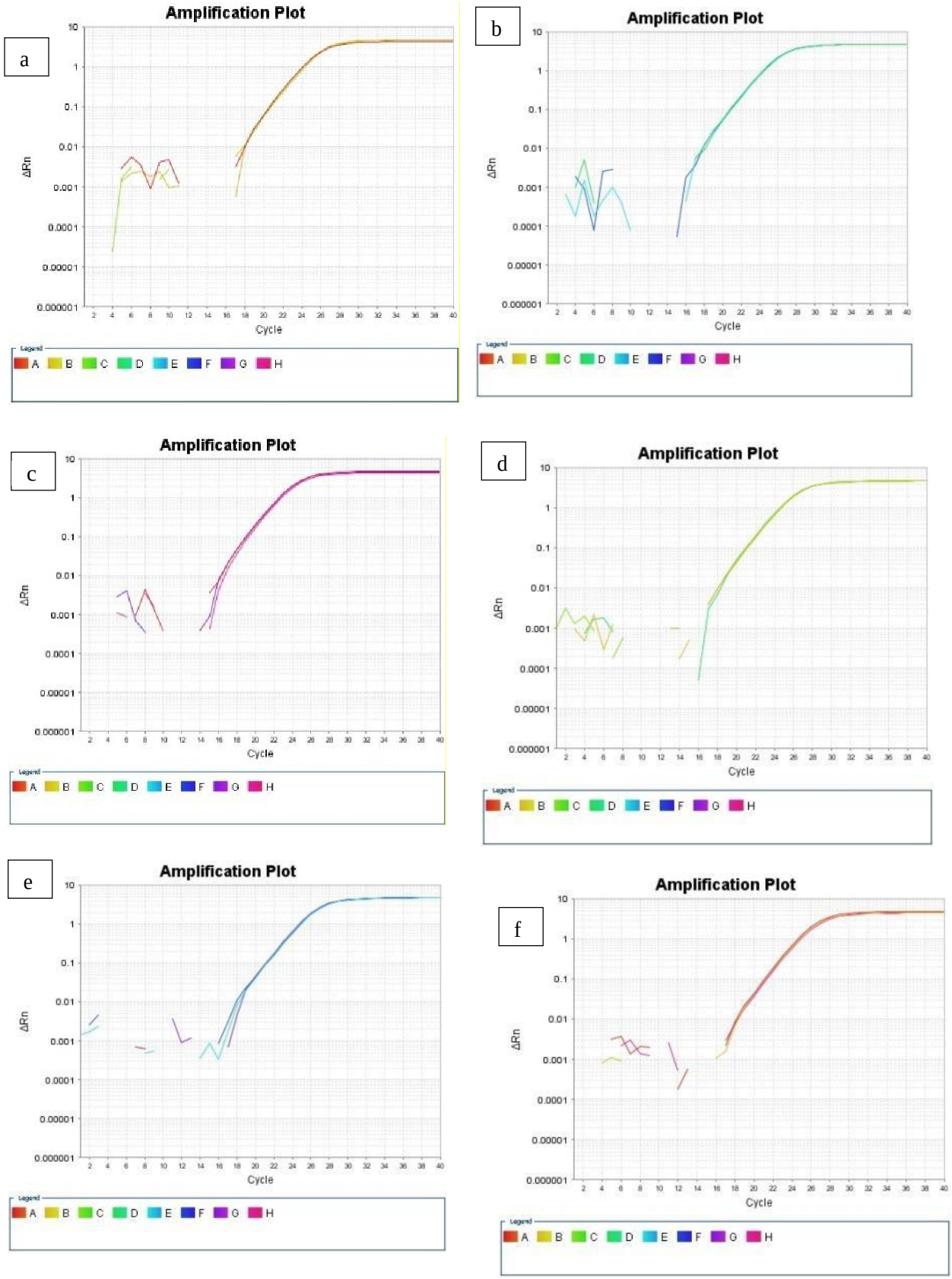



Figure 16 Actin amplification curve 1. A = S1; B = S2; C = S3; D = B1; E = B2; F = B3

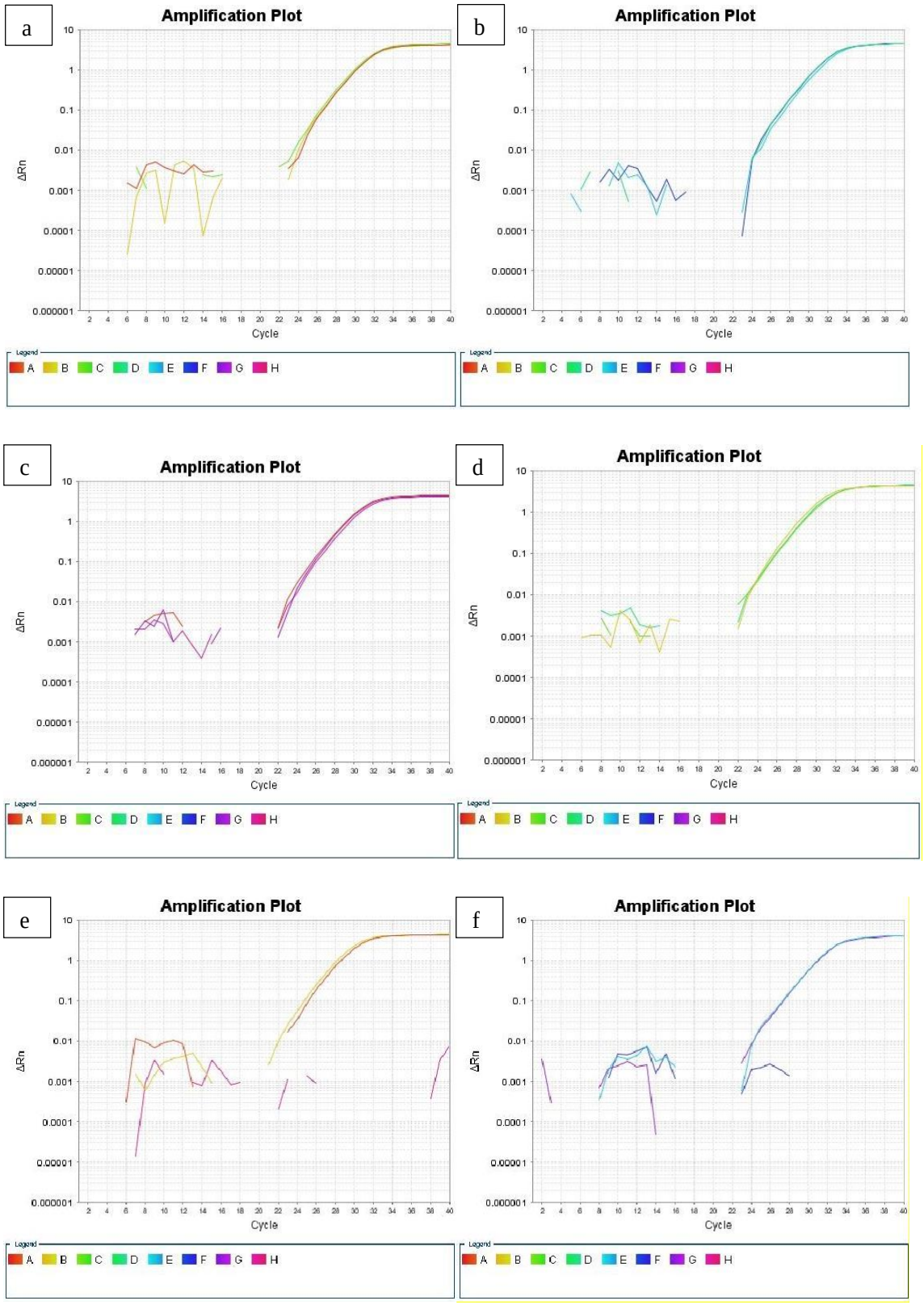



Figure 17 AAI 2 amplification curve. A = S1; B = S2; C = S3; D = B1; E = B2; F = B3

The results in Figure 16 and Figure 17 show that the amplification curve is formed quite well from all samples on AAI 2 and Actin 1 primers as seen from peak which was constant and parallel in all three replicates. Samples B2 and B3 on AAI 2 primers showed that there was 1 replication which had an unreadable curve so that the curve did not match the other replicates. Comparison of the amplification curve on November 4 and November 10 has better results compared to the previous study. The amplification curve looks tighter and neater for all samples and replicates, only that there are repeats that are not readable by the machine. This was due to the difference in temperature in the Actin 1 primer and the sample used was not too long from the start of sampling in the field. Then, the result of the melt curve cDNA samples on AAI 2 and Actin primers are shown in Figure 18 and Figure 19.

