Sample of
Master's Thesis

Graduated Master's Students

1. Nur Elia Nadhira Mohd Asmadi
2. Siti Nabilah Mohammad Sabri

Master's Program of Biotechnology
Postgraduate
UNIVERSITAS JEMBER

Homepage: https://bioteknologi.pasca.unej.ac.id

THE EXPRESSION OF RESISTANCE GENE ON INDONESIA PIGMENTED LOCAL RICE VARIETIES AGAINST BACTERIAL LEAF BLIGHT DISEASE

THESIS

Prepared by

Nur Elia Nadhira Binti Mohd Asmadi

NIM 192520101005

BIOTECHNOLOGY MAGISTER POSTGRADUATE PROGRAM STUDY

UNIVERSITY OF JEMBER

2022

STATEMENT OF AGREEMENT

The one who confirmed with the following statement:

Name : Nur Elia Nadhira bt Mohd Asmadi
NIM : 192520101005

I solemnly declare that the scientific thesis titled **"The Expression of Resistance Gene on Indonesia Pigmented Local Rice Varieties against Bacterial Leaf Blight Disease"** is entirely my own work, with the exception of the quotations for which I have cited the source, and that it has never been submitted to any institution and has never been plagiarized. In accordance with a scientific attitude that must be maintained, I am accountable for the content's validity and correctness.

As a result, I make this statement honestly, without any pressure or compulsion from anyone, and I am willing to face academic punishment if this statement is later proven to be false.

Jember, 13 January 2022
Sincerely,

Nur Elia Nadhira bt Mohd Asmadi
NIM 192520101005

THESIS

THE EXPRESSION OF RESISTANCE GENE ON INDONESIA PIGMENTED LOCAL RICE VARIETIES AGAINST BACTERIAL LEAF BLIGHT DISEASE

Prepared by

Nur Elia Nadhira Binti Mohd Asmadi

NIM 192520101005

Supervisors:

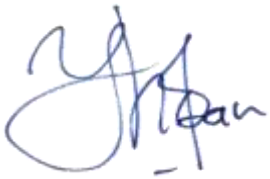
Main supervisor: Hardian Susilo Addy, S.P., M.P., Ph.D

Co - supervisor: Wahyu Indra Duwi Fanata, S.P., M.Sc., Ph.D

SUPERVISORS' APPROVAL

This thesis titled "The Expression of Resistance Gene on Indonesia Pigmented Local Rice Varieties against Bacterial Leaf Blight Disease" has been approved by:

Date and day : 13 January 2022, Thursday
Place : Department of Postgraduate, University of Jember

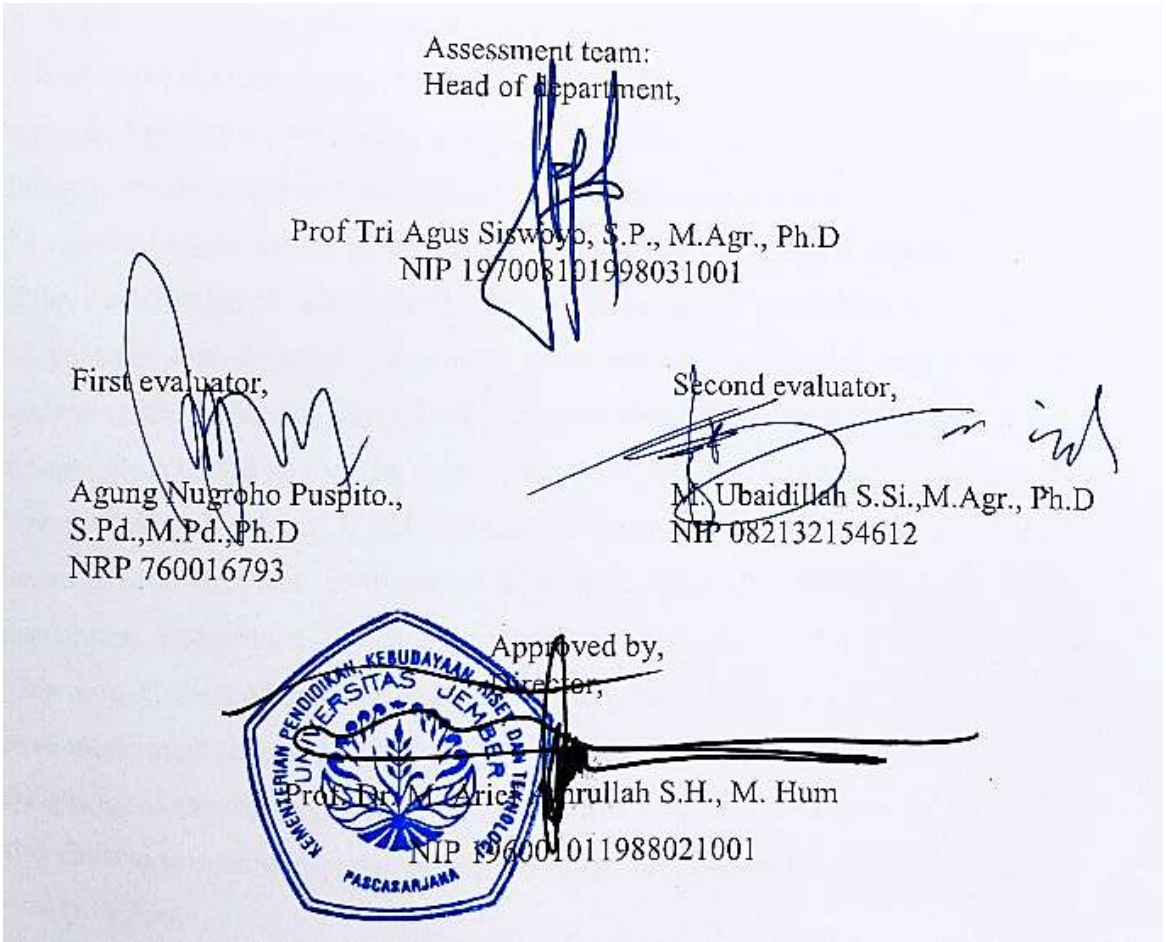
Main Supervisor,	Co – supervisor,
	
Hardian Susilo Addy, S.P., M.P., Ph.D NIP 198011092005011001	Wahyu Indra Duwi Fanata, S.P., M.Sc., Ph.D NIP 198102042015041001

APPROVEMENT

Thesis titled "The Expression of Resistance Gene on Indonesia Pigmented Local Rice Varieties against Bacterial Leaf Blight Disease", by Nur Elia Nadhira bt Mohd Asmadi, NIM 192520101005 has fulfilled the requirements of the Rector's Declaration of the University of Jember, number 1688/UN25/SP/2017, dated 1 November 2017, regarding Early Detection of Plagiarism and Prevention of Plagiarism by Scientific Lecturers, Education Personnel, and students at the University of Jember with submission ID 1739319765 and has been verified on:

Date and day : 13 January 2022, Thursday

Place : Department of Postgraduate, University of Jember



SUMMARY

The Expression of Resistance Gene on Indonesia Pigmented Local Rice Varieties Against Bacterial Leaf Blight Disease; Nur Elia Nadhira bt Mohd Asmadi;192520101005; 2022; Program Study of Magister Biotechnology, Postgraduate, University of Jember.

Oryza sativa is the scientific name for rice or rice plant. *O. sativa* which belongs to the family Gramineae and subfamily Oryzoides where generally is the second most significant cereal crop and staple food for more than half of the world's population. Pigmented rice varieties is used in this study due to high bioactive compounds found in pigmented rice such as antioxidant, anthocyanin, phenolic acids, flavonoids, pro-anthocyanidins, tocopherols, tocotrienols, coryzanol. One of the largest groups in bioactive compound is phenolics. Findings also show that the pigmented rice also used to treat many skin conditions, the abdominal pain for examples; stomach upsets, heart-burn and indigestion. Moreover, rice plants are often confronted with most destructive diseases which is bacterial leaf blight (BLB). *Xanthomonas oryzae* pv. *oryzae* (*Xoo)* is the cause of bacterial leaf blight (BLB), a very severe disease in rice-growing regions of the world which contributed up to 80% of the damage in rice production. Examining the bacterial leaf blight resistance level genes present in pigmented rice varieties and evaluating the resistance response of pigmented rice to bacterial leaf blight disease are the two goals of this study. The only *Xa* genes discussed in this research are *Xa1, Xa3, Xa4, xa5, Xa7, Xa10, xa13,* and *Xa21*. IR64, Ciherang, Cempo Merah, Inpari 24, and Hitam Bantul are five cultivars of pigmented local rice varieties used. Disease assessment categorized the three different pigmented rice into resistant varieties (Ciherang, Cempo Merah, and Hitam Bantul), while IR64 (white) and Inpari 24 (red) were moderately resistant. There was no specific pattern of possessing the *Xa* genes, the quality of expression, and the resistance level to *X. oryzae* pv. *oryzae*. The results also show that possessing resistance genes does not guarantee the resistance of a rice variety to *Xoo*.

PREFACE

I'd want to express my gratitude to Allah, Almighty God, for all of the blessing that has been granted upon me in the form of health, strength, and wisdom, allowing me to complete this study on time. This thesis, "The Expression of Resistance Gene on Indonesia Pigmented Local Rice Varieties against Bacterial Leaf Blight Disease," was prepared for a Master's degree in Biotechnology from the University of Jember in Indonesia.

On this occasion I would like to say a million thanks to Prof. Dr. Ir. Rudi Wibowo, M.S., as the Director in Department of Postgraduate, University of Jember. Also, I would like to express my gratitude to Prof. Tri Agus Siswoyo, S.P., M. Agr., PhD as the Head Department of Program Study Magister Biotechnology, University of Jember. I'd like to specially thank Dr. Hardian Susilo Addy, S.P., M.P., Ph.D., my main supervisor, for his support and supervision from the start of my studies to the completion of this thesis writing. Dr. Hardian, on the other hand, never tires of providing, monitoring and gave motivation when I’m lost at the moment. Thank you also to my co-supervisor, Dr. Wahyu Indra Duwi Fanata, who gave feedback from the research plan to help me finish my thesis and project. And thank you so much to all lectures for the knowledge since I was here.

I would like to thank especially my father, Mohd Asmadi bin Abdullah and my mother, Siti Murni bt Ajir as well as my sister, Nur Aina Natasha and also my younger sister, Nur Anis Nadia because without their support and encouragement I could not have completed my studies perfectly at the University of Jember, Indonesia. I would also like to thank my bestfriend, Wulan Arum Hardiyani and also my fiancé, Muhammad Syihan for being always been together with me going through the ups and downs of this master’s journey. Being a foreign is not as easy as one might think, but the strength I gained was from my family and friends members and my supervisors who gave too much of their support and help.

I have endeavoured to as much as possible in completing this thesis, but I am aware there are many drawbacks in terms of both content and grammar, I would like to welcome any suggestions and constructive criticism from readers for this thesis perfectly. I sincerely hope the contents of this paper would be useful in enriching the repertoire of knowledge.

Jember, 13 January 2022

Sincerely,

Nur Elia Nadhira bt Mohd Asmadi

NIM 192520101005

TABLE OF CONTENTS

Page

LIST OF FIGURES

No. Page

LIST OF TABLES

No. Page

CHAPTER 1. INTRODUCTION

1.1 Background of study

Rice is categorised as a cereal grain which is belongs to the family of Graminae. This rice plant is native to the deltas of the great Asian rivers, the Ganges, the Chang (Yantze), Tigris and Euphrates. Rice is domesticated as early as 3500 bc, and by about 2,000 years ago which is grown in almost all of the present-day cultivation areas, predominantly deltas, floodplains and coastal plains, and some terraced valley slopes. Rice is considered as the most cultivated grain in Indonesia as well as other Asian country. China is the largest rice production and it is followed by India. Even though rice plant is common in especially Asia but the methods of planting the rice plants vary for each regions and varieties. According to Hasanah *et al.,* (2009), the importance of keeping the soil unsaturated to get more air to plant roots as soil moisture still play a crucial role in rice plantation. There are several of technologies applied in the rice field sectors but still in India, they applied the traditional methods for cultivation and harvesting purposes. It is differed in Indonesia, Indonesia is also considered as a large scale rice cultivation and most of the rice field, it uses technology to maximise the production.

Biotic and abiotic factors affect the rice crop production in the field potentially reduce the crop yield. Among the biotic factors, diseases are major factor affecting the rice production (Mustafa *et al.,* 2013). In rice production, rice plants are always confronted with several major diseases such as Bacterial Leaf Blight (BLB), Leaf Blast Disease (LBD) and Sheath Blight Disease (SBD) (Kahar *et al.,* 2015). In the study of Kahar *et al.,* (2015) stated that bacterial leaf blight (BLB) is caused by *Xanthomonas oryzae pv. oryzae* where this disease causes wilting of the seedlings and yellowing and drying of the leaves. This disease is a vascular disease which spreads systemically through the xylem tissue and is a significant disease throughout many countries of rice

production (Yang *et al*, 2006). Moreover, BLB is considered as the most serious diseases of rice plants where it could trigger high yield loss that might be as high as 80% when susceptible varieties of rice are grown in situations favourable to the disease (Kahar *et al.,* 2015). The earlier the disease happens, the higher the yield loss. Due to this circumstance, planting the resistance varieties will be the great option for farmers. Quantitative resistance means the rice cultivar has many R genes in it and favoured strategy for sustainable control of plant disease (Liu *et al.,* 2018).

The other most destructive disease is leaf blast disease (LBD) is caused by the fungus *Magnaporthe grisea.* In the process of rice development, LBD is considered as a primary issue that being faced by the growers as it could arise all year long and also to every country that plants rice crops (Kahar *et al.,* 2015). In addition, during the development process especially after 15 days of planting the seeds, LBD usually occurred thus it can be classified into three different stages, which are early, middle and late stages. This disease emergence can ultimately affect approximately 100% of yield loss. Consequently, according to the study of Shafinah *et al.,* (2013), this major disease must be taken very seriously and can only be controlled during its early and middle stage. There is only a 50% chance of the plant surviving once LBD has reached the middle stages, and as it has gone into its late stage, the plant surviving become little to no chance. The spreading ways of LBD are through air, seeds and also alternative plant host (Shafinah *et al.,* 2013). However, leaf blast disease is not only spread through these three ways but it can also affect all above ground parts of a rice plant which includes the leaf, neck, collar, node, parts of panicle, and sometimes leaf sheath (Kahar *et al*., 2015). As for this disease, the key control option is planting the resistance varieties as LBD is recognised as one of the most damaging disease in rice plantation.

Next, the other main concern in rice production is Sheath Blight Disease (SBD) where it is caused by a fungal disease namely *Rhizoctonia Solani*, that infected leaves to rapidly dry out. Besides, one of the symptoms of this SBD disease that can be seen where it has resembled an ellipsoidal or an oval shape. It begins with a small white to gray oval lesions and eventually becoming greening to gray lesions accompanied with

dark green borders on the plant lesions (Kahar *et al.,* 2015). When the lesions have started to expand and combine with each other, it signals that the disease has moved on to the middle stage of the disease. When lesions are successfully covered nearly 50% and more of the leaf areas, it is already reached the last stage of SBD. These symptoms are frequently occurred at either the leaf or the stems of the rice plant. This occurrence is from the 30th – 40th – 55th day after planting the seeds of the plant. Early stages for SBD are from 1 to 4, middle stages are from 4 to 6 and late stages are from 7 to 9. According to Kahar *et al.,* (2015), the rice plants can easily be infected by sheath blight disease through several ways in particularly by water used, the ground, spores and infected from other plants. SBD is one of three of the most popular and dangerous rice diseases which can cause such a high yield loss.

1.2 Problem statement

Rice plant is one of the vital productions in world because it categorised as one of the main consumptions worldwide. The rice production rises per year but it corresponds with the increase occurrence of various destructive diseases that could eventually degrade the quality of the rice yield. The degradation of the rice quality where ultimately decreases the farmers profit will be the main concern. Moreover, the presence of this leaf blight disease especially on the susceptible plants could reduce the rice plant productions and due to the concern, many researches been done to produce tolerant and resistant rice varieties to minimise the estimated loss (Kahar *et al.,* 2015). Uncontrolled occurrence of bacterial leaf blight disease on rice plantations not only reduce the farmer's income but will dramatically causing such a high cost on importing rice from other neighbourhood countries due to high demand of rice in Indonesia.

1.3 Objectives

The major goals of this study are to examine the bacterial leaf blight resistance level genes present in pigmented rice varieties and to evaluate the resistance response of pigmented rice against bacterial leaf blight disease.