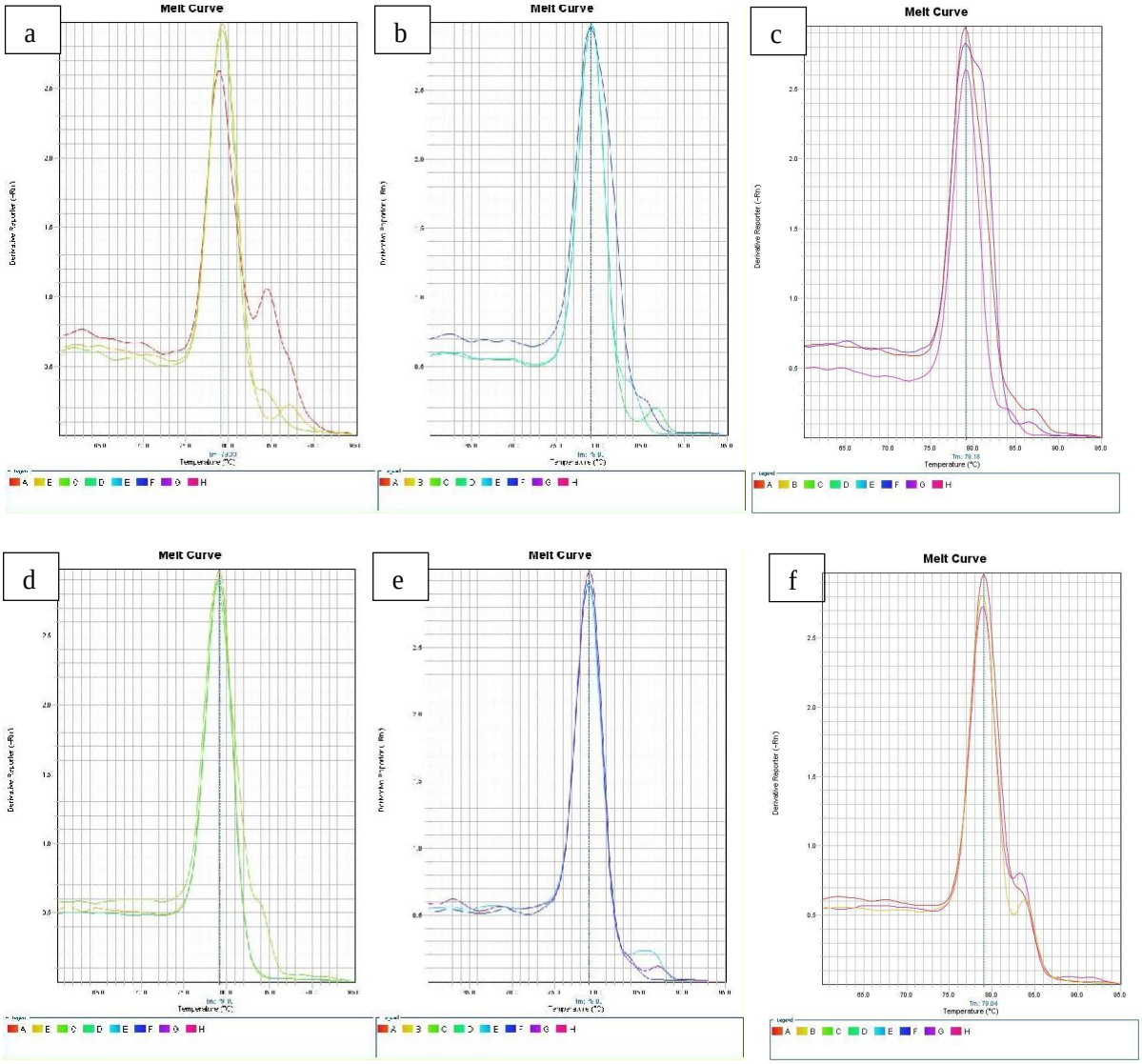



Figure 18 Meltpeak curve Actin 1. A = S1; B = S2; C = S3; D = B1; E = B2; F = B3

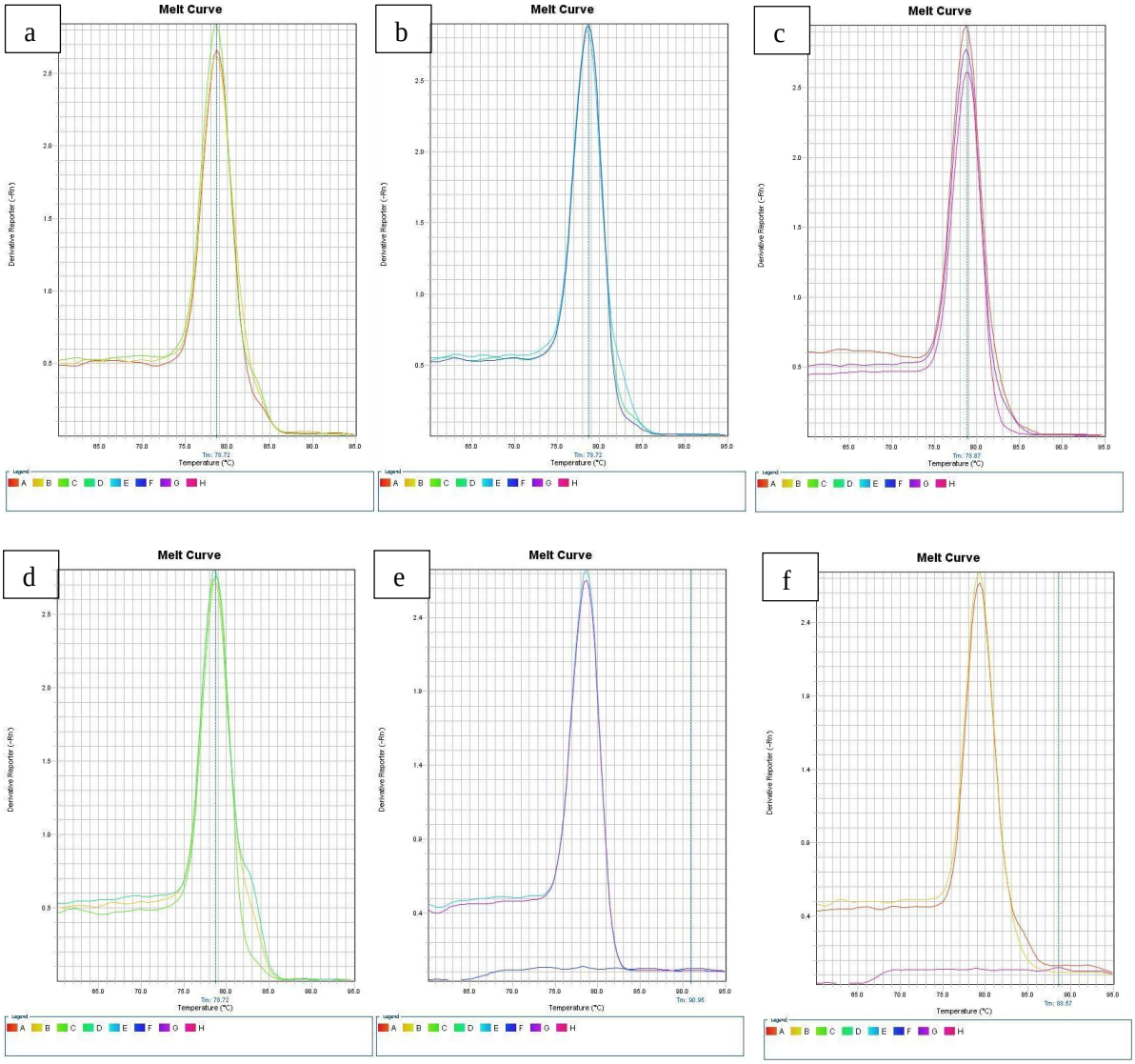



Figure 19 Meltpeak curve AAI 2. A = S1; B = S2; C = S3; D = B1; E = B2; F = B3

Meltpeak curve is the peak melt curve which can indicate the specificity of the product formed. The results in Figure 18 and Figure 19 show that meltpeak which formed in all samples and replicates on primers AAI 2 and Actin 1 were better than previous studies because they formed one peak which shows primary specificity and looks very neat and tight. Whereas meltpeak curve in the primer Actin 1 shows uniformity peak it's just that the curve is not good enough compared to primer AAI 2 and in Actin 1 there is 1 replication which double bands. When there is peak which double showed that the primer in the sample was less specific because the primer attached to 2 targets in one cDNA. This could be due to improper annealing temperature (Kartika 2018).Peak which is still not neat in the Actin 1 primer is thought to be caused by errors in reagent handling and mixing material for amplification due to method Real Time PCR is very sensitive and easily damaged.

Based on the temperature results in melting curve shows that the primary AAI 2 produces a temperature range of 85.15 ℃ – 86.64 ℃, while the primary Actin 1 showed a temperature in the range of 78.59 ℃ – 80.82 ℃. The temperature results in the second experiment showed that Actin 1 primer produced a temperature range of 79.03 ℃ – 79.33 ℃, while the primary AAI 2 produces temperatures in the range of 78.72 ℃ – 90.95 ℃. According to Koesharyani *et al.* (2017) peakmelting curve amplified below 80 °C are non-specific dimer primers. In addition to positive control, there is also a negative control (NTC) in the form of Nuclease Free Water as a substitute for cDNA templates.

The results obtained from the qPCR must be validated using electrophoresis on 4% (w/v) SFR agarose, either on AAI or Actin primers. Visualization of 4% (w/v) agarose electrophoresis on the AAI primer showed no bands on the electrophoresis results, whereas in the Actin 1 primer there was a band that appeared. This indicates that the actin primer has specific and targeted results at an annealing temperature of 61.7 °C. Meanwhile, the results obtained on AAI primers are still less specific because the results obtained are in the form of dimers. This can be caused by the concentration of cDNA templatesis still too large so that it needs to be diluted again or it can be caused by contamination during the sample processing process. Validation using electrophoresis was also carried out in the second experiment on 2% (w/v) SFR agarose, either on AAI or Actin primers. Visualization of 4% (w/v) agarose electrophoresis can be seen in Figure 20.

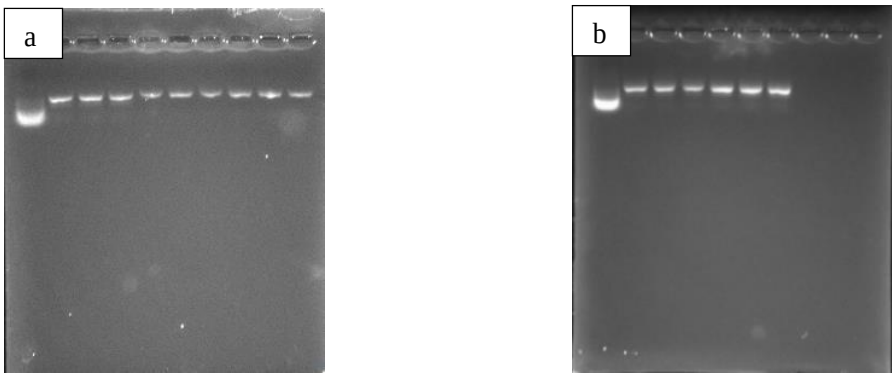



Figure 20 Results of electrophoresis visualization of RT PCR primers AAI 2 (a) and actin 1 primers (b)

Based on Figures 20a and 20b, there are bands that appear on both primers. This shows that the Actin primer has specific and targeted results at annealing temperature of 57 °C and on primer AAI 2 with annealing temperature of 61°C.

Tablel 5 $C_T$ value AAI 2 gene

| Sample | $C_T$ (Mean) gen AA1 2 | $C_T$ (Mean) $C_T$ gen Actin 1 |
|---|---|---|
| S11 | 30.9906 | 18.3613 |
| S12 | 18.8908 | 17.5669 |
| S13 | 18.8582 | 17.2138 |
| S21 | 18.8458 | 16.411 |
| S22 | 18.0006 | 16.5718 |
| S23 | 17.6944 | 16.9418 |
| S31 | 19,8024 | 16.2608 |
| S32 | 19.5621 | 15.5551 |
| S33 | 18.215 | 16.2792 |
| B11 | 18.9996 | 19.0701 |
| B12 | 18.7716 | 18.9249 |
| B13 | 19.3396 | 18.9628 |
| B21 | 17.0143 | 17.2637 |
| B22 | 19.1738 | 16.7468 |
| B23 | 20.8868 | 17.0294 |
| B31 | 18.1836 | 17.738 |
| B32 | 21.2257 | 17.4127 |
| B33 | 20.2933 | 17.4242 |

CT results on both AAI and Actin primers are shown in Table 5. Based on $C_T$ result shows that the results of $C_T$ value on actin primers (housekeeping gene) was smaller than the AAI primer. $\Delta\Delta C_T$ method obtained by comparing $\Delta C_T$ of the target gene with $\Delta C_T$ calibrator and $\Delta C_T$ normalizer (Ratnasari 2015). $C_T$ value which obtained cannot be continued to the analysis stage with $2^{-\Delta\Delta C_T}$ method due to curve melting of the Actin 1 gene formed is not good even though the CT value of all samples is below 30 and the electrophoresis visualization produces a single band and the electrophoresis results from the AAI primer have not yet appeared. The CT results of both AAI and Actin primers in the second trial are shown in Tables 5 and 6.

Table 6 $C_T$ value Actin 1 and AAI gene

| Sample | $C_T$ (Mean) AAI 2 | $C_T$ (Mean) Actin 1 | $\Delta C_T$ (Mean) | ΔΔCT | 2^(-ΔΔCt) |
|---|---|---|---|---|---|
| S11 | 29,75 | 23,88 | 6 | 0 | 1,00 |
| S12 | 29,69 | 23,88 | | | |
| S13 | 29,51 | 24,08 | | | |
| S21 | 30,24 | 24,23 | | | |
| S22 | 30,60 | 24,10 | | | |
| S23 | 30,28 | 24,16 | | | |
| S31 | 28,93 | 22,36 | | | |
| S32 | 29,21 | 22,56 | | | |
| S33 | 28,84 | 22,29 | | | |
| Average | 29,67 | 23,51 | | | |
| B11 | 28,73 | 24,48 | 5 | -1 | 2,80 |
| B12 | 29,19 | 24,32 | | | |
| B13 | 29,06 | 24,33 | | | |
| B21 | 30,58 | 24,56 | | | |
| B22 | 0 | 24,67 | | | |
| B23 | 30,64 | 24,47 | | | |
| B31 | 0 | 24,69 | | | |
| B32 | 28,19 | 24,43 | | | |
| B33 | 27,88 | 24,55 | | | |
| Average | 29,18 | 24,50 | | | |

The results in Table 6 show that the results of CT values (threshold cycle) in the primer Actin 1 with a concentration of 10 ng/µl had a lower CT value than the target gene (AAI gene). The amplification curve in the Actin 1 primer had an average CT value of 24.00 while the AAI primer (only samples B22 and B31 did not form an amplification curve so that the CT value could not be determined) was 30. Based on the CT value per sample, the samples were attacked by pests. boktor (S11, S12, S13, S21, S22, S23, S31, S32, and S33) and samples that were attacked by boktor pests (B11, B12, B13, B21, B22, B23, B31, B32, and B33) had an average CT value mean was not significantly different. This could be due to samples that were attacked by the boktor pest did not express the AAI gene at the time of sampling or the boktor pest attack was too long so that the AAI gene was under expressed. According to Christopher *et al.* (2004), gene for insect resistance in poplar (*Populus trichocarpa*) can be expressed maximally within 24 hours after the attack. Based on these results, the results on the Actin 1 primer are good enough so that it can be used for gene expression analysis, but for the AAI 2 primer, especially the B2 and B3 samples, optimization needs to be done in various fields sample concentration, temperature annealing, primary concentration and concentration fluorescent dye in order to obtain the results of the amplification curve and meltpeak curve better.

### 4.4. Analysis of gene expression in sengon plants related to tumor rust infection

The results of UBP13 gene expression analysis showed that the gene was up regulated at 1 week after inoculation and then down regulated at 2 weeks after inoculation. At the 3rd and 4th weeks after inoculation, the UBP13 gene increased its expression from the previous week. While in the seed samples, the UBP13 gene was expressed the most in tumors. The NUOR gene was highest expressed at 1 day post-inoculation and experienced a significant decrease in expression at weeks 1 to 3 and a slight increase in expression at week 4 post-inoculation. In tree samples, the NUOR gene was expressed less in the wood around the tumor than in healthy wood and had increased expression in the tumor site. The WRKY40 gene increased its expression at week 1 after inoculation, then down regulated expression at week 2 and 3, and increased expression again at week 4 post-inoculation. Meanwhile, in tree samples, the WRKY 40 gene was down-regulated in wood samples around tumors compared to healthy wood, and increased expression in tumors. The complete results of gene expression are presented in Figure 2.Ubiquitin carboxyl-terminal hydrolase 13 play a role in signaling pathway of jasmonic acid (Jeong *et al.* 2017). Gene NADH-ubiquinone oxidoreductase (NUOR) in Kant's *et al.* research (2019) is a factor that increases the susceptibility of plants of the genus Solanum against pathogens *Rhizoctonia solani*. Transcription Factor WRKY40 is a negative regulator of plant resistance (Pandey *et al.* 2010).

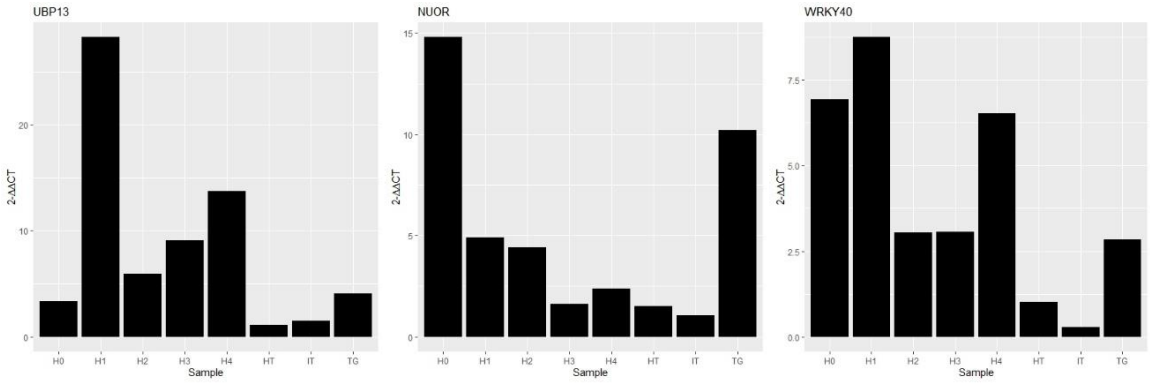



Figure 21. Gene expression results in sengon. Information H0: seedlings 1 day after inoculation, H1: 1 week post inoculation, H2: 2 weeks post inoculation, H3: 3 weeks post inoculation. H4: 4 weeks after inoculation, HT: healthy wood, IT: wood around the tumor, TG: Tumor

## 4.5. The compilation of the sengon reference genome (*Falcataria moluccana* miq.) de novo

Whole Genome Sequencing is a process of determining the complete nucleotide sequence of an individual's genome. WGS is generally carried out using a sequence approach shotgun short arranged de novo or mapped into a high quality reference genome. Short sequence fragments taken randomly are then assembled by a computer with its algorithm to obtain conclusions about the sequence of segments of the sequence (Balloux *et al.* 2018). Study on WGS according to Mei *et al.* (2016) can be used to detect single nucleotide polymorphisms (SNPs), copy number variations (CNVs), structural variation (SVs), SNP annotations, and enrichment analysis of non-synonymous SNPs. Rapid technological developments in method development Whole Genome Sequencing (WGS) such as the emergence of technology Next Generation Sequencing (NGS) will improve the quality of the results of the WGS analysis (Mukherjee *et al.* 2019). According to Tasma *et al.* (2015) sequencing using NGS will produce sequence data with relatively short size, around 50-150 bp with very large quantity. A significant increase in the number of bases produced is expected to accelerate the discovery of genomic variations of various individuals within a species so that it can support the acceleration of plant breeding programs. Success sequencing using NGS technology is highly dependent on the quality of the genome library used (Metzker 2010). A genome library is a collection of DNA sequences of an organism containing coding and noncoding regions total possession of an organism (Patterson et al et al. 2009). Activity assembly carried out to assemble the sengon reference genome in this study using several software assembler, such as Platanus, SOAP denovo, and Ray which are often used in various technologies sequencing (Segerman 2020). Platanus is assembler which works well for genome assembly with a high degree of heterozygosity while SOAP denovo works effectively for carrying out assembly reads short (short reads) (Wang *et al.* 2020). Ray is assembler which can work well on dataset reads mix so as to reduce the error rate of each sequencing platform (Biovert *et al.* 2010).

Table 7. Total contigs, the value of N50, and max length of three assembler different

| Parameter | *Assembler* | | |
|---|---|---|---|
| | Platanus | SOAP denovo | Ray |
| NC | 671 | 1051104 | 1074927 |
| N50 (bp) | 6994 | 319 | 710 |
| *Max Length* (bp) | 25663 | 9012 | 133812 |

Description : NC : Number of contig; bp :base pair

Genome coverage is important in evaluating the fraction of the genome covered by the assembly. Number of contigs, N50, and max lengthis a standard indicator that does not include error values assembly (Boisvert *et al.* 2010). Table 7 shows the results report stats of each assembly. These results indicate that Platanus has the best N50 value even though the number of contigs formed has the least amount. This N50 value indicates the length contigs which is on the median in order from longest to shortest. The greater the value of N50 indicates that the sequence is composed of contigs large (Mafireyi 2018). The interpretation of the N50 value according to Ekblom and Wolf (2014) also needs to be done carefully because a good value of the N50 statistic could be due to proximity without containing information about the accuracy of the assembly carried out.