1.4 Significant of Study

Bacterial leaf blight disease which caused by *Xanthomonas oryzae pv. oryzae*. (*Xoo*), cause serious yield losses and pose a constant threat to rice supplies (Shimono *et al.*, 2012). However, there has been little research done on identifying the gene expressions and levels of expression of all resistance genes in rice, particularly in pigmented rice rice, where much research has been done on non-pigmented rice. (Kahar *et al.,* 2015). It is crucial to ensure the plants health in order to produce yield and rate of plant disease plus irritation is one of the real phenomena faced by farmers (Kahar *et al.,* 2015). Besides, regarding from this study, growers can apply or plant the pigmented rice plants as consequence to live a healthy life. This study will focus on gene expressions in some of the pigmented local rice varieties in Indonesia with the goal of identifying the resistant variety among all five native Indonesia rice variations so that farmers and researchers can better understand and also potential of the pigmented rice varieties. In order to meet the greater grain quality standards for the food industry and good yield for farmers, improvement of pigmented rice should be accelerated and take into consideration (Sanghamitra *et al.,* 2018).

CHAPTER 2. LITERATURE REVIEW

2.1 Variety of Rice plant

Oryza sativa is the scientific name for rice or rice plant. *O. sativa* which belongs to the family Gramineae and subfamily Oryzoides where generally is the second most significant cereal crop and staple food for more than half of the world's population (Mustafa *et al.,* 2013). Moreover, rice is a grain belonging to the grass family. Surprisingly, rice also having many medicinal uses that has been proven in the study of Umadevi *et al.* (2012) said that the growing rice by-products especially for pigmented rice create various valuable and worthwhile products such as rice oil from rice bran are used to treat many skin conditions, the abdominal pain for examples; stomach upsets, heart-burn and indigestion. In addition, brown rice extract has been used to treat breast and stomach cancer, warts, indigestion, nausea and diarrhoea. Sanghamitra *et al.* (2018) found that white grain is commonly utilised for food but not for medicinal purposes, unlike coloured rice. These coloured rice types are primarily found in rice-producing Asian countries like India, Sri Lanka, the Philippines, Chinaand Japan.

Normally, for the rice plants to grow it takes around 3 – 6 months from seeds to mature plants depending on the variety and environmental conditions. There are three general growth phases involved: vegetative, reproductive and ripening. In the tropical environment, it spends about 60 days in the vegetative phase, 30 days in the reproductive phase and 30 days in the ripening phase for a 120 day variety. For the growth requirements, rice plants would demand a high light intensity, a good irrigation system and the most important requirement is the plant care to avoid any pathogen attack during the growth season. According to Watanabe & Kume (2009) consequence by the climate changes, it will directly affect the rice plant growth and within rice cultivations, alter the hydrological regime including flood patterns and water availability for irrigation and drainage. In addition of this issue, there will be changes

in air temperature, precipitation, evapotranspiration and water temperature which could affect the rice production as second most vital crop in the world. Unlike non-pigmented rice varieties, pigmented rice varieties are classified botanically to have a high endurance to unfavourable situations, such as low fertile soil, deep water, salinity and cold temperatures, but they are poor yielders (Watanabe & Kume, 2009).

On the other hands, pigmented rice is not the first option for consumers whom like to eat rice with sweet and milky taste. However, nowadays people are more concern about their health issues and become increasingly interested in pigmented rice is one of the best options to be replace with the white rice due to some studies made by Umadevi *et al.* (2012); Mau *et al.* (2018); Kaur *et al.* (2018) and Sanghamitra *et al.* (2018) which stated that there are high bioactive compounds found in pigmented rice such as antioxidant, anthocyanin, phenolic acids, flavonoids, pro-anthocyanidins, tocopherols, tocotrienols, coryzanol. One of the largest groups in bioactive compound is phenolics. According to Kaur *et al.* (2018) phenolic compounds have been known as compounds that present antioxidant, anti-inflammatory, anticarcinogenic and hypoglycaemic health benefits. In fact, preventive or nutraceutical effects must be shed the light on to more impressive health benefits for reducing the chronic disease like cardiovascular disease, type-2 diabetes, obesity, cancer etc. as it will become the main health conscious issues for thus classified as a functional food or superfood. Nevertheless, it is contrasted with the white rice where research found that there is only a small amount of phenolic compound as compared to the pigmented black, red and wild rice grains where they are rich with sources of phenolic.

2.2 Destructive diseases in rice plant

A crop failure drives the whole world into a real threat of starvation. There are some major diseases that could frequently found in the rice plant production such as blast, bacterial leaf blight, sheath blight and tungro virus (IRRI, 2019). Moreover, these diseases have such a high potential causing more than 70% of yield loss if the plants are left untreated. There are three types of a wide variety phytopathogens of

sophisticated plant defense mechanisms which are PAMP-triggered immunity (PTI), effector-triggered immunity (ETI) and systemic acquired resistance (SAR) (Moon *et al.*, 2018). Plant defense mechanisms are a second metabolism that each plant has developed to protect itself from disease infection and death.

Generally, these three diseases are the most common destructive disease in rice production. Firstly, rice blast disease caused by a fungus namely *Magnaporthe grisea* which spread abundantly form on leaves, stems, peduncles, panicles, seeds and even roots. Other than that, according to Oerke (1996) stated that the panicle blast symptom that has the greater economic impact due to leaf blast which may result in significant economic loss. Secondly, another disease that can threaten rice production is sheath blight disease. Sheath blight disease is a fungal disease which caused by *Rhizoctonia solani*. Occurrence of this disease can be clearly seen in the growing areas of temperate, subtropical and also tropical countries. According to Zhang *et al.* (1998); Saha *et al.* (2015) bacterial blight is a devastating disease of rice that can be found in both tropical and temperate climates. Economically, this disease has the largest impact, particularly in Asia's developing countries. Bacterial blight is capable of causing crop loss of more than 80% in rice rice cultivation under favourable conditions, specifically for susceptible varieties. In different countries, the disease is responsible for a 20 - 30% loss of productivity.

2.3 Bacteria Leaf Blight Disease in Rice

2.3.1 The pathogens

Bacterial leaf blight is caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) a very serious disease in rice-growing regions of the world. A flare up of *Xanthomonas oryzae* causes a potentially devastating disease (Chae *et al.,* 2014) where it is grouped in a gram-negative, non-spore forming rod 0.55 x 3.5-2.17μm, an aerobic bacterium and grows best at a temperature and pH of 25 - 30^{o}C and 6.5 - 7.5 respectively (Saha *et al.*, 2015) and monotrichous flagellate. In addition, bacterial blight is categorised as one of the most destructive rice diseases particularly in Asia and has historically been

associated with major epidemics and this bacterium can survive in soil for 1-3 months (Lee *et al.*, 2003).

International Rice Research Institute (IRRI) in 2019 states that as a precaution step to remain concern and safe, they will not endorse of releasing the new varieties unless the varieties carry the resistance of bacterial blight disease (BLB). Whenever susceptible rice varieties are grown in environments that favour bacterial blight, it could definitely create the possible accessibility for the *Xoo* causing more than 80% yield loss especially in the susceptible rice. Therefore, active breeding in national and commercial breeding programs have developed and released some hybrids and genetic molecular that have resistance to BLB.

Xoo isolates were divided into 12 pathotypes based on their pathogenicity to different rice varieties (Suparyono *et al.,* 2004). According to Sudir *et al.* (2009), pathotypes III, IV, and VIII were the most prevalent *Xoo* pathotypes in rice production hubs in Java, with *Xoo* pathotype VIII being the most dominating and having a very wide distribution area both in the lowlands and medium lands. *Xoo* pathotypes III and IV are pathotypes found in specific places, particularly in the lowlands (Suparyono *et al.*, 2004). In the IR36 rice variety, the bacterial pathotype III was also able to defeat the dominant resistance gene *Xa4* (Suparyono *et al.*, 2004). The pathotype composition varied significantly between locales indicating that locally specialized disease resistance cultivars are required in the control of this major bacterial disease in rice.

Some sign and symptoms indicate this disease for instance at the seedling stage where infected leaves turn grayish green and roll up. As the disease progresses, leaves turn yellow to straw-coloured on the leaf blades with a wavy margin. The disease assures a high causes damage at seedling stage resulting in complete wilting or death of affected tillers thus the infection at maximum tillering stage results in blighting of leaves Lee *et al.* (2003).

2.3.2 *Xoo* management and plant care

Rice diseases can be controlled by cultivation resistant varieties, cultural practices and chemical application (Mustafa *et al.,* 2013). This disease can severely infecting by potentially creates up range from 20% to 30% but may reach to 70% - 80% of yield loss (Lee *et al.* 2003; Saha *et al*. 2015). According to Nagendran *et al.* (2013) foliar spray with copper fungicides alternatively with Streptocyclin (250 ppm) to check secondary spread, application of bleaching powder in the irrigation water is recommended in the growing stage and seed treatment with 0.1 g Streptocycline and 0.1 g Copper sulphate or 0.3 g Agrimycin-100 and 0.1 g Copper Oxychloride in one litter of water for 20 minutes. These bacterial diseases can be controlled to a certain extent by agrochemicals; however, the chemicals have not always been effective method and caused public concerns (Chae *et al.,* 2014). BLB can also be controlled by the usage of pesticides yet the pesticides that are specially designed for bacterial plan infections are copped based and it is very toxic and non-environmental friendly leads to unsafe consumption. In addition, secure disease-free seed, grow nurseries preferably in isolated upland conditions, avoid clipping of seedlings during transplanting, have a balanced fertilizer, avoid excessive nitrogen application that can intensify the disease, try to skip nitrogen application at booting stage and drain the field are some of the additional methods that should be used.

As a result, a disease management strategy is a critical step in reducing the disease's development. To avoid substantial crop losses, growers are strongly advised to use resistance types for cultural and preventive management techniques, as well as to eliminate the disease or pathogen's source (Kahar *et al.,* 2015), there are no or limited means to protect rice plants from this disease (Chae *et al.,* 2014), hence planting resistance types is the best way to ensure crop security. The use of quantitative resistance cultivars is the preferred strategy for long-term plant disease control (Lee *et al.,* 2003; Liu *et al.,* 2018). Consider and plant resistant cultivars as the most successful, trustworthyand cost-efficient way for controlling BLB (Chen *et al.* 2001; Zhang *et al.* 2006; Shinta *et al.* 2014; Kahar *et al.* 2015). According to Sanghamitra *et al*. (2018),

white rice research has a large research platform, but pigmented rice research has limited reports. Resistance varieties are mostly obtained using molecular genetic processes, however some are obtained through breeding procedures, which are no longer recommended by most nations unless they contain resistance genes. However, resistance plants provide growers with low input materials and high output profit, as well as a reduction in pesticide use, all of which contribute to a more ecologically friendly environment and greatly survive any abiotic and biotic stress, including diseases (Klingeman *et al.,* 2009).

2.4 *Xa* resistance genes on pigmented rice plant

Resistance genes are present in all rice varieties, however the level of expression varies by cultivars. According to Liu *et al.* (2018) resistant hosts can be classified as either qualitative (complete) or quantitative (partial). Quantitative resistance consists of multiple genes is considered to be more broad-spectrum and durable than qualitative resistance mediated by R genes, based on its non-race specificity. These resistance towards bacterial leaf blight is controlled by R genes. Currently, recent researches have been conducted in the genetic molecular and 46 *Xa* genes which confer resistance to various *Xoo* strains have been designated in a series from *Xa1* to *Xa46* (Chen *et al*., 2021). Only eight resistance genes (R genes) would be determined in this experiment which were *Xa1, Xa3, Xa4, xa5, Xa7, Xa10, xa13* and *Xa21.*

Bacterial leaf blight disease was first discovered in Japan and in the study of Yoshimura *et al.* (1998) stated that the gene *Xa1* is involved in the pathogen recognition and resistance of Japanese race 1 to *Xoo*, the causal pathogen of bacterial leaf blight (BLB). One of the resistance genes, *Xa1* confers a high level of specific resistance to race 1 strains of *Xoo* in Japan. The deduced amino acid sequence of the *Xa1* gene product contains nucleotide binding sites (NBS) and a new type of leucine-rich repeats (LRR). Besides, *Xa3* is also one of the resistance genes that involved for rice bacterial disease (Xiang *et al.,* 2006; Susanto *et al.,* 2018). Generally, *Xa3* is genetically tightly linked *to xa26* which is also another gene for bacterial blight resistance and this gene

encode Leucine – Rich Repeat (LRR) receptor kinase-type protein. In the study of Xiang *et al.* (2006), *Xa3* and *xa26* had the same copy number of family members. Additionally, the function of *Xa3* is influenced by both genetic background and growth stage of host. Next, *Xa4* is a dominantly inherited rice resistance gene to Philippine race 1 of the bacterial blight pathogen *Xanthomonas oryzae* pv. *oryzae* in rice (Wang *et al.*, 2001). The rice gene *Xa4* encodes a wall-associated kinase and controls disease resistance and mechanical strength, possibly through a common mechanism. The other gene involved in the resistance of bacterial blight is *Xa5*. This R genes is grouped into a recessive and does not obey to one of the typical resistance gene structural classes (Iyer & McCouch, 2004). In the Aus-Boro group of rice *(Oryzae sativa* L.*)* varieties from the Bangladeshi region of Asia, x*a5* is a naturally occurring mutation and commonly found at which it is a recessive gene that encode the Y-subunit of transcription factor. The next resistance gene is *Xa7*, which provides dominant resistance against the pathogen with avirulence (Avr) gene Avr*Xa7* has significantly proved to be durably resistant to this disease (Mallikarjun & Kotasthane, 2018) and has a broad spectrum resistance. Other than that, the gene of *Xa7* has been determined in the study of Webb *et al.* (2010) where this resistance gene plays a vital role protecting the plant in high even though virulence of *Xoo* field populations increased. *Xa7* restricted disease more effectively at high than at low temperatures. Another R genes were less effective at high temperatures.

For the next resistance gene, *Xa1*0, the function of this gene *Xa1*0 confers race-specific disease resistance *to X. oryzae* pv. *oryzae* strains that deliver the corresponding transcription activator-like (TAL) effector Avr*Xa1*0. According to Mahmood & White (2019), the rice R genes *Xa1*0 has similar requirements for the transcription activation domain and nuclear localization sequence (NLS) motifs of the corresponding TALes for their induction. Likewise, in the study of Chu *et al.* (2006) *xa13* is a recessive which involved in the resistance against bacterial blight, one of the most devastating rice diseases worldwide, plays a key role in both disease resistance, pollen development and encode a novel plasma membrane protein. Suppressing expression of either the

dominant or recessive allele of x*a13* enhanced the resistance, but it caused male sterility of the plants and the product of this gene acted both as a bacterial growth dependent modulator and as an essential constituent of pollen development. Nevertheless, Ullah *et al.* (2012) said that *xa5* and *xa13* were confined to landraces only. Last gene used in this study is *Xa21*. *Xa21* is also acted as mediated resistance to bacterial blight (Liu *et al.*, 2012) and plays a crucial role in pathogen recognition. Furthermore, in the research of Xiang *et al.* (1998), the *Xa21* gene is successfully transferred to four elite Indica varieties and found significantly improved resistance to bacterial blight pathogen and this resistance is shows to be stably inherited. As to conclude that in a large scale and long term cultivation of varieties carrying only one single resistance gene will probably result in a significant shift of pathogen race frequency and causing breakdown of resistance in these cultivars (Mallikarjun & Kotasthane 2018).

CHAPTER 3. MATERIALS AND METHODS

3.1 Time and place conducting research

Research on the expression of resistance gene in Indonesia pigmented local rice varieties against bacterial leaf blight disease was conducted on October 2019 – June 2021 in the *Centre for Development of Advance Science and Technology (CDAST)*, University of Jember, Indonesia.

3.2 Materials

Five Indonesia pigmented local rice varieties were used in this study, including white, red, and black varieties (IR64, Ciherang, Inpari 24, Cempo Merah, and Hitam Bantul), micropipette and tip, mortar and pestle, eppendorf tubes, centrifuge machine, electrophoresis tools, Agarose, TBE or Tris/Borate/EDTA, DNA isolation equipment and buffers, Polymerase Chain Reaction (PCR), ReverTra Ace (Toyobo) kit, RNAprep pure kit (for plant) (Tiangen Biotech) and primer of specific target genes.

3.3 Preparation of Experiments

3.3.1 Seed germination

Seed imbibition was done in a tray for one week on all five rice varieties. The seeds were soaked for five minutes in distilled water, then in 70% alcohol for ten seconds before being rinsed with sterile distilled water. To prevent future contamination, a 70% alcohol solution was used as a seed treatment.