Ray's stats show number of contigs and value max length best of the three methods assembly used. Based on a research report from Boisvert *et al.* (2010), assembly using Ray results in better performance in assembly data short reads because it produces little to no error, like incorrect contigs, mismatches, and indels (insertion-deletions). The absence of this error then becomes important because of them is matches and indels, these can later be translated as mutations in the genome of an individual.

Table 8 Comparison of BUSCO results from SOAP denovo and Ray assembler

| Status | Complete and single copy BUSCOs (S) | | Complete and duplicated BUSCOs (D) | | Fragmented BUSCOs (F) | | Missing BUSCOs (M) | |
|---|---|---|---|---|---|---|---|---|
| Assembler | n | % | N | % | n | % | N | % |
| SOAP de-novo | 18 | 5,9 | 0 | 0,0 | 39 | 12,9 | 246 | 82,2 |
| Ray | 23 | 7,6 | 4 | 1,3 | 51 | 16,8 | 225 | 74,3 |

Based on Table 8 the BUSCO analysis of SOAP denovo and Ray showed results that were not much different, namely having missing BUSCO gene more than 220 genes (>70%). This value is due to the determination of the fragmentation status of each BUSCO gene classifier uses the result scores and length thresholds for all results based on the distribution of metrics across the species used. This causes in certain cases, such as in this study outliers in terms of length or score it is classified as a missing gene (missing) or fragmented, despite the fact that the gene is present and complete in the genome. In addition, there are problems in the process sequencing and assembly as well as the inability of pipeline annotations to be able to capture the entire complexity of the existing genes also cause genes to be classified as fragmentation or missing gene (Simão *et al.* 2015). The duplication that occurs in the BUSCO analysis can be categorized as good because it is still worth below 10%. This duplication can occur as a result of evolution under a single copy in the genome. The presence of duplication that is too high can indicate potential assembly from haplotypes different (Waterhouse *et al.* 2011).

Table 9 Recapitulation of the total results of annotated sequences from Ray Assembler

| *Database* source | Total (%) |
|---|---|
| Jumlah *contigs* | 1074927 |
| *Non-redundant protein* (nr) NCBI | 753465 (70,09) |
| Uniprot | 118829 (11,05) |

Contigs results assembly then homologated on the database Non-redundant (nr) NCBI and Uniprot to determine the genes expressed in them. Non-redundant (nr) NCBI is database managed by the National Center for Biotechnology Information (NCBI) which provides sequences with 100% identity so that there is no duplication of both user-uploaded data and data curated by NCBI (RefSeq). Based on the results of annotation analysis contigs on non-redundant database (nr) protein (NCBI) 70.09% of the total contigs matched on this database (Table 9). This is due to database is the most comprehensive because it includes the sequence of database non-curated (low quality) and curated (high quality), such as GenBank/ GenPept, trEMBL, SwissProt, RefSeq, PIR, and PDB (Bagheri *et al.* 2020).

Coding sequence (cds) is an area of DNA or RNA whose sequence determines the sequence of amino acids in a protein, where if coding sequence contains start and stop codon

then it can be called as Open Reading Frames (ORF). All cds are ORFs, but not all ORFs are cds (UniProt 2021).

Uniprot is database which works to automatically annotate protein sequences from UniProtKB/TrEMBL. Total number contigs that fits on database Uniprot as much as 11.05%. Match results contigs low on database this may be due to coverage database Uniprot lower than nonredundant (nr) NCBI, so that little sequence data can be annotated on database (Uniprot Consortium 2015).

Based on the results of the alignment (alignment) sequences containing AI from the results of WGS and transcriptomic analysis in Figure 22. It can be seen that there are three sequences containing AAI from both analyzes. The transcriptomic result sequences based on the analysis have three coding sequences (cds) containing start and stop codon (open reading frame/ORF) with the longest ORF being 1156-1225 in the sequence. Figure 2 shows the results of the alignment (alignment) sequences containing the results of the analysis of WGS (19 sequences) and transcriptomics. There are three transcriptomic sequences, namely Contig-13573, Contig-16297, and Contig-16937, each of which has an ORF of 7, 7, and 4 ORFs. Contig-13573 has the longest ORF of sequence 488-523, Contig-16297 has the longest ORF of sequence 98-118, and Contig-16937 has the longest ORF of sequence 214-251. Identification of the presence of this ORF is important because it acts as an indicator for potential protein-coding genes and increases the predictive ability of genes inshort read data (Woodcroft *et al.* 2016).

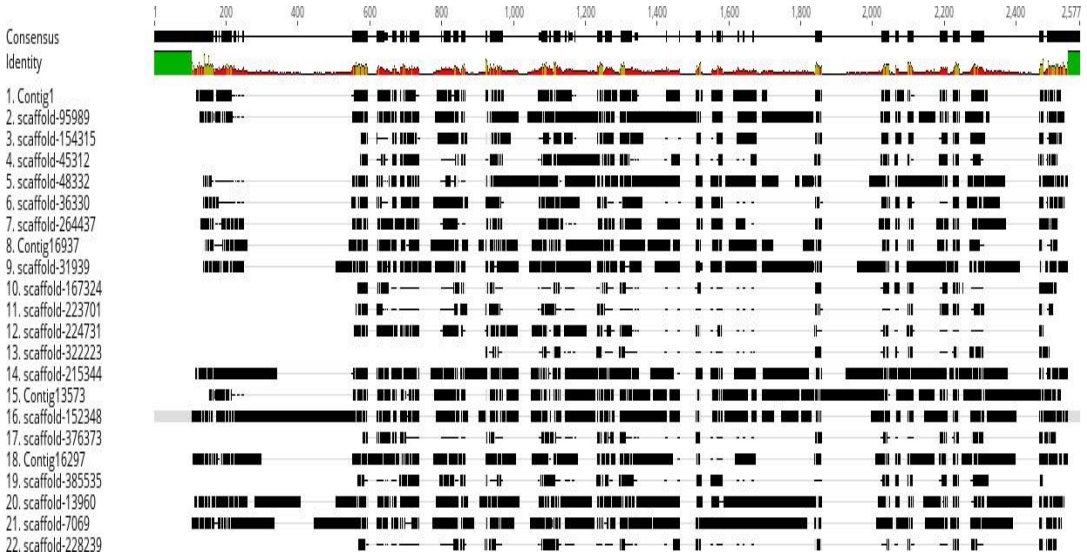



Figure 22 Alignment results TI sequences from WGS analysis and transcriptomics (contig: TI sequences resulting from transcriptomic analysis; scaffold: TI sequence WGS analysis result)

## 5. CONCLUSION

The RT-PCR results for the TI4 gene and the Actin 1 gene were good with the formation of a tight amplification curve and the melting curve only produced one peak. The electrophoresis results of TI4 and Actin 1 genes also produced a single band, which means the sample has good quality. $C_T$ value in the TI4 gene ranged from 24.33 to 26.99, while the $C_T$ value of Actin 1 gene ranged from 22.36 to 24.69. Quantification using $2^{-\Delta\Delta C_T}$ method shows the level of TI4 gene expression in sengon samples healthy (S) that is equal to 1.00 while in the sample attacked by boktor (B) of 1.91. TI4 gene expression in sengon attacked by boktor pests was higher than in healthy sengon.

Identification of gene encoding α-Amylase enzyme in plants *Falcataria moluccana* Miq, which was attacked by boktor pests, was successfully carried out. RT-PC on *Falcataria moluccana* amplified at temperature annealing 57 °C for Actin 1 and 61°C for the AAI gene. The average CT value of the Actin 1 gene that was attacked by boktor pests was 24.50 and those that were not attacked by boktor pests was 23.51. While the average CT value in the AAI 2 gene that was attacked by boktor pests was 29.18 and those that were not attacked by boktor pests was 29.67. The value of 2^(-ΔΔCt) on trees that were attacked by boktor pests had a higher value of 2.80 than those that were not attacked by boktor pests of 1.00.

The expression of tumor rust-associated genes varies. In seedlings treated with infection, there were genes whose expression increased after infection was induced and there were genes that decreased expression after infection. Meanwhile, in mature plants, the average expression of all genes was higher in tumors than in healthy wood and wood around the tumor. The higher expression of certain genes in the wood around the tumor than in healthy wood indicated that there was an indication that the gene was induced by infection with pathogens in the tumor tissue.

Sequence of the sengon reference genome de novo using Ray assembler showed better genome coverage than the two assembler other. Results assembly from SOAPd enovo and Ray showed low genome completeness due to the large number of missing BUSCO genes (>70%). The annotation results show that there are two sequences containing the AAI gene and 19 sequences containing the TI gene assembly which has been done.

## 6. TEAM CHAIRMAN AND RESEARCH MEMBER

Table 10 List of research members

| No | Name | Field | Institution | Time allocation | |
|---|---|---|---|---|---|
| | | | | day/ week | month |
| 1 | Dr. Ir. Ulfah J. Siregar (Coordinator) | Genetica Moleculer | BIOTROP | 5/4 | 9 |
| 2. | Dr.Dra. Sri N. Hartati (Researcher 1) | Biotechnology | LIPI-Bioteknologi | 1/2 | 8 |
| 3. | Fahirah Dwiyuni | Lab work | IPB student | 5/4 | 8 |
| 4. | Esti Nuraini | Lab work | IPB student | 5/4 | 8 |
| 5. | Vilda Puji D. Anita | Lab work | IPB student | 5/4 | 8 |
| 6. | Anida Lestari, S.Hut | Admin&Finance | IPB Student | 5/4 | 5 |

## 7. REFERENCES


Alrsjid H. 1973. Beberapa Keterangan tentang *Albizzia falcataria* (L) Fosberg. *Laporan No. 157*, Lembaga Penelitian Hutan, Bogor, 1973.

Andersen JR, Lubberstedt T: Functional markers in plants. Trends in Plant Science 2003, 8(11):554-560.

Amanda UD, Cartealy IC. 2015. Isolasi RNA total dari mesokarp buah kelapa sawit (*Elaeis guineenssis* Jacq. var. Tenera). Di dalam : Setyawan AD, Sugiyarto, Pitoyo A, Hernawan UE, Widiastuti A, Sutarno, Saharjo BH, Supriatna J, Jamil A, Djufri editor. *Prosiding Seminar Nasional Masyarakat Biodiveirsita Indonesia*; 2014 Des 20; Depok, Indonesia. Surakarta (ID): Masyarakat Biodiversitas Indonesia. hlm 171-176. doi:10.13057/psnmbi/m010201.

Aritonang KV, Siregar IZ, Yunanto T. 2007. Manual Analisis Genetik Tanaman Hutan di Laboratorium Silvikultur Fakultas Kehutanan Institut Pertanian Bogor. Bogor (ID): Fakultas Kehutanan IPB.

Atmosuseno BS. 1999. Budi Daya, Kegunaan, dan Prospek Sengon, Cetakan V. Jakarta: Penebar Swadaya.

[BPS] Badan Pusat Statistik. 2019. Statistik Produksi Kehutanan 2018. Jakarta: Badan Pusat Statistik.

Bagheri H, Dyer R, Severin AJ, Rajan H. 2020. Comprehensive analysis of non redundant protein database. *Research Square* : 2-9. doi: 10.21203/rs.3.rs-54568/v1.

Baskorowati L. 2014. Budidaya Sengon Unggul (Falcataria moluccana) Untuk Pengembangan Hutan Rakyat. Bogor: IPB Press.

Balloux F, Brynilsrud OB, van Dorp L, Shaw LP, Chen H, Harris KA, Wang H, Eldholm V. 2018. From theory to practice: translating Whole-Genome Sequencing (WGS) into the clinic. *Trends in Microbiollogy* 26(12) : 1035-1048. doi: 10.1016/j.tim.2018.08.004.

Barakat A, DiLoreto DS, Zhang Y, Smith C, Baier K, Powell WA, Wheeler N, Sederoff R and Carlson JE. 2009. Comparison of the transcriptomes of American chestnut (*Castanea dentata*) and Chinese chestnut (*Castanea mollissima*) in response to the chestnut blight infection. BMC Plant Biology 9:51 [http://www.biomedcentral.com/1471-2229/9/51].

Barbazuk WB, Emrich SJ, Chen HD, Li L, Schnable PS: SNP discovery via 454 transcriptome sequencing. Plant Journal 2007, 51(5):910-918.

Bouck A, Vision T: The molecular ecologist's guide to expressed sequence tags. Molecular Ecology 2007, 16(5):907-924.

Budelman A., 2005. *Paraserianthes falcataria* - Southeast Asia's Growth Champion. Winrock International.

Boisvert S, Laviolette F, Corbeil J. 2010. Ray: simultaneous assembly of reads from a mix of high-throughput sequencing technologies. *J. Comput. Biol.* 17(11):1519–1533.doi:10.1089/cmb.2009.0238.

Chandrawijaya Y, Tajuddin T, Pancasakti HK, Budiharjo A. 2013. Pelacakan fragmen gen penyandi enzim ß-ketoasil-acp Sintase II (Kas II) dari mesokarp kelapa sawit (*Elaeis guineensis* Jacq. L.). Jurnal Biologi 2(2):45– 56.

Christopher ME, Miranda M, Major IT, Constabel CP. 2004. Gene expression profiling of systemically wound-induced defenses in hybrid poplar. *Planta* 219: 936–947.

Dangl JL and Jones JDG. 2001. Plant pathogens and integrated defence responses to infection. Nature 411:826–833.

Darwiati W, Anggraeni I. 2018. Serangan boktor (Xystrocera festiva Pascoe) dan karat tumor (*Uromycladium tepperianum* (Sacc.) McAlpine) pada sengon (*Falcataria moluccana* (Miq.)) di Perkebunan Teh Ciater. Jurnal Sains Natural Universitas Nusa Bangsa 8(2) : 59-69. doi: 10.31938/jsn.v8i2.119.

Doungsa-ard C, McTaggart AR, Geering ADW, Dallsay TU, Ray J, Shivas RG. 2014. Uromycladium falcatarium sp. nov., the cause of gall rust on Paraserianthes falcataria in south-east Asia. Australasian Plant.Pathol. 44(1):25 – 30

Ekblom R, Wolf JBW. 2014. A field guide to whole-genome sequencing, assembly and annotation. *Evol. Appl.* 7(9): 1026–1042. doi:10.1111/eva.12178.

Emrich SJ, Barbazuk WB, Li L, Schnable PS: Gene discovery and annotation using LCM-454 transcriptome sequencing. Genome Research 2007, 17:69-73.

Farrell RE. 2005. RNA methodologies: A laboratory guide for isolation and characterization. 3rd ed. Burlington (US): Elsevier Academic Pr.

Finkeldey R. 2005. Pengantar Genetika Hutan Tropis (*An Introduction to Tropical Forest Genetics*) terjemahan oleh E. Djamhuri, IZ Siregar, UJ Siregar dan AW. Kertadikara. Proyek AUNP, IPB-Universitas Gottingen.244pp.

Gao Q, Yue G, Li W, Wang J, Xu J, Yin Y. 2012. Recent progress using high-throughput sequencing technologies in plant molecular breeding. *Journal of Integrative Plant Biology* 54 (4): 215–227. doi: 10.1111/j.1744-7909.2012.01115.x.

Graham R, Liew M, Meadows C, Lyon E, Wittwer CT. 2005. Distinguishing different DNA heterozygotes by high-resolution melting. Clin Chem. 51(7):1295-1298. doi: 10.1373/clinchem.2005.051516.

Haris N, Aswidinnoor H, Suwanto A, Suhartono MT, Toruan-MAthius N, Purwantara A. 2005. Konstruksi pustaka cDNA dari daun klon karet AVROS 2037 yang diinfeksi patogen *Corynespora cassiicola*. *J Menara Perkebunan* 73(2):44-46.

Hartati NS. 2002. Konstruksi pustaka cDNA sengon (*Paraserianthes falcataria* L. Nielsen) yang terinduksi serangan hama boktor (*Xystrocera festiva*) [tesis]. Bogor (ID): Institut Pertanian Bogor.

Huang X, Madan A. 1999. CAP3: a DNA sequence assembly program. Genome Res. 9: 868 – 877. doi: 10.1101/gr.9.9.868.

Jenkins, Rowena, Sarah M. 2019. Bacteriology methods for the study of infectious diseases. Academic Press. 135-164.

Jeong JS, Jung C, Seo JS, Kim JK, Chua NH. 2017. The deubiquitinating enzymes UBP12 and UBP13 positively regulate MYC2 levels in jasmonate responses. Plant Cell. 29(6):1406–1424.

Kajitani R, Yoshimura D, Okuno M, Minakuchi Y, Kagoshima H, Fujiyama A, Kubokawa K, Kohara Y, Toyoda A, Itoh T. 2019. Platanus-alle is a de novo haplotype assembler enabling a comprehensive acces to divergent heterozygous regions. *Nature Communications* 10(1702) : 1- 15. doi: 10.1038/s41467-019-09575-2.

Kartika AI. 2018. Optimasi annealing temperature primer mRNA RECK dengan metode One Step qRT-PCR. Jurnal Labora Medika 2(1):22-31.

Kant R, Tyagi K, Jha G. 2019. Host alternative NADH-Ubiquinone Oxireductase serves as a susceptibility factor to promote pathogenesis of Rhizoctonia solani in plants. Phytopathol. 109(10): 1741–1750

Kinjo S, Monma N, Misu S, Kitamura N, Imoto J, Yoshitake K, Ikeo K. 2017. Maser: A cloud-based platform to manage, analyze, and visualize NGS data for basic researches in life science. (in prep). http://cell-innovation.nig.ac.jp/maser/.

Koesharyani I, Gardenia L, Mufidah T, Santika A. 2017. Aplikasi Real Time- Quantitative Polymerase Chain Reaction (RT-qPCR) dalam kuintifikasi koi herpes virus pada ikan mas (*Cyprinus carpio*). *Media Akuakultur* 12(1): 45-53.

Kubista M. 2006. Nucleic acid-based technologies: application amplified. Pharmacogenomics 5(6): 767–773.

Larosa B. 2020. Analisis transkriptomik pohon sengon (Falcataria moluccana Miq.) rentan dan resisten terhadap hama boktor (Xystrocera festiva Pascoe)[skripsi]. Bogor : Institut Pertanian Bogor.

Li R, Zhu H, Ruan J, Qian W, Fang X, Shi Z, Li Y, Li S, Shan G, Kristiansen K, *et al.* 2010. De novo assembly of human genomes with massively parallel short read sequencing. *Genome Research* 20 : 265-272. doi : 10.1101/gr.097261.109.

Li W, Godzik A. 2006. Cd-hit: a fast program for clustering and comparing large sets of protein or nucleotide sequences. *Bioinformatics*. 22: 1658 – 1659. doi: 10.1093/bioinformatics/btl158.

Mafireyi G. 2018. A case study for the de novo genome assembly of plant genomes from Illumina short reads[tesis]. Pretoria (ZA) : University of Pretoria.

Mazzucotelli E, Mastrangelo AM, Crosatti C, Guerra D, Stanca AM, Cattivelli L. 2008. Abiotic stress response in plants: When post-transcriptional and post-translational regulations control transcription. *Plant Science* 174: 420-431. doi: 10.1016/j.plantsci.2008.02.005.

Metzker ML. 2010. Sequencing technologies—the next generation. *Nat. Rev. Genet.* 11 : 31–46. doi: 10.1038/nrg2626.

McDowell JM and Woffenden BJ. 2003. Plant disease resistance genes: recent insights and potential applications. Trends Biotechnol 21(4):178–183.

Michelmore RW and Meyers BC. 1998. Clusters of resistance genes in plant evolve by divergent selection and a birth-and-death process. Genome Res 8:1113–1130.

Miftakhurohmah, Mariana M, Wahyuno D. 2016. Deteksi *piper yellow mottle virus* (PYMoV) penyebab penyakit kerdil pada tanaman lada secara *polymerase chain reaction* (PCR). *Buletin Penelitian Tanaman Rempah dan Obat.* 27(1): 77-84.