3.3.2 Growing seed

After one week of seed imbibition, all seedlings were transplanted into rice soil in containers. Each container was planted with 6 germinated seeds. The samples were kept in a greenhouse for three weeks. All of the growth requirements were met, resulting in a well-developed and uniform plant. There were five pigmented local rice

varieties that been used in this research where two varieties from red pigmented rice, two varieties from white pigmented rice and one variety from black pigmented rice.

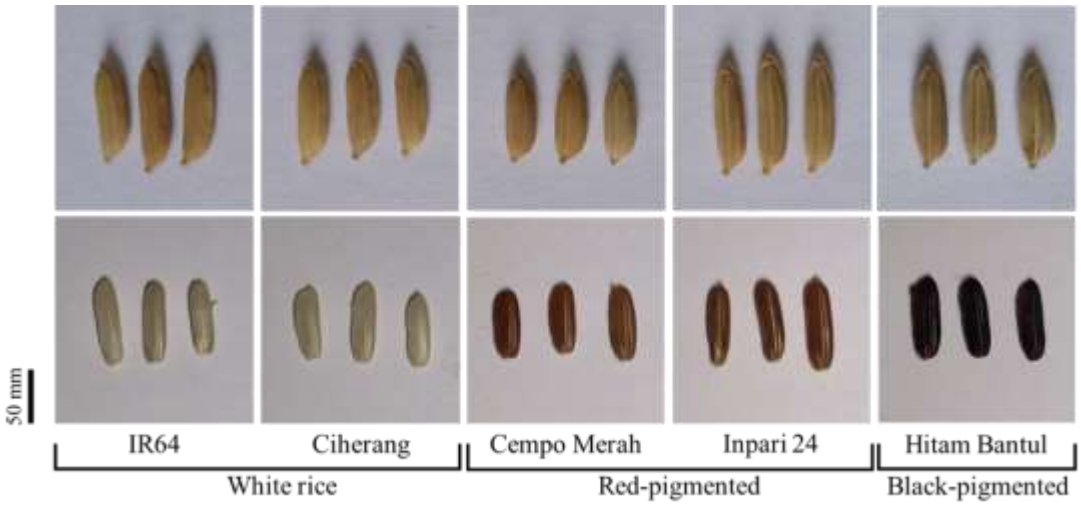


Figure 1. Five pigmented local rice varieties been used in this experiment.

3.3.3 Pathogen inoculum preparation

The *Xanthomonas oryzae* strain XooJ2 which obtained in the study of Rejeki *et al.* (2020) that had been isolated from Indonesian rice. Bacterial XooJ2 was cultured in the media of Yeast Dextrose Peptone Agar medium (YDPA) and Yeast Dextrose Carbonate Agar medim (YDCA). The bacterium was cultured in the agar media using streak plate method. The cultured media were then kept in the 28^{o}C incubator for 48 hours. A single colony of 48 hours old *X. oryzae* was taken and further cultured in the liquid of Yeast Dextrose Broth (YDB) using erlenmeyer flask. The bacteria was then kept in the shaker for 48 hours. After 48 hours, the optical density of *X. oryzae* was set until 1.0.

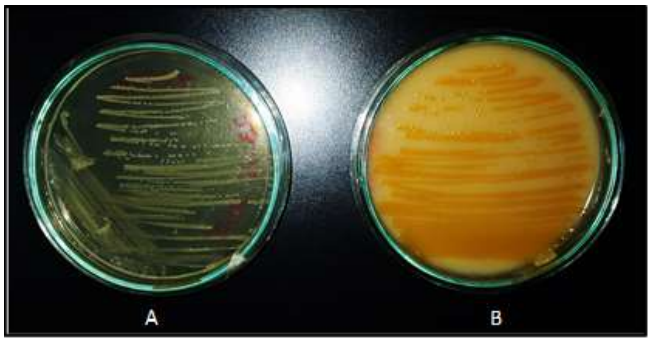


Figure 2. *Xanthomonas oryzae* strain XooJ2 were cultured in a media A and B where A) Yeast Dextrose Peptone Agar medium (YDPA); B) Yeast Dextrose Calcium Agar medium (YDCA) by using streak plate method. The bacterial cultures were left for 48 hours to grow.

3.4 Preparation of Experiments

3.4.1 Pathogen Inoculation

Xanthomonas oryzae pv. *oryzae* (*Xoo*) is a bacterium that cause the bacterial leaf blight disease in rice plant. In the pathogen inoculation method, leaf clipping method is the best way for inoculation method of *Xoo* (Kauffman *et al.,* 1973) at the booting stage. The five rice varieties were split into two groups (Non *Xoo*-inoculated and *Xoo*-inoculated). All leaves were cut at the tip using a sterile scissor as the entrance for bacteria invasion. Disease severity and disease incidence had been observed for 14 days after inoculation and recorded. To begin the pathogen inoculation process, the bacterial suspension was centrifuged and added with sterile distilled water before inoculation process. After bacterial preparation, inoculation process began with the rice plants were cut tip using sterile scissors that were dipped into bacterial suspension with optical density 1.0. The signs and symptoms of each plant were monitored and recorded for a period of 14 days (Figure 3).

3.4.2 DNA harbouring *Xa* genes

The DNA isolation was followed the protocol of Sambrook & Rusell (2001) Phenol:Chloroform with modification where the leaves of all seedling varieties were collected and weighted for about 2g for each variety and thus were grinded using

nitrogen liquid. 800 µl of lysis buffer was added in a centrifudge tubes containing samples. The samples were then vortex and incubated for 10 minutes at 65°C. The samples were incubated in -20°C for 1 hour and centrifuged at 10,000 rpm, 10 minutes, 20°C. The supernatant was then transferred into new centrifudge tubes and Phenol Chloroform Isoamyl (PCI) were added at the same amount of supernatant taken. Centrifuged at 10,000 rpm, 10 minutes, 20°C and again the supernatant were taken and transferred into a new centrifudge tubes. Sodium acetate was then added at 10% from the volume of transferred supernatant. After that, 1 ml of absolute ethanol was added into each tubes. The samples were then incubated in -20°C for 1 hour and centrifuged at 10,000 rpm, 10 minutes, 20°C. After centrifugation, the supernatant were then discarded and 500 µl ethanol 75% was added. All samples were centrifuged at 10,000 rpm, 10 minutes, 20°C. TE buffer was added after the samples were air dried. The DNA obtained can be kept at -20°C and until use.

After the DNA extraction done for all varieties, confirmation of all R genes on Indonesia pigmented local rice varieties were done. The purpose of this process to know the presence of the *Xa* gene(s) harbouring variety. The R genes involved were *Xa1, Xa3, Xa4, xa5, Xa7, Xa10, xa13* and *Xa21*.

3.4.3 Monitoring the *Xa* genes expression

Leaves were harvested three days after pathogen inoculation and used for RNA isolation. The 65 mg of frozen leaves were ground into a powder in a pre-cooled mortar with liquid nitrogen. The level of gene expression for each of the cultivars was determined using specific target genes after RNA (Ribonucleic Acid) was isolated. The isolation of RNA was carried out for all varieties three days after pathogen inoculation. The isolation of RNA was using RNAprep pure kit (for plant) (Tiangen Biotech). Firstly, 450 µl Buffer RL containing a final concentration of β-mercaptoethanol (β-ME) of 1% (added 10 µl β-ME to 1 ml of Buffer RL) into the crushed and homogenised sample using nitrogen liquid. The lysate was the transferred into the RNase-Free CS Filter Column placed in a 2 ml collection tube and centrifuged at 12,000 rpm for 2-5

minutes. The resulting supernatant was then transferred to a new centrifuge tube. Supernatant was used for the following. Then 0.5 volumes of ethanol (96-100%) was added to the cleaned lysate and mixed immediately with a pipette until homogeneous. After that, the samples were transferred to the RNase-Free Spin Column CR3 column placed in 2 ml collection tubes and centrifuged 12,000 rpm for 30-60 seconds. Discarded the flow-through. 350 µl of RW1 buffer was added to the CR3 spin column and centrifuged at 12,000 rpm for 30 - 60 seconds. Discarded the flow-through. Then the working solution of DNase I was prepared by adding 10 µl of DNase I solution to 70 µl of the RDD buffer and mix with the inverting of the tube slowly. DNase I work solution (80 µl) was added directly into the centre of the CR3 spin column and place it on the table (20 - 30ºC) for 15 minutes. Again, 350 µl of Buffer RW1 was added to spin column CR3. The lid was properly closed and centrifuge at 12,000 rpm for 30 - 60 seconds. Discarded the flow-through. 500 µl of Buffer RW was added to the CR3 spin column (ethanol had been added to the Buffer RW before used) then placed at room temperature for 2 minutes and centrifuge at 12,000 rpm for 30 - 60 seconds. Discarded the flow-through. Step of adding 500 µl RW Buffer to the CR3 spin column until centrifuged was repeated once. Discarded the flow-through. Then centrifuge at 12,000 rpm for 2 minutes to dry the spin column membrane. Next, placed the CR3 spin column in a new 1.5 ml collection tab. Finally, 50 µl of RNase-Free water was added directly to the spin column membrane and placed it at room temperature for 2 minutes and centrifuged at 12,000 rpm for 2 minutes to elute RNA. Purified RNA was stored at -80ºC.

3.4.4 cDNA conversion

The Reverse-Transcription Polymerase Chain Reaction (RT-PCR) was widely used to measure mRNA expression and convert mRNA to complementary DNA (cDNA) (Freeman *et al.,* 1999). Furthermore, RNA analysis using Reverse-Transcription Polymerase Chain Reaction (RT-PCR) was a sensitive and powerful technology. With a total volume of 20 µl, the ReverTra Ace (Toyobo, Japan) kit was

used to synthesise cDNA from mRNA. The amount of 5x RT master mix, nuclease-free water, and RNA template added to each tube varied between varieties and RT-PCR was performed at 37°C for 1 hour, 98°C for 5 minutes, and 4°C for 10 minutes. The results of the RT-PCR were then used to carry out a PCR with a specific target genes (Table 2).

3.4.5 Detection of *Xa* genes

According Handoyo & Rudiretna (2000), Polymerase Chain Reaction (PCR) is an in vitro method for amplifying a specific DNA region without transferring it into living cells. It's a powerful approach since it can reach a million-fold amplification in just a few hours. Eight resistance genes were used as some specific target genes which were *Xa1, Xa3, Xa4, xa5, Xa7, Xa10, xa13* and *Xa21* (Table 2). (1) Pre-denaturation at 94ºC for 3 minutes; (2) Denaturation at 94ºC for 1 minute; (3) Annealing at a temperature calibrated to the primer used for 1 minute based on Table 2; (4) Elongation at 72º C for 1 minute; (5) Post-elongation at 72º C for 10 minutes. After all, the PCR results were visualised through electrophoresis and Gel Imaging System (GelDoc) and gene expression was determined by examining the DNA bands under UV transilluminator in the gel documentation CCD image system (Major Science, Saratoga, CA, USA).

3.5 Experimental data collection

3.5.1 Disease Assessment

The symptoms of BLB was assessed from day 0, 7, and 14 days after pathogen inoculation. For disease severity, the length (cm) of lesion was observed and recorded on the day 7 and day 14 after pathogen inoculation for all cultivars. In addition, the

disease incidence was aim to evaluate the percentage of BLB disease after the pathogen invasion. The disease incidence of all varieties were calculated using this formula (Rasmiyana *et al.*, 2019):

Disease Incidence:

$$\text{DI} = \frac{n}{N} \times 100\%$$

DI : Disease Incidence,
n : Number of symptomatic plant,
N : Total number of plant observed. .

After calculating the disease severity and disease incidence, all the cultivars were categorised according to International Rice Research Institute (IRRI, 2013) as following. By measuring the length of the lesion, the disease was evaluated using the standard evaluation method (SES) defined by the International Rice Research Institute (IRRI 2013). Plants were classified as resistant if the average lesion length was less than 3.0 cm, moderately resistant if it was between 3.0 and 6.0 cm, moderately susceptible if it was between 6.0 and 9.0 cm, and susceptible if it was greater than 9.0 cm.

Table 1. The disease severity and disease incidence responses.

Disease severity		**Disease Incidence**	
Response	Size of lesion	Response	Percentage
Resistant	≤ 5 cm	Highly resistant	0 - 3 %
Medium resistant	5 – 10 cm	Resistant	4 – 6 %
Medium susceptible	10 – 15 cm	Medium resistant	7 – 12 %
Susceptible	≥ 15 cm	Medium susceptible	13 – 50 %
		Susceptible	51 – 75 %
		Highly susceptible	≥ 75%

Table 2. Resistance genes target, sequences of primer, annealing temperatures, and product sizes.

No.	Target R genes	Primer sequences	Annealing (°C)	Product size (bp)
1	*Xa1*	F: 5'-ACTGCCCTCTTGCACACGCCTTTGG-3' R:5'-CCGGTACATCAGTATTGTCCATCGG-3'	66	447
2	*Xa3*	F:5'CCACAATGCCATGTCAGGTGGCATCCCTGCA-3' R: 5'-AGGTGTTGGAGGATTGGCAT-3'	67	255
3	*Xa4*	F: 5'- ATCGATCGATCTTCACGAGG3' R : 5'-TGCTATAAAAGGCATTCGG-3'	53	150
4	*Xa7*	F:5'-CGATCTTACTGGCTCTGCAACTCTGT-3' R :5'-GCATGTCTGTGTCGATTCGTCCGTACGA-3'	65	1,170 or 294
5	*Xa10*	F: 5'-CAACGCCTATCTTCTGCATTTC-3' R :5'-GTGACCCTAGTTTCTGGTTATG-3'	53	604
6	*xa5*	F:5'-AGCTCGCCATTCAAGTTCTTGAG-3' R :5'-TGACTTGGTTCTCCAAGGCTT-3'	57	200, 300, or 400
7	*xa13*	F:5'-GGCCATGGCTCAGTGTTTAT-3' R :5'-GAGCTCCAGCTCTCCAAATG-3'	55	400 or 200
8	*Xa21*	F: 5'-CGATCGGTATAACAGCAAAAC-3' R :5'-ATAGCAACTGATTGCTTGG-3'	50	1400

3.6 Data analysis

After the preparation of experiment, the data were collected from the genomic DNA and RNA isolation and visualisation. Also, the calculation of length of lesion for day 7 and day 14 (Figure 4) post-pathogen inoculation and calculation of disease incidence using the formula. In this study, the data analysis were in the form of descriptive data and bar graph was from the calculation of disease severity.

CHAPTER 4. RESULTS AND DISCUSSION

4.1 Bacterial leaf blight disease assessment on pigmented local rice varieties

The disease assessment was conducted to examine the severity and the disease incidence for all pigmented local rice varieties. The seedlings began to show signs and symptoms of bacterial infection starting from 7 days after pathogen inoculation. After the inoculation process, the slightly symptom can be seen on day three. A grayish lesion formed as a symptom and the infected leaves started drying (Figure 3). The data for disease severity (Figure 4) and disease incidence (Table 3) were calculated and recorded.

Figure 3. The pathogen symptoms on day 14 after pathogen inoculation. The grayish lesion began from the cut tip and spread across the entire leaf and plant.

The disease severity was analysed and data was collected in the Figure 4. The lesion of the 3 weeks seedlings were calculated on day 7 and day 14 after pathogen inoculation. The purpose for the disease evaluation which act as a vital measure for determining disease severity and as a result, can be used to estimate yield and make treatment recommendations. The disease incidence was calculated using formula (Rasmiyana *et al.*, 2019).

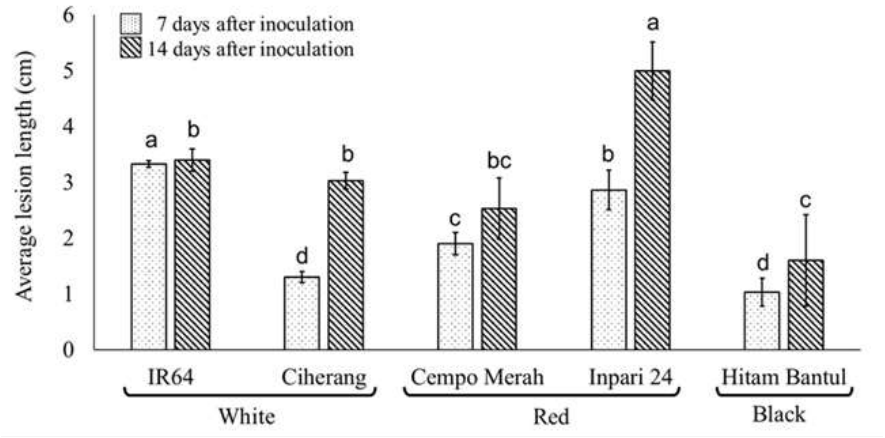


Figure 4. The lesion lengths were measured at 7 and 14 days post-inoculation (B). The error bars represent the standard deviation of the lesion lengths repeated for 10 leaves. Not significantly different (Duncan Multiple Range Test) among the same observation indicated by the notation above the same shaded-bar indicated.

According to the following bar graph (Figure 4), cultivar Inpari 24 had the highest disease severity on the 14th day after pathogen inoculation, followed by IR64, Ciherang, Cempo Merah, and Hitam Bantul. Ciherang cultivar showed a very rapid increased in disease severity on day 7 after pathogen inoculation, whereas Cempo Merah and Hitam Bantul cultivars showed a comparatively moderate increased in disease severity from day 7 to 14.

Table 3. The cultivars, resistance genes, disease severity, disease incidence and responses.

No.	Cultivars	Resistance genes exhibited	Disease severity (cm)	Response	Disease Incidence (%)	Response
1	IR64	*Xa4, Xa10, xa13, Xa21*	5.7	MR	61.4	S
2	Ciherang	*Xa4, Xa10, xa13*	3.0	R	22.7	MS
3	Cempo Merah	*Xa10, xa13*	2.5	R	42.3	MS
4	Inpari 24	*Xa1, Xa4, Xa7, Xa10, xa13*	4.8	R	42.9	MS
5	Hitam Bantul	*Xa4, Xa10, xa13*	1.6	R	50.0	MS

*Key: R: resistance; MR: medium resistance; S: susceptible; MS: medium susceptible

Table 3 showed the resistance genes exhibited after pathogen inoculation.IR64 cultivar exhibited four *Xa* genes post-inoculation and had a response of medium resistance in disease severity and susceptible in disease incident however the rest of the cultivars were categorised as resistance in disease severity and medium susceptible in disease incidence. *Xa4, Xa10* and *xa13* genes were contained in all cultivars.

4.2 Presence of *Xa* related genes on pigmented local rice varieties

DNA isolation and extraction on three week seedlings were used to evaluate the presence of *Xa* genes in each of the five cultivars. Only the existing *Xa* genes will be further analysed in the *Xa* genes profiling because not all *Xa* resistance genes were present in all varieties. The DNA for all cultivars were analysed using PCR with all the stated annealing temperatures in Table 1. Moreover, the PCR analysis were then visualised under UV transilluminator using Gel Imaging System (Major Science, Saratoga, CA, USA).

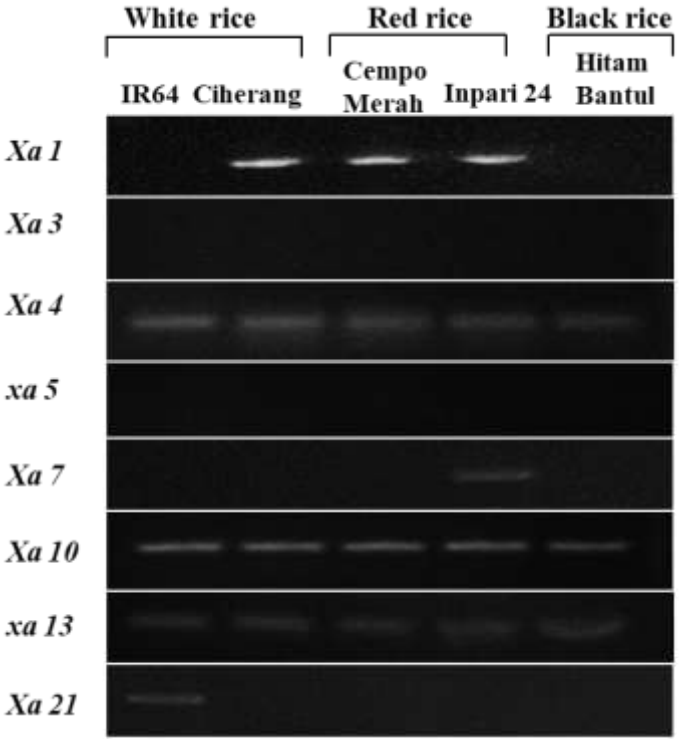


Figure 5. The result of PCR analysis in DNA isolation and extraction for all pigmented varieties using the specific target genes.

Only cultivar Inpari 24 (*Xa1, Xa4, Xa7, Xa10* and *xa13*) carried five *Xa* resistance genes, while cultivar Hitam Bantul had three (*Xa4, Xa10* and *xa13*). Four *Xa* resistance genes were found in the other cultivars. Resistance genes *Xa4, Xa10* and *xa13* were found in all five cultivars, while *Xa3* and *xa5* were not. *Xa21* resistance genes were only found in IR64. In RNA isolation and extraction, the existing *Xa* genes in each variety were examined for their expression.

4.3 The expression of *Xa* genes after pathogen inoculation

The *Xa* gene analysis was perform as a gene profiling to evaluate the expression of the resistant gene(s) with correlation to the *Xa* gene confirmation from DNA. The results of DNA from PCR in *Xa* genes detection were used to undertake the *Xa* genes analysis. Because RNA is a byproduct of the transcription and translation of DNA, only

a few alterations were done for each specific target gene(s) based on the PCR result in Figure 5.

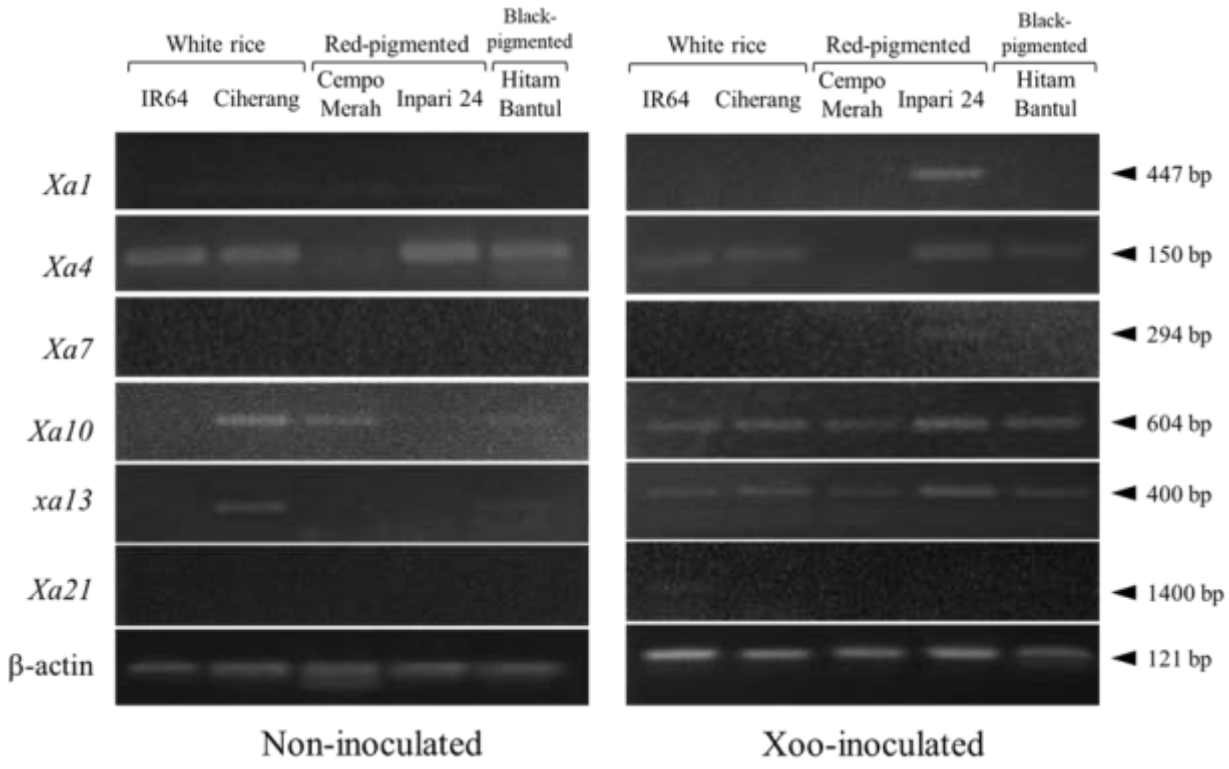


Figure 6. Agarose gel electrophoresis of RT-PCR products from the total RNA of five (white, red-pigmented, and black-pigmented) rice varieties to detect the qualitative expression of *X. oryzae* pv. *oryzae* resistance-related genes three days after *Xoo* inoculation using specific *Xa* genes primers (listed in Table 1). White rice varieties used in this study were IR64 (lane 1) and Ciherang (lane 2); red rice, Cempo Merah (lane 3) and Inpari 24 (lane 4); black rice, Hitam Bantul (lane 5). The non-inoculated leaf treatment was sterile water.

Because there was no DNA band for genes *Xa3* and x*a5* during DNA analysis in Figure 5, none were presented in this figure. Each cultivar had a different level of expression, as shown in the figure above. Actin was utilised as a consecutive gene because its expression was unaffected by any of the conditions. Cultivar Inpari 24 expressed five *Xa* genes which were *Xa1, Xa4, Xa7, Xa10* and *xa13* while IR64

expressed four *Xa* genes (*Xa4, Xa10, xa13, Xa21*), Ciherang and Hitam Bantul had the expression of three *Xa* genes which were *Xa4, Xa10* and *xa13*. In contrast, cultivar Cempo Merah only expressed two *Xa* genes which were *Xa10* and *xa13.*

4.4 Discussion

Rice bacterial blight is a lethal bacterial disease that is one of the most damaging diseases to cultivated rice. Bacterial leaf blight (BLB), caused by the bacteria *Xanthomonas oryzae* (*Xoo*), is one of the most significant diseases of rice, affecting productivity in irrigated and rain-fed lowland ecosystems across the world. According to Yuliani *et al.* (2014), Kresek was a symptom that appeared when a plant in the vegetative phase or which less than 30 days old after planting, however the blight symptoms appear in generative phase plants. The leaves turn grey, folded, rolled, and finally dried as a result of Kresek symptoms and blight. Plant photosynthetic capacity was diminished as a result of leaf damage and the grain filling process was disrupted, resulting in an empty grain production. Study conducted by Fatimah *et al*. (2021) stated that in the rice plant during the vegetative phase, the BLB disease intensity was higher than in the generative phase, indicating that the vegetative phase was more susceptible.

The symptoms of BLB disease could be seen on the leaves and when inoculated during the seedling or vegetative phase, the leaf tissue was still relatively immature, allowing pathogens to easily spread and symptoms to appear very quickly. Moreover, the severity of the disease implies that the disease progresses more quickly at the younger the plant. Otherwise, as rice plants mature, the *Xoo* pathogen spreads more slowly, slowing the rate of infection (Khaeruni *et al.*, 2014). Rice genotypes influence disease progression; the more resistant a plant is, the less severe the disease develops and the disease progresses will become slower. However, sunlight and temperature at which the disease develops, the genetic of cultivar, inoculum concentration and the pathogen pathotypes were the most important elements influencing plant resistance to

a pathogen. Climate and favourable environment affect pests and pathogens' life also the population to attack on rice fields, according to Somantri (2016).

The development of BLB disease was fast in the vegetative growth phase, which occurs 5 – 8 weeks after transplantation, compared to the other growth phases, however the disease slows or stops in the generative growth phase (Khaeruni *et al.*, 2014). In addition, the wax layer and cuticle thickness on plant cells epidermis may boost plant resistance to diseases penetrating directly through the epidermal layer. Low disease severity during the generative phase could be linked to the plant's cell structure, as well as the concentration of a secondary metabolic compound generated by the plant, which varies depending on the plant's age. However, studies were stated that rice cultivars were very vulnerable during the vegetative phase whereas highly resistance rice cultivars would withstand and minimised its severity.

The percentage of relevant host tissues or organs that were covered by a symptom or size of lesion infected by the disease was referred as disease severity. According to Pharmawati & Wrasiati (2018) stated that plants under biotic stress will perform molecular, biochemical, physiological, anatomical and morphological adaptations. Wilting, yellowing and drying of leaves were the morphological responses of plants to BLB. When the leaves dried out and died, they developed distinctive grey white lesions that signal the ending of the infection. Bacterial leaf blight usually kills infected seedlings within two to three weeks of infection; adult plants could survive, but rice yield and quality degraded (Madden & Hughes, 1999). In this study showed that cultivars IR64 (white rice) and Inpari 24 (red rice) had longer lesions compared to the other three cultivars (Figure 3) of 5.7 cm and 4.8cm respectively.

Based on Figure 4, lesion length for disease severity were taken on days seven and fourteen post-inoculation. Cultivar IR64, Inpari 24 and Ciherang all showed a significantly increased in lesion size. The pigmented cultivars possessed the lowest disease severity. In this study found that Inpari 24 cultivar possessed five *Xa* genes (Figure 5) and exhibited all *Xa* genes (Figure 6) when treated with *Xoo* pathogen. Theoretically, rice plants with more *Xa* genes in one crop have stronger resistance,

therefore plant resistance can be longer, according to Swamy *et al.* (2006). According to Van der Plank (1996), the rate at which disease spreads were slowed by horizontal resistance otherwise for vertical resistance, it minimized the amount of inoculum used to start an epidemic. In this study showed that the average lesion of Inpari 24 was above the average and had the highest lesion length even though it possessed 5 *Xa* genes after *Xoo* inoculation. Therefore, Inpari 24 had a possibility that it lacks of vertical genes that helped to reduce the amount of *Xoo* inoculum. Vertical resistance refers to a type of resistance found in plant varieties that was effective against some pathogen races but not others. As a result, vertical resistance was extremely specific. Pathotype specificity in vertical resistance means that the host has a gene for vertical resistance that was only attacked by pathotypes that have a virulent gene directed at that resistance gene. Vertical resistance in plants were unstable and less durable. Also, while Inpari 24 and IR64 had 5 and 4 *Xa* genes respectively, those cultivars lacked vertical genes that inhibited the *Xoo* inoculum, and the vertical genes were easily influenced by the environment. Cultivars that have lost their horizontal resistance were more susceptible to new strains. In the pathogen population, the frequency of virulence genes capable of breaking host resistance that increased in recent years.

In addition, Inpari 24 cultivar had been commercially and widely grown since 2012, as indicated by Yuliani & Rohaeni (2017) a long-term growth of the same variety was not recommended since it can accelerate the resistance breakdown and induced the production of more new virulent *Xoo* pathotypes. Selection from cultivars resistant to certain pathotype was thought to produce virulent *Xoo* pathotypes that were compatible with their hosts. In a study conducted by Khaeruni *et al.* (2016) said that some *Xa* genes that are easily broken by certain *Xoo* phathotypes, for example *Xoo* pathotype IV have virulence genes that can break resistance *genes Xa1, Xa2, Xa4, Xa7, Xa10, Xa11, and Xa4* in rice plants, pathotype VIII can break the resistance *genes Xa1, Xa2, Xa3, Xa4, Xa7, Xa10, Xa11* and *Xa14* while pathotype X can break the resistance *genes Xa1, Xa2, Xa4, Xa7, Xa10, Xa11, Xa14, and Xa21. Xoo* had a great ability to evolve into a new strain rapidly.

Environmental factors such as seasonal fluctuations and the presence of resistance genes in rice plants could impact the population structure of *Xoo* (Joko *et al.*, 2019). The resistance of each rice crop was influenced by its genotype, environment and ability to undergo second metabolism. Table 3 showed that only IR64 had that categorised in medium resistance in disease severity and showed susceptible response in disease incidence. In contrary, the rest of the cultivars were considered resistant in disease severity and medium susceptible disease incidence. Cultivar IR64 had the most severe symptoms of BLB (Fatimah *et al.*, 2021) where in susceptible plants, pathogen detection could be blocked or the response was too slow, allowing pathogens to spread quickly and cause extensive symptoms (Aderem and Ulevitch 2000). In disease severity, Hitam Bantul cultivar had the lowest disease severity. Interestingly, in the study conducted by Susanto *et al.* (2018) stated that many local black rice varieties exist in Indonesia, which can be used as a genetic resource to help breeding programs to produce resistant cultivars. Screening for BLB resistant black rice cultivars could provide genetic resources for breeding to improve BLB resistance. According to Khaeruni *et al.* (2016), a plant has an ability to produce phytoalexins, which limit bacterial growth, makes it resistant to BLB disease. High amounts of phytoalexins can limit the area where pathogens can infect. Rice plants have a resistant response to BLB disease because they quickly develop the phytoalexin sakuratenin, which was a toxic to plant pathogens. Phytoalexin chemicals somehow may help to prevent bacterial leaf blight.