Mukherjee S, Cai Z, Mukherjee A, Longkumer I, Mech M, Vupru K, Khate K, Rajkhowa C, Mitra A, Guldbrandtsen B, *et al.* 2019. Whole genome sequence and de novo assembly revealed genomic architecture of Indian Mithun (*Bos frontalis*). *BMC Genomics* 20(617): 1-12. doi: 10.1186/s12864-019-5980-y.

Okanti JR, Putri DH, Fathoni A. 2020. Analysis of ZDS and LCYb Enzyme Coding Gene Related to Beta Carotene Bioshynthesis in Cassava (*Manihot esculenta* Crantz) Using Reverse Transcription PCR (RT-PCR). *Bioscience*. 4(1): 1-10.

Namroud MC, Beaulieu J, Juge N, Laroche J, Bousquet J: Scanning the genome for gene single nucleotide polymorphisms involved in adaptive population differentiation in white spruce. Molecular Ecology 2008, 17(16):3599-3613.

NFTA, 1989. Paraserianthes falcataria, Southeast's Asia Growth Champion.

Novaes E, Drost DR, Farmerie WG, Pappas GJ Jr, Grattapaglia D, Sederoff RR, Kirst M: High-throughput gene and SNP discovery in Eucalyptus grandis, an uncharacterized genome. BMC Genomics 2008, 9:312.

Pattersson E, Lundeberg J, Ahmadian A. 2009. Generations of sequencing technologies. *Genomics* 93: 105–111. doi: 10.1016/j.ygeno.2008.10.003.

Pandey SP, Roccaro M, Schon M, Logemann E, Somssich IE. 2010. Transcriptional reprogramming regulated by WRKY18 and WRKY40 facilitates powdery mildew infection of Arabidopsis. Plant J 64:912-23. DOI: 10.1111/j.1365-313X.2010.04387.x.

Polosoro A, Enggarini W. 2016. Keragaman jumlah salinan transgen galur T0 padi kultivar Nipponbare berdasarkan analisis qPCR dengan penanda gen hptII. *Jurnal AgroBiogen*. 12(2): 73-80.

Promega. 2011. Technical Manual Assembly of Restricition Enzyme Digestion. USA: Promega Corp.

Program Geneious Prime®2021.1 (http://www.geneious.com/) digunakan dalam kegiatan penjajaran sekuens hasil WGS dan transkriptomik.

Puspitarini. 2006. Sebaran diameter pohon sengon (*Paraserianthes falcataria* (L) Nielsen) mendapat serangan *Xystocera festiva* Pascoe pada berbagai umur tegakan [skripsi]. Bogor (ID) : Fakultas Kehutanan, Institut Pertanian Bogor.

Putri KP, Bramasto Y. 2017. Pengendalian cendawan Uromycladium tepperianum pada bibit sengon (Falcataria moluccana (Miq.) Barneby & J.W. Grimes) di persemaian. Jurnal perbenihan Tanaman Hutan 5(1): 13-22

Qiagen 2012. DNeasy Plant Handbook. Centre Mainz (DE): Qiagen.

Rahmawati D. 2016. Morphology, Phytochemical, Genetic Evaluation of Sengon (*Paraserianthes Falcataria*) Resistant and Susceptible To Gall Rust Disease (Indonesian). Master Thesis, Graduate School IPB.

Rahayu S, Ab. Shukor NA, LEE SS and SALEH G. 2008. Responses of *Falcataria moluccana* seedlings of Different Seed Sources to Inoculation with *Uromycladium tepperianum*. Silvae Genetica 58:1–2.

Ratnasari T. 2015. Stabilitas transgen dan ekspresi gen B11 pada tanaman tembakau transgenik generasi $T_3$ [tesis]. Sekolah Pasca Sarjara. Bogor (ID): Institut Pertanian Bogor.

Rimbawanto, A. 2006. Keragaman Genetik dan Ketahanan Hama Penyakit pada Tanaman.

Sambrook J, Russel DW. 2001. Molecular Cloning: A Laboratory Manual 3rd Ed. Volume 3. Newyork (US): Cold Spring Harbor.

Schmittgen TD, Livak KJ. 2008. Analyzing real-time PCR data by the comparative CT method. Nature Protoc. 3:1101-1108

Shabrina H, Siregar UJ, Matra DD, Siregar IZ. 2019. The dataset of De novo transcriptome assembly of *Falcataria moluccana* cambium from gall-rust (*Uromycladium falcatarium*) infected and non-infected tree. Data in Brief. 26: 104489.

Segerman B. 2020. The most frequently used sequencing technologies and assembly methods in different time segments of the bacterial surveillance and refseq genome databases. *Front. Cell. Infect. Microbiol.* 10:527102.doi:10.3389/fcimb.2020.527102.

Seppey M, Manni M, Zdobnov EM. 2019. BUSCO: Assessing genome assembly and annotation completeness. Di dalam: Kollmar M, editor. *Gene Prediction. Methods in*

*Molecular Biology*. Volume 1962. New York: Humana Press. doi: 10.1007/978-1-4939-9173-0_14.

Shabrina H. 2020. Pendekatan genomika ekologi untuk pemuliaan sengon (*Falcatarian moluccana*) resisten karat tumor (*Uromycladium falcatariae*)[disertasi]. Bogor : Institut Pertanian Bogor.

Simão FA, Waterhouse RM, Ioannidis P, Kriventseva EV, Zdobnov EM. 2015. BUSCO: assessing genome assembly and annotation completeness with single-copy orthologs. *Bioinformatics*. 31(19):3210–3212.doi:10.1093/bioinformatics/btv351.

Siregar UJ and Hartati SN. 2015. RNA Isolation and Construction of cDNA Library of Sengon (*Paraserianthes falcataria)* Resistant to Boktor Pest II: Sequencing and Genomic Analysis. Final Report BIOTROP-SEAMOLEC.

Siregar UJ and Hartati SN. 2016. Study on Sengon (*Falcataria moluccana)* Resistance to Boktor Pest (*Xystrocera festiva*) and Gall Rust (*Uromycladium tepperianum*) PART I. Final Report BIOTROP-SEAMOLEC.

Siregar UJ. 2009. Laporan akhir PU-IPB: Perakitan Klon Sengon Tahan Hama Boktor Dalam Rangka Pengembangan Social Forestry. Dirjen DIKTI-IPB.

Siregar, UJ dan Hartati SN. 2014. Isolasi RNA dan Konstruksi cDNA Sengon (*Paraserianthes falcataria)* Resisten Terhadap Hama Boktor. Laporan Akhir Penelitian BIOTROP-SEAMOLEC.

Takara Bio Inc. 2016. SYBR® Premix DimerEraser™ (Perfect Real Time) Product Manual [internet]. Tersedia pada https://www.takarabio.com/assets/documents/User%20Manual/RR091A_e.v1611Da.pdf. Diakses pada 21 Maret 2019.

Tasma IM. 2015. Pemanfaatan teknologi sekuensing genom untuk mempercepat program pemuliaan tanaman. *J. Litbang Pert.* 34(4) : 159-168. doi: 10.21082/jp3.v34n4.2015.p159-168.

Tasma IM, Satyawan D, Rijzaani H. 2015. Pembentukan pustaka genom, resekuensing, dan identifikasi SNP berdasarkan sekuen genom total genotipe kedelai Indonesia. *Jurnal AgroBiogen* 11(1) : 7-16. doi: 10.21082/jbio.v11n1.2015.p7-16.

Tooy DC, Bernadus JB, Sorisi A. 2016. Deteksi Plasmodium falciparum dengan menggunakan metode real-time Polymerase Chain Reaction di daerah Likupang dan Bitung. *eBiomedik*. 4(1).

The Fagaceae Genomic Tools Project [http://www.fagaceae.org]

UniProt. 2021. What are UniProtKB's criteria for defining a CDS as a protein?[artikel]. [diakses 2021 Mei 26]. https://www.uniprot.org/help/cds_protein_definition.

UniProt Consortium. 2015. UniProt: a hub for protein information. *Nucleic Acids Research* 43 : D204-D212. doi: 10.1093/nar/gku989.

Van K, Rastogi K, Kim KH, Lee SH. 2013. Next-generation sequencing technology for crop improvement. *SABRAO J. Breed. Genet.* 45(1): 84–99.

Vera JC, Wheat CW, Fescemyer HW, Frilander MJ, Crawford DL, Hanski I, Marden JH: Rapid transcriptome characterization for a nonmodel organism using 454 pyrosequencing. Molecular Ecology 2008, 17(7):1636-1647.

Wang W, Wang F, Hao R, Wang A, Sharshov K, Druzyaka A, Lancuo Z, Shi Y, Feng S. 2020. First de novo whole genome sequencing and assembly of the bar-headed goose. *PeerJ*. 8:e8914.doi:10.7717/peerj.8914.

Waterhouse RM, Zdobnov EM, Kriventseva EV. 2011. Correlating Traits of Gene Retention, Sequence Divergence, Duplicability and Essentiality in Vertebrates, Arthropods, and Fungi. *Genome Biol. Evol.* 3:75–86.doi:10.1093/gbe/evq083.

Woodcroft BJ, Boyd JA, Tyson GW. 2016. OrfM: a fast open reading frame predictor for metagenomic data. Bioinformatics 32(17) : 2702- 2703. doi: 10.1093/bioinformatics/btw241.
Yahya SA. 2019. Optimasi Metode Deteksi dan Ekspresi Gen α-Amilase Inhibitor pada Sengon (*Falcataria moluccana* Miq) [skripsi]. Bogor (ID): Institut Pertanian Bogor.