Transcription is the process of turning DNA into RNA, while translation is the process of turning RNA into proteins. According to Grosjean (2009) cells read out or express the genetic instructions in their genes through transcription and translation. In DNA transcription which shares certain similarities with DNA replication, produces all of the RNA in a cell. Certain *Xa* genes were done for the process of PCR in *Xa* gene profiling, as shown in the RNA expression result (Figure 6). This was due to the fact that transcription could only take place when DNA was present in specific target genes. On the other hand, when stress attacked, specific resistance genes were produced

causing the plant to engage the second metabolism. As the second metabolism begins, specifically resistance genes will express as a defense strategy to keep the plants from dying.

The expression of all *Xa* genes were highly visible in Figure 6. Throughout the Figure 6 of gene expression, there were only *Xa4, Xa10* and *xa13* genes were exhibited the DNA band while for *Xa1*, *Xa7* and *Xa21* genes were only exhibited in certain cultivars. Recorded in the study of Susanto & Sudir (2015), *Xoo* had the potential to create new strains (pathotypes) that can break down the resistance and compatible to rice plant. In this study shown that *Xa1* was expressed only in Inpari 24 cultivars while other cultivars were lacks of *Xa1* either in *Xoo*-inoculated and non *Xoo*-inoculated. *Xa1* was involved in pathogen recognition and improving the efficacy of interactions with the avr gene (Rasmiyana *et al.*, 2019). *Xa1* were existed in Ciherang and Cempo Merah as well as Inpari 24 in Figure 5 however only Inpari 24 exhibited *Xa1* after pathogen inoculation. Expression of resistant genes influence by several factors; this was corresponding in the study of Yuriah *et al.* (2016), the genotype of the host plant, the virulence of the *Xoo* race and environmental factors all determine the intensity of *Xoo* attacks. Generally, Inpari 24 cultivar resistant to pathotype III bacterial leaf blight, moderately resistant to pathotype IV, and susceptible to pathotype VIII. Moreover, there was a positive expression in *Xa7* (Figure 6) exhibited by Inpari 24 cultivar. According to Setiawan & Purwestri (2021) each *Xa* gene produces different resistance to each *Xoo* pathotype for instance *Xa7* gene at which this gene responsible in giving a long lasting resistant (durable), broad spectrum and withstand in extreme condition. There was a positive response in IR64 where it exhibited *Xa21* after *Xoo* inoculation. The *Xa21* gene was found to be one of the most efficient gene against *Xoo* resistance in a study conducted by Swamy *et al.* (2006). Yoshimura *et al* (1998) reported that *Xa21* could be ineffective against *Xoo* if protein fail to recognize specific pathogen ligands for defense response activation. The *Xa21* gene confers resistance to *Xoo* races III and VIII pathogens, as well as a moderate level of resistance to race IV pathogens

(Yuriah *et al.,* 2016). Moreover, the dynamics of *Xoo* pathotype changes in the field were rapid, genes that were previously resistant to BLB can be disrupted in a short period of time. The genes *xa5, Xa7* and *Xa21* were initially effective against *Xoo*, according to Setiawan & Purwesti (2021), but a new strain of *Xoo* evolved that was able to overcome and break the combination of the three resistance genes. This indicates that the *Xoo* virulence strain was becoming more diverse and required to develop specific rice breeding strategies, particularly those related to BLB resistance, so that rice genotypes with greater resistance to changes in the *Xoo* strain population in the field can be obtained.

The present of resistant genes were depends on their genotype. *Xa3* responsible in recognising the Avr protein carried by *Xoo* and triggered the plant response while *xa5* had a function in encoding the Y-subunit of transcription IIA. Plants had inherited their resistant gene from their parents and these R genes were controlled by R protein in plants (Yuliani & Rohaeni, 2017). Throughout this study found that all cultivars were absent of *Xa3* and *xa5* respectively. According to Yuriah *et al.* (2016) gene pyramiding enables the cultivars with greater resistance characteristics, making them more successful in combating pathogen attacks that enhance their pathogenic characteristics over time. Nowadays, gene pyramiding was widely used to produce more than one *Xa* gene in one variety. However, not all gene pyramiding effects showed a positive response to the resistance level. Gene pyramiding was one of the technique to produce rice plants with greater BLB resistance where cultivars that had been demonstrated to be resistant should be propagated and monitored to anticipate changes in the pathotype and genotype of the local *Xoo* population to enhance the effectiveness and longevity of single and pyramiding *Xa* genes. Although *xa5* and *Xa21* showed long-lasting resistance, broad-spectrum resistance in Taiwan, virulent strains have evolved rapidly in the *Xoo* population (Yuliani & Rohaeni, 2018).

Throughout Figure 6, the expression of were highly *Xa4*, *Xa10* and *xa13* visible in most of the cultivars. In *Xa4*, the band were clearly visible in both inoculated plants however, only Cempo Merah lacks of *Xa4* in both non *Xoo*-inoculated and *Xoo*-inoculated plants. Inoculated rice had lower quality *Xa4* expression, indicating loss of expression (Figure 6). This study indicates that following pathogen inoculation, the cell wall was weakened. Because *Xa4* was linked to cell wall production, its expression will make cell walls stronger (Hu *et al.*, 2017). According to Gonzales *et al.* (2012), *Xoo* causes disease symptoms on rice leaves by releasing enzymes via the type two secretion system (T2SS). Almost all rice crops which particularly Asian rice rely on the *Xa4* gene to retain resistance (Chen *et al.* 2001; Jiang *et al.* 2004; Luo *et al*. 2005; Zhang *et al.* 2006; Luo *et al.* 2012). Moreover, *Xa4* resistance gene was most widely used in breeding programs because it had a long-lasting effect in many rice cultivars. Rasmiyana *et al.* (2019) found that *Xa4* gene could increase in number of compatible reaction between host and also pathogen. In this case, the *Xa4* resistance gene for the Cempo Merah cultivar was likely to have been broken by the rapidly evolving *Xoo* pathogen. If the conditions were favourable, the pathogen will attack cultivars with high resistant traits and thus cause economic damage. The R gene does not disappear or change; it only loses its economic value (Setiawan & Purwesti, 2021). The expression of *Xa10* were clearly visible in *Xoo*-inoculated plants while only Ciherang, Cempo Merah and Hitam Bantul expressed *Xa10* in non-*Xoo* inoculated plants. *Xa10* was a gene for an executor R protein that promotes disease resistance by triggering the hypersensitive response (Wang *et al.*, 2017). Avr*Xa10* from pathogen interacts to the effector binding site in the R gene promoter and activates its expression in response to bacterial infection. According to Susanto & Sudir (2015), the *Xa4* and *xa5* genes can produce a resistant response to BLB strain III, but must be in the right combination of gene. The *Xa10* gene had a different mechanism of resistance than the *Xa4* and *xa5* genes, so that the rotation of varieties that have these different genes can provide a longer resistance (durable). A rice genotype with a pathotype *Xoo* resistant gene does not eliminate the possibility of infection; *Xoo* can infect but does not produce severe

BLB disease in that genotype (Susanto & Sudir, 2015). The result of gene expression found that all cultivars exhibited resistant gene *xa13* when inoculated with pathogen *Xoo*. The *xa13* gene was completely recessive, with resistance conferred only when homozygous. *xa13* responsible to encodes a plasma membrane protein that varies by one amino acid from the dominant (susceptible) allele *xa13*, which mediates race-specific resistance *to Xanthomonas oryzae* pv. *oryzae* (*Xoo*). In Figure 6 clearly showed that there was a positive increase of expression in *Xa10* for IR64 and Inpari 24 in *Xoo*-inoculated plants after the pathogen inoculation. Meanwhile in *xa13* there was also a positive increased of expression for cultivar IR64, Cempo Merah, Inpari 24 and Hitam Bantul in *Xoo*-inoculated cultivars where *xa13* resistant gene was exhibited when stress by *Xoo* pathogen occurred. The positive response in gene expression was visibly seen in *Xa1*, *Xa10* and *xa13*. In contrast, there were several resistance genes, such as *Xa1* (Ciherang and Cempo Merah) and *Xa4* (Cempo Merah) had a high possibility that the R genes were broken by *Xoo*, as shown in Figure 6. These genes were not expressed either *Xoo*-inoculated or non *Xoo*-inoculated plants.

The R protein induced the particular resistant genes in order to protect the plant from severe infection. However, the virulence of *Xoo* was determined by two key factors: a host plant and favourable environment. When one of these components changes, it triggers and influences the pathogen's pathogenicity. In plants, there were two layers to the plant defense system. Basal resistance (basal immunity) was the first layer, which was the phase of plant identification of pathogenic molecules. The second layer reacts to pathogen virulence factors in the presence of a plant resistance gene (R gene) (Dangl & Jones, 2001). However, the plant's first reaction when infected with a disease was to try to recognise the "foreign substance" using pathogen pattern recognition molecules (pathogen associated molecular-pattern = PAMP). The elicitor recognised subsequently activated plant resistance mechanisms such as induce higher ROS activity and expression of resistant genes without causing tissue death when endurance was affected (Boller & Felix, 2009).

Each *Xa* gene in plants confers different resistance to each *Xoo* pathotype. The bacterium *Xoo* that cause bacterial leaf blight adapt quickly and rice types that were resistant to BLB only last for six growing seasons (Yuriah *et al.,* 2016). Moreover, *Xoo* pathogens can spread between regions in addition to pathotype evolution. Despite regional differences in pathogen populations, Yuliani & Rohaeni (2018) found that the main strains in a collection of pathogens from Indonesia and the Philippines were closely related. This demonstrates that diseases can spread across regions as a result of germplasm exchange. *Xoo* infections were seed-borne pathogens that can survive when transplanted to a new location. Planting resistant cultivars was such an important part in reducing the risk of BLB disease. To anticipate changes in *Xoo* strains and to ensure that resistant varieties last longer, rotation of resistant cultivars should be considered (Susanto & Sudir, 2015). Planting the same varieties without improving their genotype, on the other hand, will result in the emergence of new strains of the *Xoo* pathogen. As a result, even though some rice have a higher resistance genes, the symptoms are however more severe than in rice with a lower resistance gene. So when a pathogen was inoculated, RT PCR will identify all of the genes but this was only for detection of active gene expression, however additional steps must be taken to ensure that the genes will develop resistance pathways or not during *Xoo* infection.

CHAPTER 5. CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

In a nutshell, the induction of pathogenic stress by the bacterium *Xoo* resulted in a positive increase exhibited in *Xa1*, *Xa7*, *xa13* and *Xa10* for some cultivars, whereas there were some other cultivars which their resistant genes were possibly be broken by virulent *Xoo* such as *Xa1, Xa4* and *Xa21*. Environmental conditions and host plants had a huge impact on phythotypes of virulent *Xoo* to evolve. In the case of the Inpari 24 cultivar, which had a high resistance gene yet also had a high disease severity, the resistance gene may have been successfully broken by *Xoo,* which was becoming more virulent. Finally, this study found that the quantity of resistance genes in plants does not guarantee the disease resistance.

5.2 Recommendations

My suggestion to future researchers is to look into the level of gene expression in *Xanthomonas oryzae* bacteria that are 24 hours old, 48 hours old and 96 hours old and infect the pigmented rice cultivars as well as white rice, because there are very limited study on the level of gene expression in different ages of *Xanthomonas oryzae* bacteria. In addition, I propose that study be conducted to see if specific resistance genes are expressed differently depending on age and growth phase. This is because some genes in this study are not expressed during the vegetative phase, but other studies have shown that genes that are not expressed during the vegetative phase are expressed during the generative phase.

REFERENCES

Aderem A, Ulevitch RJ (2000) Toll-Like Receptors in the Induction of the Innate Immune Response. Nature 406(6797):782–787

Boller, T., & Felix, G. (2009). A Renaissance of Elicitors: Perception of Microbe-Associated Molecular Patterns and Danger Signals by Pattern-Recognition Receptors. *Annual review of plant biology*, 60, 379-406.

Chae, J. C., Hung, N. B., Yu, S. M., Lee, H. K., & Lee, Y. H. (2014). Diversity of Bcteriophages infecting *Xanthomonas oryzae* pv. *oryzae* in Rice Fields and its Potential to Control Bacterial Leaf blight of Rice. *J Microbiol Biotechnol*, 24(6), 740-747.

Chen S, Xu C, Lin X, Zhang Q (2001) Improving Bacterial Blight Resistance of '6078', an Elite Restorer Line of Hybrid Rice, by *Molecular Marker-Assisted Selection. Plant Breed* 120:133–137

Chen, X., Liu, P., Mei, L., He, X., Chen, L., Liu, H., & Ma, B. (2021). *Xa7*, a new executor R gene that confers durable and broad-spectrum resistance to bacterial blight disease in rice. *Plant communications*, 2(3), 100143

Chu, Z., Yuan, M., Yao, J., Ge, X., Yuan, B., Xu, C., & Zhang, Q. (2006). Promoter Mutations of an Essential Gene for Pollen Development Result in Disease Resistance in Rice. *Genes & Development*, 20(10), 1250-1255.

Dangl, J. L., & Jones, J. D. (2001). Plant Pathogens and Integrated Defence Responses to Infection Nature, 411(6839), 826-833.

Deepak, S. A., Kottapalli, K. R., Rakwal, R., Oros, G., Rangappa, K. S., Iwahashi, H., & Agrawal, G. K. (2007). Real-Time PCR: Revolutionizing Detection and Expression Analysis of Genes. *Current Genomics*, 8(4), 234-251.

Disease- And Pest- Resistant Rice. (2019, January 30). Retrieved From https://www.irri.org/disease-and-pest-resistant-rice.

Fatimah, F., Rangkuti, S. M. S., Mustopa, A. Z., & Suryani, S. (2021, May). Molecular Detection of *Xanthomonas oryzae* pv. *oryzae* from West Java and in Vivo Pathogenicity Test. In *AIP Conference Proceedings* (Vol. 2353, No. 1, P. 030088). *AIP Publishing LLC.*

Freeman, W. M., Walker, S. J., & Vrana, K. E. (1999). Quantitative RT-PCR: Pitfalls and Potential. *Biotechniques*, 26(1), 112-125.

González JF, Degrassi G, Devescovi G, De Vleesschauwer D, Höfte M, Myers MP, Venturi V. 2012. A proteomic study of *Xanthomonas oryzae* pv. *oryzae* in rice xylem sap. J *Proteomics*. 75(18):5911-5919. doi: 10.1016/j.jprot.2012.07.019.

Grosjean, H. (2009). DNA and RNA Modification Enzymes: Structure, Mechanism, Function and Evolution. Crc Press.

Gu, K., Sangha, J. S., Li, Y., & Yin, Z. (2008). High-Resolution Genetic Mapping of Bacterial Blight Resistance Gene *Xa10*. *Theoretical and Applied Genetics*, *116*(2), 155-163.

Handoyo, D., & Rudiretna, A. (2000). Prinsip Umum dan Pelaksanaan Polymerase Chain Reaction (PCR) [General Principles and Implementation of Polymerase Chain Reaction]. *Unitas*, 9(1), 17-29.

Hasanah, N. A. I., Setiawan, B. I., Arif, C., & Widodo, S. Sri (2019) Rice Growth Simple Prediction Model.

Iyer, A. S., & Mccouch, S. R. (2004). The Rice Bacterial Blight Resistance Gene *Xa5* Encodes a Novel Form of Disease Resistance. *Molecular Plant-Microbe Interactions*, 17(12), 1348-1354.

Jiang G, Xu C, Tu J, Li X, He Y, Zhang Q (2004) Pyramiding of Insect- and Disease-Resistance Genes into an Elite Indica, Cytoplasm Male Sterile Restorer Line of Rice, 'Minghui 63'. *Plant Breed* 123:112–116

Joko, T., Kristamtini, K., Sumarno, S., & Andriyanto (2019), R. The Resistance of Local Pigmented Rice Varieties against Bacterial Leaf Blight. *Jurnal Perlindungan Tanaman Indonesia*, 23(2), 205-210.

Kahar, M. A., Mutalib, Sofianita & Abdul-Rahman, Shuzlina (2015). Early Detection and Classification of Rice Diseases with Neural Networks and Fuzzy Logic. In Proceedings of the 17th International Conference on Mathematical and Computational Methods in Science and Engineering, Macmese (Pp. 248-257).

Kauffman, H.E., A.P.K. Reddy, S.P.Y. Hsieh and S.D. Merca. 1973. An Improved Technique for Evaluating Resistance of Rice Varieties to *Xanthomonas Oryzae*. *Plant Disease Report*, 57: 537-541.

Kaur, P., Singh, N., Pal, P., & Kaur, A. (2018). Variation in Composition, Protein and Pasting Characteristics of Different Pigmented and Non-Pigmented Rice (*Oryza Sativa L*.) Grown In Indian Himalayan Region. *Journal of Food Science and Technology*, 55(9), 3809-3820.

Khaeruni, A., Najamuddin, E., Wijayanto, T., & Syair, S. (2016). Ketahanan Berbagai Kultivar Padi Lokal terhadap Penyakit Hawar Daun Bakteri. *Jurnal Fitopatologi Indonesia*, *12*(3), 89-89.

Khaeruni, A., Taufik, M., Wijayanto, T., & Johan, E. A. (2014). Perkembangan Penyakit Hawar Daun Bakteri Pada Tiga Varietas Padi Sawah yang diinokulasi Pada Beberapa Fase Pertumbuhan. *Jurnal Fitopatologi Indonesia*, 10(4), 119-119.

Klingeman, W. E., Pettis, G. V., & Braman, S. K. (2009). Lawn Care and Landscape Maintenance Professional Acceptance of Insect and Disease Resistant Ornamental Plants. *Hortscience*, 44(6), 1608-1615.

Lee, K. S., Rasabandith, S., Angeles, E. R., & Khush, G. S. (2003). Inheritance of Resistance to Bacterial Blight in 21 Cultivars of Rice. *Phytopathology*, 93(2), 147-152.

Liu, Q., Li, X., Yan, S., Yu, T., Yang, J., Dong, J., Liu, B. (2018). Oswrky67 Positively Regulates Blast and Bacteria Blight Resistance by Direct Activation of PR Genes In Rice. *BMC Plant Biology*, 18(1). Doi: 10.1186/S12870-018-1479-Y

Luo Y, Wang S, Li C, Wu S, Wang D, Du S (2005) Improvement of Bacterial Blight Resistance by Molecular Marker Assisted Selection in a Wide Compatibility Restorer Line of Hybrid Rice. *Chin J Rice Sci* 19:36–40

Luo, Y., Sangha, J. S., Wang, S., Li, Z., Yang, J., & Yin, Z. (2012). Marker-Assisted Breeding of *Xa4, Xa21* and *Xa27* in the Restorer Lines of Hybrid Rice for Broad-Spectrum and Enhanced Disease Resistance to Bacterial Blight. *Molecular Breeding*, 30(4), 1601-1610.

Madden, L. V., & Hughes, G. (1999). Sampling for Plant Disease Incidence. *Phytopathology*, 89(11), 1088-1103.

Mahmood, T., & White, F. F. (2019). Disease Resistance and Susceptibility Genes to Bacterial Blight of Rice. In Protecting Rice Grains In The Post-Genomic Era. Intech Open.

Mallikarjun, M., & Kotasthane, A. S. (2018). Fine Mapping of *Xa7*, a Dominant Bacterial Blight Resistance Gene in Rice. *Int. J. Curr. Microbiol. App. Sci*, 7(2), 3281-3287.

Mau, Y. S., Ndiwa, A. S. S., Markus, J. E. R., Oematan, S. S., Nasution, A., Handoko, D. D., & Makbul, K. (2018). Blast Resistance Levels of Red and Black Upland Rice Local Cultivars from Indonesia. *Asian Journal of Crop Science*, 10(2), 53–65. Doi: 10.3923/Ajcs.2018.53.65

Moon, S. J., Park, H. J., Kim, T. H., Kang, J. W., Lee, J. Y., Cho, J. H., & Shin, D. (2018). Ostga2 Confers Disease Resistance to Rice against Leaf Blight by Regulating Expression Levels of Disease Related Genes via Interaction with Nh1. *Plos One*, 13(11), E0206910.

Mustafa, A., Yasin, S. I., Mahmood, S., Hannan, A., & Akhtar, M. (2013). Field Evaluation of New Fungicides against Rice (*Oryza Sativa*) Diseases. *Pakistan Journal of Phytopathology*, 25(2), 141-145.

Nagendran, K., Karthikeyan, G., Peeran, M. F., Raveendran, M., Prabakar, K., & Raguchander, T. (2013). Management of Bacterial Leaf Blight Disease in Rice with Endophytic Bacteria. *World Appl Sci J*, 28(12), 2229-2241.

Oerke, E. C. (1996). Rice Blast Disease: Edited By RS Zeigler, SA Leong and PS Teng. Cab International, Wallingford, UK, In Association with the International Rice Research Institute, Los Banos, Philippines, 1994. 626.Isbn 0 85198 935 7. *Agricultural Systems*, 51(3), 367-369.

Pharmawati, M., Wirasiti, N. N., & Wrasiati, L. P. (2018). Respon Morfologis dan Ekspresi Gen Aquaporin pada Padi IR 64 yang Mengalami Cekaman Kekeringan pada Fase Reproduktif (Morphological Responses and Aquaporin Gene Expression in Rice IR64 under Drought Stress at the Reproductive Stage). *JURNAL BIOS LOGOS*, *7*(2), 60-66.

Rasmiyana., Addy, H. S., & Narulita, E. (2019). Detection of Genes Resistant to Bacterial Leaf Blight in Rice Cultivars from Situbondo and Jember, Indonesia. *Jurnal Hama dan Penyakit Tumbuhan Tropika*, 19(2), 127-134.

Saha, S., Garg, R., Biswas, A., & Rai, A. B. (2015). Bacterial Diseases of Rice: An Overview. *J Pure Appl Microbiol*, 9(1), 725-736.

Saharan, G. S., & Mehta, N. (2008). Sclerotinia Diseases of Crop Plants: Biology, Ecology and Disease Management. *Springer Science & Business Media*.

Sambrook, J., & Russell, D. W. (2001). Molecular Cloning-Sambrook & Russel-Vol. 1, 2, 3. *Cold Springs Harbour Laboratory Press*.

Sanghamitra, P., Sah, R. P., Bagchi, T. B., Sharma, S. G., Kumar, A., Munda, S., & Sahu, R. K. (2018). Evaluation of Variability and Environmental Stability of Grain Quality and Agronomic Parameters of Pigmented Rice (*O. Sativa L.). Journal of Food Science and Technology*, 55(3), 879-890.

Sari, V. K., & Murti, R. H. (2015). An Effective Method for DNA Extraction of Mature Leaf of Sapodilla (*Manilkara Zapota (L.) Van Royen*). Agrivita, *Journal of Agricultural Science*, 37(1), 18-23.

Setiawan, A., Sebastian, A., & Purwestri, Y. A. (2021) Deteksi Gen Ketahanan Hawar Daun Bakteri Xa21 pada Padi (*Oryza sativa L*.) Hitam dan Merah Lokal Indonesia. *Vegetalika*, *10*(2), 120-132.

Shafinah, K., Sahari, N., Sulaiman, R., Yusoff, M. S. M., & Ikram, M. M. (2013). A Framework of an Expert System for Crop Pest and Disease Management. *Journal of Theoretical & Applied Information Technology*, 58(1).

Shimono, M., Koga, H., Akagi, A. Y. A., Hayashi, N., Goto, S., Sawada, M., & Kaku, H. (2012). Rice Wrky45 Plays Important Roles in Fungal and Bacterial Disease Resistance. *Molecular Plant Pathology*, 13(1), 83-94.

Shinta, S., Indriyani, S., & Arisoesilaningsih, E. (2014). Morphological Variation of Six Pigmented Rice Local Varieties Grown in Organic Rice Field at Sengguruh Village, Kepanjen Subdistrict, Malang District. *Journal of Tropical Life Science*, 4(2), 149-160.

Singh Ak, Dharmraj E, Nayak R, Singh Pk, & Singh Nk. 2015. Identification of Bacterial Leaf Blight Resistance Genes in Wild Rice of Eastern India. *Turk. J. Bot.* 39(6): 1060–1066.

Somantri, S. D. R. U. (2016). The use of improved varieties resistant to pests and diseases to increase national rice production. *Jurnal Penelitian dan Pengembangan Pertanian*, *35*(1), 25-36.

Sombunjitt, S., Sriwongchai, T., Kuleung, C., & Hongtrakul, V. (2017). Searching For and Analysis of Bacterial Blight Resistance Genes from Thailand Rice Germplasm. *Agriculture and Natural Resources*, 51(5), 365-375.

Suparyono, S., Sudir, S., & Suprihanto, S. (2004). Pathotype profile of *Xanthomonas oryzae* pv. *oryzae* isolates from the rice ecosystem in Java.

Susanto, F. A., Wijayanti, P., Retnoningrum, M. D., Nuringtyas, T. R., Joko, T., & Purwestri, Y. A. (2018). Screening of Resistant Indonesian Black Rice Cultivars against Bacterial Leaf Blight. *Euphytica*, 214(11), 199.

Susanto, U., & Sudir, S. (2015). Ketahanan Genotipe Padi terhadap Xanthomonas oryzae pv. oryzae Patotipe III, IV, dan VIII. *Jurnal Penelitian Pertanian Tanaman Pangan*, 31(2), 108-116.

Swamy, P., Panchbhai, A. N., Dodiya, P., Naik, V., Panchbhai, S. D., Zehr, U. B., Char, B. R. 2006. Evaluation of bacterial blight resistance in rice lines carrying multiple resistance genes and Xa21 transgenic lines. *Current Science*. 90(6):818–824.

Ullah, I., Jamil, S., Iqbal, M. Z., Shaheen, H. L., Hasni, S. M., Jabeen, S., & Akhter, M. (2012). Detection of Bacterial Blight Resistance Genes in Basmati Rice Landraces. *Genet Mol Res*, 11(3), 1960-1966.

Umadevi, M., Pushpa, R., Sampathkumar, K. P., & Bhowmik, D. (2012). Rice-Traditional Medicinal Plant in India. *Journal of Pharmacognosy and Phytochemistry*, 1(1), 6-12.

Van Der Plank, J. E. (1966). Horizontal (polygenic) and vertical (oligogenic) resistance against blight. *American Potato Journal*, 43(2), 43-52.

Velusamy, P., Immanuel, J. E., Gnanamanickam, S. S., & Thomashow, L. (2006). Biological Control of Rice Bacterial Blight by Plant-Associated Bacteria Producing 2, 4-Diacetylphloroglucinol. *Canadian Journal of Microbiology*, 52(1), 56-65.

Wang, G., Sun, Y., & Wang, J. (2017). Automatic Image-Based Plant Disease Severity Estimation Using Deep Learning. *Computational Intelligence and Neuroscience*, 2017.

Wang, J., Tian, D., Gu, K., Yang, X., Wang, L., Zeng, X., & Yin, Z. (2017). Induction Of *Xa10*-Like Genes in Rice Cultivar Nipponbare Confers Disease Resistance to Rice Bacterial Blight. *Molecular Plant-Microbe Interactions*, 30(6), 466-477.

Wang, W., Zhai, W., Luo, M., Jiang, G., Chen, X., Li, X., & Zhu, L. (2001). Chromosome Landing at the Bacterial Blight Resistance Gene *Xa4* Locus Using a Deep Coverage Rice Bac Library. *Molecular Genetics and Genomics*, 265(1), 118-125.

Watanabe, T., & Kume, T. (2009). A General Adaptation Strategy for Climate Change Impacts on Rice Cultivation: Special Reference to the Japanese Context. *Rice and Water Environment*, 7(4), 313.

Webb, K. M., Ona, I., Bai, J., Garrett, K. A., Mew, T., Vera Cruz, C. M., & Leach, J. E. (2010). A Benefit of High Temperature: Increased Effectiveness of a Rice Bacterial Blight Disease Resistance Gene. *New Phytologist*, 185(2), 568-576.

Xiang, Y., Cao, Y., Xu, C., Li, X., & Wang, S. (2006). *Xa3,* Conferring Resistance for Rice Bacterial Blight and Encoding a Receptor Kinase-Like Protein, is the same as *Xa26*. *Theoretical and Applied Genetics*, 113(7), 1347-1355.

Yang, B., Sugio, A., & White, F. F. (2006). Os8n3 is a Host Disease-Susceptibility Gene for Bacterial Blight of Rice. Proceedings of *The National Academy of Sciences*, 103(27), 10503-10508.

Yen, Hugo. "Rice Bacterial Blight". Encyclopedia Britannica, 1 Apr. 2020, https://www.britannica.com/science/rice-bacterial-blight. Accessed 3 July 2021.

Yoshimura, S., Yamanouchi, U., Katayose, Y., Toki, S., Wang, Z. X., Kono, I., & Sasaki, T. (1998). Expression of *Xa1,* A Bacterial Blight-Resistance Gene in Rice, is induced by Bacterial Inoculation. Proceedings of *The National Academy of Sciences*, 95(4), 1663-1668.

Yuan, M., Chu, Z., Li, X., Xu, C., & Wang, S. (2009). Pathogen-Induced Expressional Loss of Function is the Key Factor in Race-Specific Bacterial Resistance Conferred by a Recessive R Gene *xa13* in Rice. *Plant and Cell Physiology*, 50(5), 947-955.

Yuliani, D., & Rohaeni, W. R. (2017). Heritabilitas, sumber gen, dan durabilitas ketahanan varietas padi terhadap penyakit hawar daun bakteri. *Jurnal Penelitian dan Pengembangan Pertanian*, *36*(2), 99-108.

Yuliani, D., & Rohaeni, W. R. (2018). Evaluasi Ketahanan Galur International Rice Bacterial Blight terhadap Hawar Daun Bakteri dan Identifikasi Patotipe Xanthomonas oryzae pv. oryzae Provinsi Jawa Tengah Berdasarkan Galur IRBB. *Jurnal Ilmu Pertanian Indonesia*, *23*(1), 52-59.

Yuliani, D., Wening, R. H., & Sudir, S. (2014). Selection Resistance of Rice Germplasm Accessions to Bacterial Leaf Blight. *Buletin Plasma Nutfah*, *20*(2), 65-76.

Yuriyah, S., Nurani, S., Utami, D. W., & Silitonga, T. S. (2016). Evaluasi dan Identifikasi Marka Penanda Gen Ketahanan Penyakit Hawar Daun Bakteri pada Padi Lokal Sulawesi Selatan.

Zhang J, Li X, Jiang G, Xu Y, He Y (2006) Pyramiding of *Xa7* and *Xa21* for The Improvement of Disease Resistance to Bacterial Blight in Hybrid Rice. *Plant Breed* 125:600–605

Zhang, S., Song, W. Y., Chen, L., Ruan, D., Taylor, N., Ronald, P., & Fauquet, C. (1998). Transgenic Elite Indica Rice Varieties, Resistant to *Xanthomonas Oryzae* Pv. *Oryzae*. *Molecular Breeding*, 4(6), 551-558.

EFFICIENT CALLUS FORMATION AND REGENERATION OF RICE (*Oryza sativa* L.) IN THEIR EPIGENETIC REGULATION

THESIS

By:

SITI NABILAH MOHAMMAD SABRI
NIM. 192520101004

PROGRAM OF MASTER STUDY IN BIOTECHNOLOGY
POSTGRADUATE SCHOOL
UNIVERSITY OF JEMBER
2021

EFFICIENT CALLUS FORMATION AND REGENERATION OF RICE (*Oryza sativa* L.) IN THEIR EPIGENETIC REGULATION

THESIS

submitted to complete thesis and meet one of the requirements to complete the Program Master (S2) and achieve a Master Degree in Biotechnology

By:

SITI NABILAH MOHAMMAD SABRI
NIM. 192520101004

PROGRAM OF MASTER STUDY IN BIOTECHNOLOGY
POSTGRADUATE SCHOOL
UNIVERSITY OF JEMBER
2021

ACKNOWLEDGEMENT

By praising the presence of Allah SWT, the Most Merciful and the Most Compassionate, for the blessings of His grace and guidance so that I can complete the writing of this thesis that I present for:

1. Both parents, Mohammad Sabri and Fatimah Rejab, thank you for all the sacrifices, love, prayers, and endless support. Beloved sisters who lovingly accompany all struggles undertaken;
2. Our teachers, from kindergarten until university, who have poured their knowledge and patiently educated me;
3. Almamater Program of Master Study in Biotechnology, Postgraduate school, University of Jember.