## ATTACHMENT

Appendix 1 Result of cDNA concentration test with nanophotometer machine

| **Sampel** | **Conc. (ng/µl)** | **Method** | **1/∑** | **A230** | **A260** | **A280** | **λ** | **Abs.** | **A260/A280** | **A260/A230** | **Dilution** |
|---|---|---|---|---|---|---|---|---|---|---|---|
| S1 | 614.60 | RNA | 40.00 | 22.46 | 15.46 | 7.834 | 320 | 0.095 | 1.985 | 0.687 | 15 |
| S2 | 597.60 | RNA | 40.00 | 13.05 | 15.04 | 7.283 | 320 | 0.100 | 2.080 | 1.154 | 15 |
| S3 | 542.60 | RNA | 40.00 | 8.505 | 13.60 | 6.451 | 320 | 0.035 | 2.114 | 1.640 | 15 |
| B1 | 1194.2 | RNA | 40.00 | 34.57 | 30.00 | 15.01 | 320 | 0.146 | 2.008 | 0.867 | 140 |
| B2 | 737.40 | RNA | 40.00 | 19.71 | 18.65 | 9.459 | 320 | 0.215 | 1.994 | 0.946 | 140 |
| B3 | 301.36 | RNA | 40.00 | 7.253 | 7.575 | 3.677 | 320 | 0.041 | 2.072 | 1.045 | 15 |

Appendix 2 Base arrangement of primers used in RT-PCR experiments

| Sumber | Primer F (5'- 3') | Primer R (5'- 3') |
|---|---|---|
| Actin 1 | TTGACTGCGCTTCATCACCC<br>Tm: 60.5 ºC | GGCTGGTTTTGCTGGTGATG<br>Tm: 60.5 ºC |
| Actin 2 | ATCTTGCAGGGCGTGATCTC<br>Tm: 60.5 ºC | TTCTCGCTCAGCAGTTGTGG<br>Tm: 60.5 ºC |
| Tubulin B | GCAGATGACCTCCAGAACTTG<br>Tm: 64 ºC | GCACCGAAATGAGAGAAATCCCC<br>Tm: 64.7 ºC |
| Tubulin A1 | AAGGCTCAAATGCGCTGTTG<br>Tm: 58.4 ºC | TGCACCAGTTATCTCGGCTG<br>Tm: 60.5 ºC |
| EF1 | CTGAAGCGTGGTTATGTTGCC<br>Tm: 61.6 ºC | TGTGTGAAGTGTGGCAGTCC<br>Tm: 60.5 ºC |
| TI1 | TCTACGTCGGCCAGGAAAAC<br>Tm: 60.5 ºC | TCACGACGTTGTCTTCTCCG<br>Tm: 60.5 ºC |
| TI2 | ACCTTCAATTGCCTGACCTCC<br>Tm: 61.3 ºC | TCTCTTTGCCATCACCACGG<br>Tm: 60.5 ºC |
| TI3 | TCGTTTCCTGTCGCAGCAAG<br>Tm: 60.5 ºC | TCTCTTTGCCTTCATCGCCTC<br>Tm: 61.3 ºC |
| TI4 | GACAGGAAACGAAACTTGCCC<br>Tm: 61.3 ºC | ACGAAATTTTCCATGGCAAGCC<br>Tm: 60.3 ºC |
| TI6 | TCTTCAGCAGCCTCCAAACC<br>Tm: 60.5 ºC | TGACATTCTCACCGGCCAAG<br>Tm: 60.5 ºC |

Appendix 3 Types of primers and temperatures used during RT-PCR experiments

| Primer | Temperature | Curve result |
|---|---|---|
| Actin 1 | 55°C | Not good |
| TI 6 | 55°C | Not good |
| Tubulin B | 55°C and 56°C | Graphics are less uniform |
| TI1 | 60,5°C | AC and MC good |
| TI2 | 60,5°C | AC is there, MC not good |
| TI3 | 60,5°C | AC is there, MC not good |
| TI4 | 60,5°C | AC is there, MC good |
| TI6 | 60,5°C | AC and MC not good |
| Tubulin B | 60,5°C | AC and MC not good |
| TI2 | 60°C, 61°C, 62°C | 60°C AC good, MC not good<br>61°C AC and MC not good<br>62°C AC and MC not good |
| TI3 | 60°C, 61°C, 62°C | 60°C AC good enough, MC good enough<br>61°C AC good and MC not good<br>62°C AC good and MC not good |
| Tubulin B | 60°C, 61°C, 62°C | 60°C AC and MC not good<br>61°C AC and MC not good<br>62°C AC and MC not good |
| EF1 | 60,5°C; 61°C; 61,5°C | Cannot read the results |
| TI1 | 60,5°C; 61°C; 61,5°C | Cannot read the results |
| Actin1 | 57°C, 58°C, 59°C, 60°C | 57°C AC and MC good<br>58°C AC good, MC not good<br>59°C AC good and MC not good<br>60°C AC good and MC not good |
| TI1 | 57°C, 58°C, 59°C, 60°C | 57°C AC and MC not good<br>58°C AC and MC not good<br>59°C AC and MC not good<br>60°C AC and MC not good |
| TI1 | 61°C, 62°C, 63°C, 64°C | 61°C AC and MC not good<br>62°C AC and MC not good<br>63°C AC and MC not good<br>64°C AC and MC not good |

| | | |
|---|---|---|
| TI4 | 61°C | AC and MC good (a little more good) |
| TI4 | 60°C; 60,8°C; 61,7°C; 62,7°C | 60°C AC and MC good<br>60,8°C AC and MC good<br>61,7°C AC and MC very good<br>62,7°C AC and MC good |
| Tubulin A1 | 60°C | AC good, MC not good |
| Actin1 | 61°C; 61,7°C; 62,7°C; 63,7°C | 61°C AC good, MC not good<br>61,7°C AC and MC good<br>62,7 AC good, MC not good<br>63,7°C AC and MC good |
| Actin 1 | 61,7°C | AC and MC are good enough |
| TI4 | 61,7°C | AC and MC are good enough |
| Actin 1 | 61,7°C | AC good enough, MC not good |
| TI4 | 61,7°C | AC and MC are good enough |
| Actin | 62°C | AC very good, MC not good |
| Actin 1 | 62°C | AC very good, MC not good |
| Actin | 62,5°C; 63°C | 62,5°C AC good, MC not good<br>63°C AC good, MC not good |
| Actin 1 | 62,5°C | AC good, MC not good |
| Actin 2 | 61°C; 61,5°C; 62°C | AC and MC are not good at all temperatures test |
| Actin 1 | 61,7°C; 61,9°C; 62°C | 61,7°C AC good, MC not good<br>61,9°C AC good, MC good<br>62°C AC and MC not good |
| Actin | 61,7°C; 62°C | 61,7°C AC good, MC not good<br>62°C AC and MC not good |
| Actin 1 | 60,8°C; 60,9°C | AC and C are not good at both temperatures |
| Actin 1 | 61,8°C; 61,9°C | 61,8°C AC good, MC not good<br>61,9°C AC and MC not good |
| Actin 1 | 61,8°C | AP good enough, MC not good |
| TI4 | 61,7°C | AP good, MC good enough |
| Actin 1 | 61,7°C | AP good, MC good enough but |

| | | |
|---|---|---|
| | | not too close |
| Actin | 61,7°C | AP good, MC is not good because all the test are double peak |
| Actin 1 | 54°C; 54,5°C; 55°C; 55,5°C; 56°C; 56,5°C | The air conditioner looks good on all temperature tests. MC doesn't look good on all temperature tests |
| | 57°C;57,5°C; 58°C; 58,5°C; 59°C; 59,5°C | The air conditioner looks good on all temperature tests. MC doesn't look good on all temperature tests |
| Actin 1 | 60°C; 60,5°C; 61°C; 61,2°C; 61,5°C; 61,8°C | AC looks good on all temperature test. MC doesn't look good on all temperature tests |

AC: Amplification curve, MC: Melting curve

Appendix 4 C Nilai ValueT TI gene

| Sample | $C_T$ (Mean) | $\Delta C_T$ (Mean) | $\Delta\Delta C_T$ | RQ |
|---|---|---|---|---|
| S11 | 18.7347 | -0.4546 | 0 | 1 |
| S12 | 18.4601 | -0.0814 | 0.3732 | 0.7721 |
| S13 | 18.9822 | 0.0842 | 0.5388 | 0.6883 |
| S21 | 18.5621 | -0.4716 | -0.017 | 1.0118 |
| S22 | 18.746 | 1.1364 | 1.5911 | 0.3319 |
| S23 | 18.8997 | 1.7184 | 2.1731 | 0.2217 |
| S31 | 23.6456 | 4.5386 | 4.933 | 0.0314 |
| S32 | 22.4035 | 3.8648 | 4.3194 | 0.0501 |
| S33 | 17.9838 | -1.4084 | -0.9538 | 1.9369 |
| B11 | 18.2046 | -0.1044 | 0.3502 | 0.7845 |
| B12 | 18.6107 | 0.2933 | 0.7479 | 0.5955 |
| B13 | 18.5775 | 0.0089 | 0.4635 | 0.7252 |
| B21 | 18.5659 | -0.3386 | 0.116 | 0.9228 |
| B22 | 17.62 | -1.1747 | -0.7201 | 1.6473 |
| B23 | 17.7941 | -1.2109 | -0.7563 | 1.6892 |
| B31 | 18.2192 | -1.2368 | -0.7821 | 1.7197 |
| B32 | 17.7241 | -2.7651 | -2.3105 | 4.9606 |
| B33 | 18.137 | -1.0394 | -0.5847 | 1.4998 |