MOTTO

"Whoever treads a path seeking knowledge, Allah will make easy for him the path to Paradise"

(reported by Ibn Majah and others, fulfilling the conditions of Imam al Bukhari and Imam Muslim)

DECLARATION

I hereby,

Name : Siti Nabilah Mohammad Sabri

NIM : 192520101004

Stated that thesis entitled **"Efficient Callus Formation and Regeneration of Rice *(Oryza sativa L.)* in Their Epigenetic Regulation"** is an original piece of writing. I certainly that this thesis has never been submitted for any other degree or any publication. I certify to the best of my knowledge that all the sources during the composition of this thesis have been acknowledge.

I am fully responsible for the validity of this thesis's content and the academic ethics which I strongly uphold. Accordingly, this statement is made truthfully without any coercion of any part and I am willing to accept academic penalties if I deliberately violate such academic integrity.

Jember, 07 December 2021

Writer,

Siti Nabilah
NIM. 192520101004

THESIS

EFFICIENT CALLUS FORMATION AND REGENERATION OF RICE (*Oryza sativa* L.) IN THEIR EPIGENETIC REGULATION

By:
SITI NABILAH MOHAMMAD SABRI
NIM. 192520101004

Supervisor

First Supervisor	: Tri Handoyo, SP., Ph.D. NIP 197112021998021001
Member of Supervisor	: Moh. Ubaidillah, S.Si., M.Agr., Ph.D. NIP 198612112019031008

APPROVAL BY SUPERVISOR

The thesis entitled "Efficient Callus Formation and Regeneration of Rice (*Oryza sativa* L.) in Their Epigenetic Regulation" by Siti Nabilah, NIM 192520101004 that has been checked and validated on:

Day and Date : Friday, 26 November 2021
Place : Pascasarjana, University of Jember

First Supervisor	Member of Supervisor
Tri Handoyo, S.P., Ph.D.	M. Ubaidillah, S.Si., M.Agr., Ph.D.
NIP 197112021998021001	NIP 198612112019031008

THESIS VALIDATION

The thesis entitled "Efficient Callus Formation and Regeneration of Rice (*Oryza sativa* L.) in Their Epigenetic Regulation" by Siti Nabilah, NIM 192520101004 has met the requirements of the of the Decree of the Rector of the University of Jember, number 26887/UN25/SP/2017, tanggal 1 November 2017, on the Early Detection and Prevention of Plagiarism Scientific Works of Lecturers, Educators, and Students of the University of Jember, with *submission* ID 1712059108, and has been tested and approved on:

Day and Date : Friday, 26 November 2021
Place : Pascasarjana, University of Jember

Thesis examiner:

First Thesis examiner	Second Thesis Examiner
Dr. Ir. Parawita Dewanti, MP.	Prof. Tri Agus Siswoyo, Ph.D.
NIP 196504251990022002	NIP 197008101998031001

The sign
The Director,

Prof. Dr. Ir. Rudi Wibowo, M.S
NIP 1952070619760310

SUMMARY

Efficient Callus Formation and Regeneration of Rice (*Oryza Sativa* L.) in Their Epigenetic Regulation; Siti Nabilah Mohammad Sabri; 192520101004; Program of Master Study in Biotechnology, Postgraduate School, University of Jember.

Somatic embryogenesis could successfully develop the reproductive system in rice using mature embryos dependent on genotype. It involves hormone activity, transcription factors, and epigenetic regulation as a complex mechanism. Several primary transcription factors respond to hormone signals to establish the cell-fate transition, including genome-wide changes in gene expression, regulated by epigenetic pathways. Transcription and epigenetic factors also control the activity of hormones through their biosynthesis and signalling effects, forming feedback networks. Epigenetics is the process of genetic changes in gene expression that are independent of DNA sequence variation. Almost all organisms contain approximately the same DNA and cell types but have different functions due to qualitative and quantitative differences in their gene expression. Thus, regulation of gene expression is at the heart of differentiation and development. Gene expression patterns that characterize differentiated cells are established during development and are maintained as the cells divide by mitosis. By applying biotechnology, especially crop improvement with tissue culture techniques, optimization of the use and development of rice potential was carried out. Furthermore, these biological variables may offer future knowledge for rice breeding and understanding rice biology. Rice requires *SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASE* (*SERK*), *LEAFY COTYLEDON* (*LEC*), and *WUSCHELL* (*WUS*) in somatic embryogenesis. These genes discovered that early somatic embryogenesis was thoroughly studied, while somatic embryo development was not. So, we define its importance. We discovered that *OsSERK* was significantly expressed in somatic embryo development in rice, whereas

OsWOX4 and *OsLEC1* were absent or expressed at low levels using modified combination hormones, T2: 2 mg L^{-1} kinetin + 1 mg L^{-1} NAA, and T3: 2 mg L^{-1} kinetin + 1 mg L^{-1} BAP + 1 mg L^{-1} NAA. Moreover, *OsSERK*, *OsLEC1*, and *OsWOX4* were expressed in somatic embryo development on the 28th day and demonstrated a positive response to plant morphology, as indicated on the GogoNiti II variety on the 45th day in plant regeneration media. As a result, rice regeneration is the most significant in vitro in somatic embryo development in the knowledge of the efficacy of high cell transformation, particularly in specific rice cultivars and their epigenetic process. Previously, studies reported that the source of callus cells became the key to transforming DNA into the cell in order to turn a new genetically modified rice into increasing cell transformation and that the induction of callus and regeneration of the rice were most important in understanding the high efficiency of cell transformation, especially in some of the rice cultivars, including their epigenetic process. Moreover, the study of the epigenetic process is still very limited in rice.

FOREWORD

Praise Allah SWT, for His mercy and blessing I can finish the research and writing a thesis entitled " Efficient Callus Formation and Regeneration of Rice (*Oryza sativa* L.) in Their Epigenetic Regulation" as a one of the requirements to complete the Program Master (S2) and achieve a Master Degree in Biotechnology.

This paper (thesis) cannot be completed without the support and motivation of all parties and the author would like to thank:

1. Prof. Dr. Ir. Rudi Wibowo, M.S. as the Director of the Postgraduate School of the University of Jember.
2. Prof. Tri Agus Siswoyo, Ph.D. as Head of Program of Master Study in Biotechnology (S2) who has guided the author.
3. Tri Handoyo, S.P., Ph.D., as a main supervisor, M. Ubaidillah, S.Si., M.Agr., Ph.D., as a member of supervisor who has provided opportunities, guidance, knowledge, experience, and support in completing this thesis.
4. Dr. Ir. Parawita Dewanti MP and Prof. Tri Agus Siswoyo, Ph.D. as a thesis examiner who provides suggestions and input in completing the thesis.
5. My parents and as well as families in Malaysia who always provide full support.
6. All students and staff at the Lab of Agriculture and Center for Development of Advanced Science and Technology (CDAST), University of Jember.
7. The Rice team and fellow students in Program of Master Study in Biotechnology, University of Jember.

The author also accepts criticism and suggestions from all parties for the perfection of this thesis. Finally, the authors hope this thesis can be useful.

Jember, 07 December 2021

Author

TABLE OF CONTENT

LIST OF TABLES

LIST OF FIGURES

Page

LIST OF APPENDICES

CHAPTER 1. INTRODUCTION

1.1 Background of the study

Rice (*Oryza sativa* L.) is the world's most widely consumed grain. As a result, it significantly influences the diets of more than half of the world's population, primarily in Asia (Tiwari *et al.*, 2020). As the world's population grows, farmers must provide enough food for a population expected to reach nearly 10 billion people by 2050 (Searchinger *et al.*, 2019). Additionally, 771 million tons of rice will be used globally over the next decade (Badawi, 2004).

As a result of this demand, several research projects focused on *Oryza sativa* L. genomic and transcriptome data, contributing to a better knowledge of rice biology. In addition, somatic embryogenesis could successfully develop the reproductive system in rice using mature embryos dependent on genotype (Siddique *et al.*, 2014). Thus, this plant could be used in the future to obtain high quality and quantity of output employing in-vitro biotechnology processes.

In the future, we will need to use biotechnology, like plant transformation, to improve the quality and quantity of local Indonesian rice. However, the problem is that not all the local Indonesian rice can regenerate well into plantlets. Some are easy, and some are difficult. It is often challenging to get the transformants because different plant varieties have different abilities to regenerate callus into plantlets. Process transformation will be successful when the callus can regenerate well after being given agrobacterium. So, in this study, we need to identify the factor that is difficult to regenerate and find a solution for the best combination of hormones and other ways to increase the callus' ability to regenerate and improve the callus characteristics. Then, we need to discover which genes are active and can regenerate well into plantlets.

Plant tissue cultivation is a part of biotechnology and a successful technique for modern agriculture. It has contributed to the development of agricultural sciences in recent years, providing plants needed to meet ever-increasing global demand in a shorter period than conventional crop improvement methods.

Under controlled and aseptic conditions, the rice potential can be optimized to develop, preserve, and proliferate cells, tissues, organs, and whole plants. Precisely, when somatic embryogenesis is integrated with conventional breeding programs and molecular and cell biological techniques, it provides a valuable tool for the genetic improvement of the crop (Quiroz-Figueroa *et al.*, 2006).

Somatic embryogenesis is an asexual reproduction process found in many plant species. This is a great model for embryo development and clonal propagation and transformation (Salaün *et al*., 2021). There are some morphological and biochemical changes in somatic embryogenesis in response to modifications in gene expression patterns (de Oliveira Santos *et al*., 2005). Somatic embryogenesis involves hormone activity, transcription factors, and epigenetic regulation as a complex mechanism. Despite this, differences in developmental stage and regeneration capacity may be attributable to active genes expressed during somatic embryogenesis (Mahdavi-Darvari *et al.*, 2015). In this work, we have focused on the role of *Somatic Embryogenesis Receptor-like Kinase* (*SERK*), *Leafy Cotyledon1* (*LEC1*) and *Wuschel-Related Homeobox4 (WOX4)* genes in rice (*Oryza sativa* L.) as a totipotency markers expressed in several plants, including rice.

In *Arabidopsis thaliana*, *WUSCHEL* (*WUS*) is responsible for activating *LEC* genes. In *Gossypium hirsutum*, *WUS* activates *GhLEC1, GhLEC2,* and *GhFUS3* genes. Each stimulation takes place to develop somatic embryogenesis and induce cell differentiation. In addition, high expression of the *WUS* gene indicates that it is helpful as a valuable gene marker for the initiation of embryogenesis (Kumar and Van Staden, 2017). In addition, both zygotic and somatic embryogenesis requires *LEC* genes, *LEC1*, *LEC2* and *FUS3*. Functional loss in *LEC* altered embryonic development substantially (Gulzar *et al.*, 2020). *SERK* was expressed during the regeneration process in the rye (*Secale cereale* L.) cultured immature embryos. Nevertheless, its expression is suppressed in later stages (Gruszczyńska and Rakoczy-Trojanowska, 2011). The current study uses

this information to understand further somatic embryogenesis regulation in rice and gene expression in *SERK*, *LEC1*, and *WOX4* to develop somatic embryos.

The exogenous hormone causes morphological changes in stimulated tissues during callus cell development. Transcriptional and epigenetic factors regulate hormone activity through their biosynthesis and signalling effects, generating feedback networks. As a result, cell-fate transition and genome-wide gene expression changes as several primary transcription factors respond to exogenous hormone signals. Epigenetic pathways regulate them simultaneously. In addition, an increasing number of studies have revealed a strong link between plant hormone signalling and the epigenetic process (Yang and Zhang, 2010).

Epigenetics is a process of genetic changes in gene expression but not in DNA and cell types (Springer and Schmitz, 2017). Almost all organisms contain approximately the same DNA and cell types but have different functions due to qualitative and quantitative differences in their gene expression. Thus, regulation of gene expression is at the heart of differentiation and development. Gene expression patterns that characterize differentiated cells are established during development and are maintained as the cells divide by mitosis. The study of epigenetic regulation may provide precious details information for biotechnology in gene transformation in plants.

Many types of stress may induce plant cells to change their cellular and molecular programs from previous studies. Plant hormone are one type of stress that can alter plant cell and molecular processes (Mariani *et al*., 1998; Debbarma *et al.*, 2019). In addition, exogenous hormone exposure during the development of an organism can cause morphological changes in induced tissues (Méndez-Hernández *et al.*, 2019). We believe these factors influenced the rice varieties in significant ways. Similar to the findings of Zhao *et al.* (2017), signals from the environment increase endogenous hormone concentrations, and gene expression all trigger stages of somatic embryogenesis.

Consequently, this study's novelty depends on understanding the morphological analysis of somatic embryo development on the 14th and 28th days associated with expressed genes in rice. Epigenetic information on local rice is an

opportunity to improve the efficiency of plantlet regeneration. Thus, genetic diversity needs to be studied to obtain clear response information on culture media through callus induction and plant regeneration. Unfortunately, there are still no epigenetic studies on local Indonesian rice. Therefore, each rice variety had various genetic characteristics using molecular analysis of the epigenetic process in the somatic embryogenesis. However, it has not been studied thoroughly in Javanica rice. The cell source was the key to converting the cell's DNA to generate a new genetically modified rice to enhance cell transformation (Gelvin, 2003). Therefore, callus induction and rice regeneration are most significant in understanding the effectiveness of high-cell transformation, especially in some rice cultivars, related to their epigenetic process.

1.2 Problem statement

1. How is the response of plant regeneration through different concentrations of hormones with different local Indonesian rice in vitro?
2. How is the relationship between gene expression and morphological changes in plant regeneration using different varieties and combinations of hormones related to epigenetic regulation?
3. In the biotechnology sector, how to improve the overall quality and quantity of local Indonesian rice?

1.3 Objectives of research

1. To study the response of plant regeneration through different concentrations of hormones with different local Indonesian rice in vitro.
2. To study the relationship between gene expression and morphological changes in plant regeneration using different varieties and combinations of hormones related to epigenetic regulation.
3. To improve the overall quality and quantity of local Indonesian rice in the biotechnology sector.

1.4 Benefits from research

To provide information on the response of plant regeneration through different hormone concentrations with different local Indonesian rice varieties to gene expression in somatic embryogenesis in plant tissue culture techniques and improve the overall quality and quantity of local Indonesian rice in the future.

CHAPTER 2. LITERATURE REVIEW

2.1 Potential local Indonesia rice

Rice is one of the oldest cultivated crops in the Gramineae family (Paudel *et al*., 2012). Rice can be classified into Indica and Japonica subspecies. The Japonica sub-species are made up of Temperate and Tropical Japonica (called Javanica), mainly Javanica, which originated in Indonesia (Deshmukh, 2015). Moreover, Indonesians are the world's largest nation consuming rice as a staple food, making it a key carbohydrate source. Rice consists of 8000 varieties due to its diversity. The physical shape and size of the grain are different for each rice variety (Utami *et al.*, 2019).

This study used local Indonesian rice varieties, Cigeulis, GogoNiti II, Ketan Hitam 1, and TN1, as a control. The Cigeulis variety is a cross between Ciliwung and Cikapundung, with selection number S3429-4D-PN-1-1-1, which is essential for increasing yields and a key component in controlling pests and diseases, also interested among farmers. The plant is erect, 100–110 cm, and 115–125 days old, with productive seedlings of 14–16 stems. This variety was resistant to brown planthopper biotype 2, susceptible to biotype 3, and resistant to bacterial leaf blight strain IV (Mirsam and Sudartik, 2017). Although GogoNiti II and Ketan Hitam I were pigmented rice with high phenolic, flavonoids, and phytochemical pigment, with higher antioxidant and metal ion chelating properties than non-pigmented rice (Pradipta *et al.,* 2020). The market demand is increasing due to the rice's nutrient content, but the huge demand has been constrained by the generally low productivity of pigmented rice among farmers. In economic terms, it is a profitable agricultural product and can be developed because it is very different from the glutinous rice consumed by the general public in terms of both taste and aroma. That is why, pigmented need to get high productivity by using biotechnology through plant transformation to get maximum results.