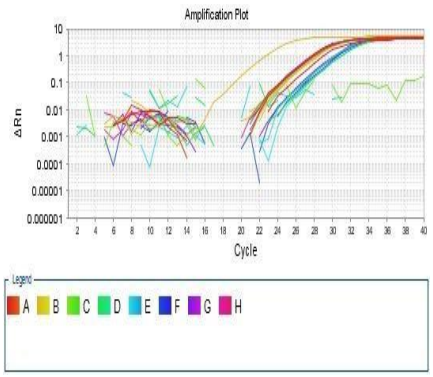
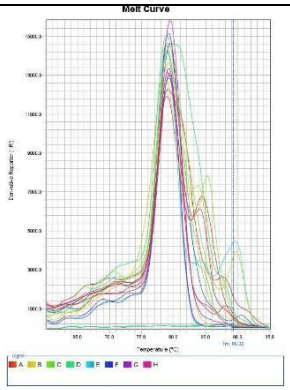
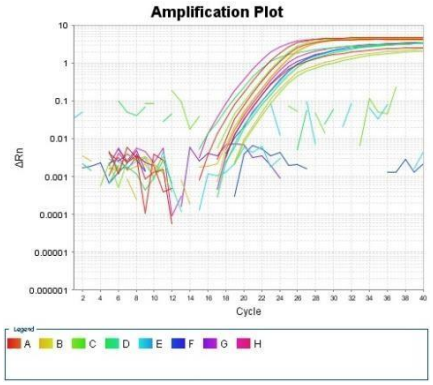
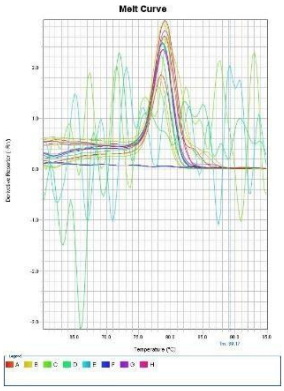
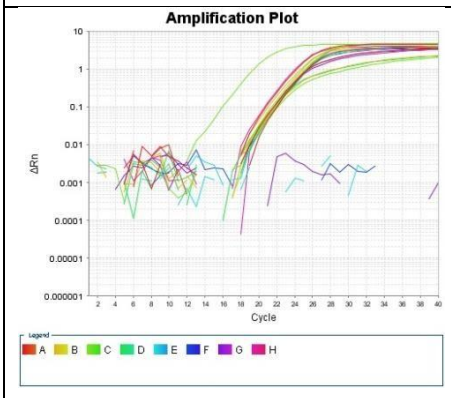
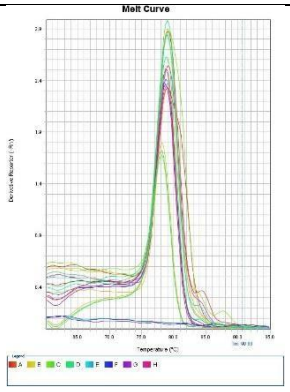
Appendix 5 C ValueT Actin 1 gene

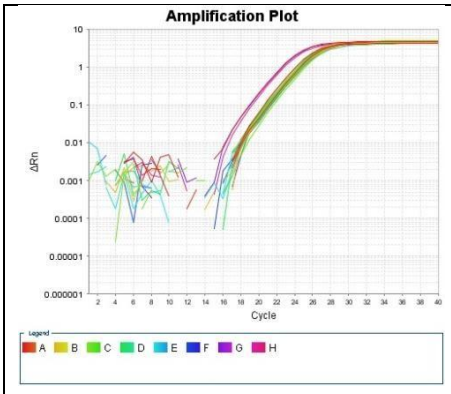
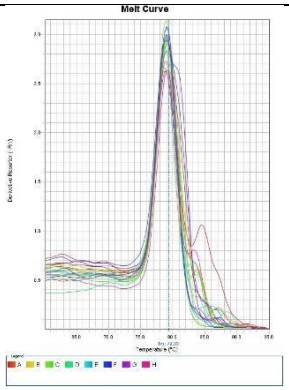
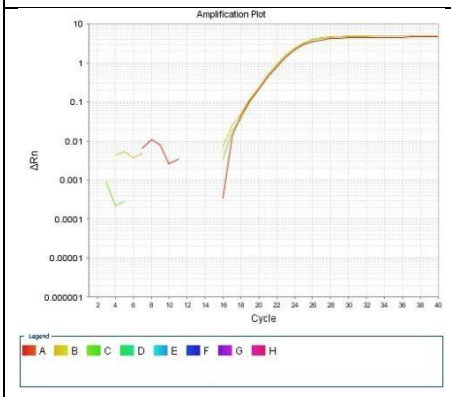
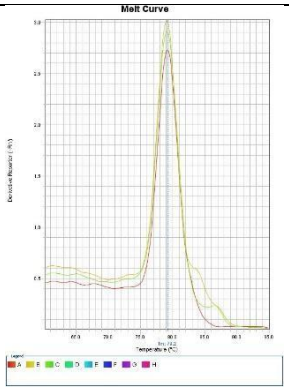
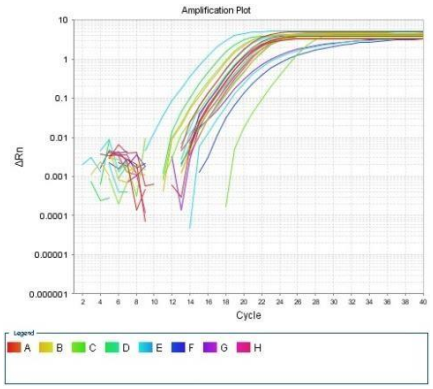
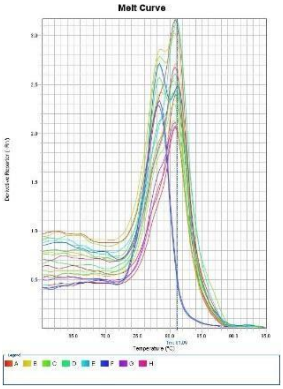
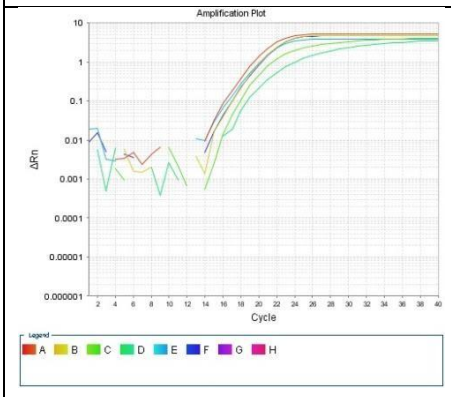
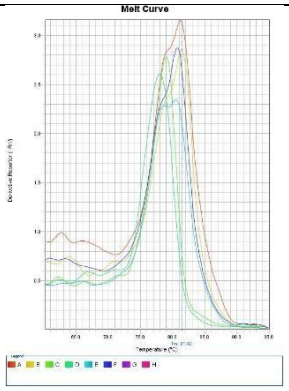
| Sample | $C_T$ (Mean) | $\Delta C_T$ (Mean) | $\Delta\Delta C_T$ | RQ |
|---|---|---|---|---|
| S11 | 18.3613 | -12.6293 | -12.6999 | 6635.535 |
| S12 | 17.5669 | -1.3239 | -1.3945 | 2.6289 |
| S13 | 17.2138 | -1.6444 | -1.715 | 3.2829 |
| S21 | 16.411 | -2.4348 | -2.5054 | 5.678 |
| S22 | 16.5718 | -1.4288 | -1.4993 | 2.8271 |
| S23 | 16.9418 | -0.7526 | -0.8232 | 1.7693 |
| S31 | 16.2608 | -3.5416 | -3.6122 | 12.2287 |
| S32 | 15.5551 | -4.007 | -4.0775 | 16.8834 |
| S33 | 16.2792 | -1.9358 | -2.0063 | 4.0176 |
| B11 | 19.0701 | 0.0706 | 0 | 1 |
| B12 | 18.9249 | 0.1533 | 0.0827 | 0.9443 |
| B13 | 18.9628 | -0.3768 | -0.4474 | 1.3636 |
| B21 | 17.2637 | 0.2494 | 0.1788 | 0.8834 |
| B22 | 16.7468 | -2.427 | -2.4975 | 5.6473 |
| B23 | 17.0294 | -3.6774 | -3.748 | 13.4353 |
| B31 | 17.738 | -0.4456 | -0.5161 | 1.4301 |
| B32 | 17.4127 | -3.8131 | -3.8836 | 14.76 |
| B33 | 17.4242 | -2.8691 | -2.9397 | 7.6724 |

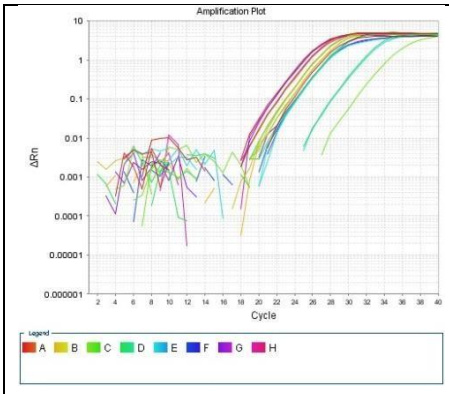
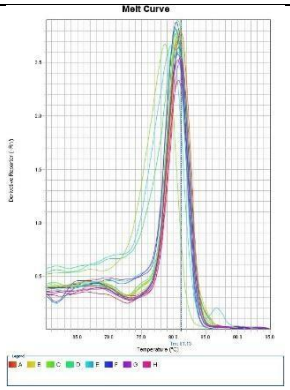
Appendix 6 List of primers for RT-PCR for gall rust

| No | Gene | Code | Primer Forward | Primer Reverse |
|---|---|---|---|---|
| 1 | Ubiquitin carboxyl-terminal hydrolase 13 | UBP13 | CAGGATGATAACCAGCCATTTC | ATGTCGGAAGGCTCTTTGTG |
| 2 | NADH-ubiquinone oxidoreductase | NUOR | CATGAGCATCATGGCATAGG | GAGTGCAGGTTCGGTGATTC |
| 3 | WRKY transcription factor 40 | WRKY 40 | CCAGCATCAGCAACAACAAC | TTGCTTTGTTGGTGGCTTCC |
| 4 | Actin | Actin | CCTTTGCAGGTATTGTGTTGG | AAGACGAAGGATGGCATGAG |

**RT-PCR Results**

| Result | | Information |
|---|---|---|
| **Amplification Curve** | **Melting Curve** | |
|  |  | AC and MC pretty good on all sample however not yet uniform. Multiple MC still have 2 peak. |
|  |  | AC and MC good on all samples except sample B2 (curve no formed). Another curve who does not formed is negative sample. |
|  |  | AC and MC good on all samples except sample B2 (curve still not formed) |

| | | |
|---|---|---|
|  |  | AC and MC good on all samples, sample only S1 test 1 still formed double peak. |
|  |  | AC curve already very good, curve MC is good |
|  |  | AC curve and enough MCs good however not yet uniform. MC curve Sample S2 still not yet good and formed two peak |
|  |  | AC and MC good on all replays but curve still apart |

| | | |
|---|---|---|
|  |  | AC and MC good on all replay, curve meeting and uniform. 2 the AC curve that further iscontrol negative. |