On the other hand, local Indonesian rice varieties are still limited in the study, especially in vitro. Through plant tissue culture, according to preliminary experiments, GogoNiti II had higher regeneration rates. In contrast, Cigeulis and

Ketan Hitam I had lower regeneration rates. That is why we chose that local Indonesian rice to study the characteristics of morphology in high and low regeneration and to study gene expression in rice related to epigenetic regulation. In the future, we need to use biotechnology, like plant transformation, to improve the quality and quantity of local Indonesian rice. However, the problem is that not all the local Indonesian rice can regenerate well into plantlets. Some are easy, and some are difficult. So, we need callus, which is easily viable for plant regeneration. It is often challenging to get the transformants because different plant varieties have different abilities to regenerate callus into plantlets. Process transformation will be successful when the callus can regenerate well after being given agrobacterium. So, we need to identify the problematic factors to regenerate and find a solution for the best combination of hormones and other ways to increase the callus' ability to regenerate and improve the callus characteristics. Some genes are active, and some are not. After we know that factor, we can activate the gene by inducible use of that hormone or other ways. Then, we can do plant transformation in biotechnology. For success, one must use callus media, which can then be able to regenerate into plants.

TN1 was used as a control in this study because it was a model in-vitro in the genetic background of rice and had the potential to produce numerous calli after subcultures. In a previous study, TN1 could produce thousands of embryogenic calli from 50 seeds in ten weeks (Sivamani et al., 1996).

2.2 Plant propagation through tissue culture

Rice responds mainly to tissue cultivation and can be regenerated in vitro. The ability of rice seeds to callogenesis is greater than the node or tip (Ganeshan *et al.*, 2003). The production of high-frequency callus induction is a crucial phase in improving crop or fast biotechnological propagation (Upadhyaya *et al.*, 2015). Plant regeneration by the callus of various rice explants, such as immature embryos, immature young inflorescences, and roots. Dehusked rice cultivation is an innovative strategy for using somaclonal varieties and enhancing grain performance. Somatic embryogenesis is the development, growth, and evolution of somatic cell embryos. Moreover, somatic embryo induction is one of the most

important in vitro propagation techniques in plant growth (Von Arnold *et al.*, 2002). The first pro embryogenic phase begins with the transition of the embryogenic forms. Dicotyledons and monocotyledons differ in this form. In dicotyledonous plants, embryogenic structures are globular, heart-shaped, torpedo and cotyledonary, whereas in monocotyledonous plants they are globular, scutellar and coleoptilar.

2.3 Breeding techniques of biotechnology using explant

The basic principles of biotechnology in plant improvement activities include plant breeding, the application of Mendell's law, the application of genetic engineering and gene transformation (Dudley, 2002). For example, rice breeding has made considerable strides towards higher yields, improved efficiency, short-growing productivity, increased disease and insect resistance, and tolerance for problem soil (Khush, 1987). Biotechnology is the solution to conventional breeding practices. However, incorporating genetic engineering methods for enhancing rice requires an essential procedure for in vitro culture (Escobar-Guzmán *et al.*, 2009). Tissue cultivation is an efficient method for producing better varieties than conventional breeding methods (Suraiya and Alina, 2018). Rice yields have grown into the cultivation of plant tissue and are used to produce breeding and agricultural yields such as rice (Kadhimi *et al.*, 2016).

2.4 Background genetic and tissue culture

Successful use of the tissue culture method involves developing several different cells or tissues within the stated culture conditions and the distribution of several cells. Tissue culture is recognised as a practical approach to producing genetic diversity that can boost breeding programs by adding new genetic variability (Sakulsingharoj *et al.*, 2014). The callus is a mass of tissue influenced by unregulated development of different elements such as plant growth regulators (PGRs) that show significant characteristics for cell development, such as cytokinin/auxin plant hormones, which have an essential role in the growth and differentiation of callus cells (Kadhimi *et al.* 2016). However, the genetic basis of

the explant has demonstrated considerable limitations in evaluating the progress of the regeneration of the rice plant tissue culture. Genetic differences in the number of hormone levels have influenced the distribution of the callus (Deo *et al.*, 2010). stated that genetic factors significantly contribute to the in vitro reaction of cultivated tissues (Wani *et al.*, 2010). Differences in the production of embryogenic calli and regenerated plantlets have been observed, depending on the genotype and source of the explant. However, the induction of rice callus and the factors that affect it, such as genotypes and growth regulators, are insufficient to produce the best results. Different genotypes would produce different responses in tissue culture (Suraiya and Alina, 2018). Therefore, somatic embryogenesis's success depends on many factors, including plant growth regulator, genotype, basal salt, explant, cultural conditions, and developmental stages of the mother plants.

2.5 Genetic function to potency of callus formation and regeneration

Changes in morphological and biochemical are happening across the formation of induced tissues during somatic embryogenesis to form plant regeneration from callus. It is directly connected to alterations in gene expression.

2.5.1 *Somatic embryogenesis receptor kinase* (*SERK*)

Somatic embryogenesis receptor kinase, also referred to as *SERK*, acts as an integral part of the induction of somatic embryogenesis at the molecular level, belonging to a small family of genes encoded for a transmembrane protein involved in signal transduction. In addition, the expression of the *SERK* gene begins with the activation of an embryogenic stage to the globular stage of somatic embryos but not in the non-embryogenic stages. In embryogenic tissues and specific undifferentiated cells, *SERK* is highly expressed, preceding and referring to early somatic embryogenesis (Santos and Aragão, 2009). For instance, during the regeneration process in the rye (*Secale cereale* L.) cultivated immature embryos, the expression of *SERK* was expressed. However, its expression is suppressed in later stages (Gruszczyńska and Rakoczy-Trojanowska, 2011a).

2.5.2 Leafy cotyledon (LEC)

Leafy cotyledon (*LEC*) genes play a crucial role in regulating many factors of plant embryogenesis and transcriptional encoding. There are two primary regulators for the growth of plant embryos, which are *LEC1* and *LEC2,* which play a crucial role in embryo morphogenesis and maturation processes (Guo *et al.*, 2013). Moreover, *LEC* genes, which are *LEC1, LEC2* and *FUS3,* are also necessary for both zygotic and somatic embryogenesis induction. Loss of function mutation in *LEC* greatly affected embryonic development (Gulzar *et al.,* 2020).

2.5.3 *Wuschel* (*WUS*)

Wuschel, also known as *the WUS*-related homeobox (*WOX*) gene family, is a class of homeodomain (HD) transcription factors that play a significant role in regulating the transcription of genes involved in the early stages of embryogenesis (Gambino *et al.*, 2011). Besides, it is also the founding member of the family of *WOX* genes that allow somatic embryos to develop if expressed. For example, in Arabidopsis, *WUS* is responsible for activating *LEC* genes, while in *Gossypium hirsutum, WUS* is also responsible for activating *GhLEC1, GhLEC2,* and *GhFUS3* genes. Each stimulation occurs for the development of somatic embryogenesis and the induction of cell differentiation. In addition, high expression of the *WUS* gene indicates that it is helpful as a valuable gene marker for initiation of embryogenesis (Kumar and Van Staden, 2017).

2.6 Epigenetic generally forms a genetic function

Epigenetics means genetic changes in the expression of genes that are independent of DNA sequence variation. In-plant regeneration, somatic embryogenesis plays a vital role and involves complex cellular, molecular, and biochemical pathways for embryo initiation and expression of plant epigenetics. The expression of different genes is involved in the *Auxin Response Factor (ARF7, ARF19)* and *Protein Regulator of Cytokinesis (PRC1)* related to stress and exogenous hormones. After the cell has reached dedifferentiation ability, *Leafy Cotyledon* (*LEC1* and *LEC2*) genes are expressed and increase the endogenous

auxin level, which consequently upregulates the expression of *Curly Leaf* (*CLF*), *Wuschel* (*WUS*), and *Somatic Embryogenesis Receptor Kinase* (*SERK*). Epigenetic processes are highly complex acts that regulate the expression of genes via DNA methylation, chromatin remodelling, and small RNAs. The modulation of epigenetic pathways has recently been identified as an essential phenomenon during somatic embryogenesis in plants. A few studies have shown that in embryogenic cells under in-vitro environments, the degree of DNA methylation may shift. Changes or variations in DNA methylation patterns are related to regulatory mechanisms implicated in the initiation and growth of somatic embryogenesis in plants by different candidate marker genes (Osorio-Montalvo *et al.*, 2018).

Furthermore, the explant's metabolic patterns, gene expression, and epigenetic processes (DNA methylation, histone modifications, and microRNAs) may affect different conditions (endogenous or exogenous to the explant). They can cause a cell (or a group of cells) to change its nature. The transcription factor genes, *BABY BOOM1 (BBM1*) and *LEAFY COTYLEDON1* (*LEC*1), which are critical for activation of somatic embryogenesis and cell differentiation, are epigenetically regulated by H3K27me3 (Horstman *et al.*, 2017). Moreover, *LEC1* is an essential regulatory gene for embryogenesis that helps to trigger somatic embryogenesis through expression (Suer *et al.*, 2011). Although the repressive mark *H3K9me2* regulates *WUSCHEL RELATED HOMEOBOX4 (WOX4)* by Chromatin Immunoprecipitation (ChIP) assays, the expression of *WOX4* is necessary to promote the differentiation of procambium in Arabidopsis and tomato (Nic-Can *et al.*, 2013). In conclusion, in recent years, due to its potential for food and biotechnological applications, epigenetic control has been one of the most critical pathways in modern plant biology. Awareness of epigenetic processes during somatic embryogenesis may help develop new approaches to plant breeding and crop development. The use and epigenetic effects of in vitro-related plants are a crucial biotechnological method for plant development and crop breeding programs.

2.7 Combination of hormone to epigenetic regulation

An increasing number of studies indicate a strong link between plant hormone signalling and epigenetic regulation. Actin alone or in combination, hormones have essential effects on chromatin compaction mediated by DNA methylation and histone post-translational modifications, which suggest important influence on epigenetic modulation through play in hormonal flowering promoting flowering through epigenetic regulation, such as gibberellins, jasmonic acid, abscisic acid, and plant hormone applications, including 2,4-D, NAA, and kinetin, have helped preserve dedifferentiation status and hypermethylation of promoter regions, suggesting that plant hormones can facilitate DNA methylation by modifying chromatin structure (Campos *et al.,* 2016). The auxin and cytokinin combinations preserve the dedifferentiated state of callus cells by retaining the promoter region of many genes, including *MAPK12, GSTU10,* and *BXL1*, with DNA hypermethylation and histone hypoacetylation status. Nowadays, scientists have started investigating how stress-mediated histone changes and DNA methylation regulate the transcription of stress-responsive genes and have focused on the epigenetic role in ABA biosynthesis, cognition and signalling (Zhu, 2010). Some phytohormones have been shown to influence epigenetic alterations. For example, the plant hormone auxin is identified by *TRANSPORT INHIBITOR RESPONSE1 (TIR)/AUXIN SIGNALING F BOX PROTEINS (AFBs)* nuclear auxin receptors, leading to the activation of *AUXIN RESPONSE FACTORS (ARFs)* (Yamamuro, Zhu and Yang, 2016). These transcriptional factors activate auxin-induced gene expression. Emerging research suggests that the induction of auxin-response-gene based on ARF-dependent is modulated by microRNAs (miRNAs) and epigenetic factors such as histone modifications and chromatin remodelling components *PICKLE (PKL)*. In conclusion, proper epigenetic regulation of plant hormone signalling is essential for the correct transition from the vegetative to the reproductive stage. A greater understanding of global epigenetic control mechanisms will lead us to grow plants that respond to new ecosystem challenges, including DNA methylation, histone post-translational modification, and miRNAs.

2.8 Hypothesis

1. There is the potency of callus in plant regeneration determined by an epigenetic specific mechanism and hormone specific.
2. There are genetic differences that affect plantlet regeneration.
3. There are hormone factors that have specific functions.

CHAPTER 3. RESEARCH METHODOLOGY

3.1 Time and place

The study entitled "Efficient callus formation and regeneration of rice (*Oryza sativa* L.) in their epigenetic regulation" was carried out at the Laboratory Center for Development of Advanced Sciences and Technology (CDAST) and at the Faculty of Agriculture's Laboratory, University of Jember, Indonesia. The study was conducted from April 2021 until July 2021.

3.2 Materials and apparatus

3.2.1 Materials

The materials for "Efficient callus formation and regeneration of (*Oryza sativa* L.) rice in their epigenetic regulation" were seeds varieties (TN1, Cigeulis, GogoNiti II, and Ketan Hitam I), 2 mg L^{-1} 2,4-D (2,4-Dichlorophenoxyacetic acid), 1 mg L^{-1} BAP, 2 mg L^{-1} Kinetin, 1 mg L^{-1} NAA, NaOH 1 N or 1 N HCL, 0.3 g L^{-1} casamino acid, 5.2 g L^{-1} phytagel, 30 g L^{-1} sucrose, Murashige-Skoog (MS vitamin), alcohol, GoTaq® Green Master Mix and Green Master Mix kit (Promega).

3.2.2 Apparatus

The apparatus for "Efficient callus formation and regeneration of rice (*Oryza sativa* L.) in their epigenetic regulation" was LAF (Laminar Air Flow), autoclave, pH meter, filter paper, Bunsen burner, micropipette, spatula, plastic wrap, light microscope, microwave, Petri dish, measuring cylinder, beaker glass, scalpel, forceps, tissues, paper labels, and NEXprep™ Plant RNA Mini Kit (NEXT™ Diagnostics), Nanodrop (TECAN® Infinite M200 Multi Detection Microplate Reader Part), electrophoresis, and UV transilluminator.

3.3 Research Procedures

3.3.1 Sterilization of apparatus and space

All the apparatus was sterilized by autoclave at 121°C with a pressure of 17.5 psi. In addition, the room was sterilized by cleaning the inside of the LAF

(Laminar Air Flow Cabinet) using alcohol, then irradiated with UV light under closed conditions for 30 minutes before use. The mature seeds of four varieties (Figure 4.1), Cigeulis, GogoNiti II, Ketan Hitam I, and TN1, were dehusked by shaking for 30 minutes at 120 rpm in 50% Clorox bleach (5.25 % hypochlorite, The Clorox Company, Indonesia), then rinsed with sterile distilled water five times, after which they were allowed to dry thoroughly on sterile filter paper.

3.3.2 Making of media

This research was conducted using four types of germplasm, namely Cigeulis, GogoNiti II, Ketan Hitam I, and TN1. 30 g L^{-1} Sucrose, 2 g L^{-1} casein hydrolysate, 1.0 mg L^{-1} BAP, 2 mg L^{-1} kinetin, 1.0 mg L^{-1} NAA, 0.1 g L^{-1} Myo-Inositol and 5.2 g L^{-1} phytagel were prepared. The medium was measured using a pH meter before it was combined with pytagel as a compactor. For cultural media, the optimum pH is 5.8. The pH was adjusted by adding a few drops of 1N NaOH or 1N HCl. The prepared medium was then sterilized at 17.5psi in an autoclave and put into a Petri dish for 30 minutes at 121°C. The Petri dish was then wrapped in plastic wrap and then kept incubated for three days before it was ready to be utilized to determine medium contamination. The procedures for producing regeneration media were the same as for preparing induction media.

2,4-D is a growth regulator with higher activity than IAA and NAA. 2 mg L^{-1} of 2,4-D is explicitly indicated as the optimal concentration of embryogenic calluses at rice seed maturity (Revathi and Pillai, 2011; Nic-Can *et al.*, 2013). Therefore, a concentration of 2 mg L^{-1} of 2,4-D is preferred in mature rice seeds and researchers have widely used it for the primary inducement of calluses and the formation of embryogenic calluses (Libin *et al.*, 2012; Hoque *et al.*, 2013).

A hormone such as kinetin was used to accelerate the formation of plantlets and produce growth of callus and plantlets normally (Abrahamian and Kantharajah, 2011). The use of kinetin aims to enhance somatic embryogenesis by impacting cytokinesis, total protein synthesis, lignin biosynthesis, mitosis, vascular differentiation and the differentiation of mature chloroplasts from protoplasts (Abe and Futsuhara, 1986). Initial division of cells can promote by BAP, Kinetin and

integral part of somatic embryogenesis and plantlet regeneration NAA has been consistently used in all media. Regenerated plantlet was weak without NAA (Rueb *et al.*, 1994).

3.3.3 Preparation of callus induction

Healthy mature rice seeds were dehusked and cleaned by immersing them in 12.5 mL of distilled water and 12.5 mL of Clorox bleach (5.25 % hypochlorite, The Clorox Company, Indonesia) for 30 minutes. The seeds stored in a falcon tube were placed in an orbital shaker for 5-15 minutes. Broken, unhealthy, or black-lined seeds were discarded. Then, the seeds were sterilized at least five times with sterile distilled water to remove residue from the bleach solution. The seeds were then blot dried on sterilized filter paper (90 mm) before callus induction. Finally, the sterilized seeds were placed on a callus induction medium for two weeks (20 seeds per Petri dish) and incubated at 27°C under darkness for another two weeks to facilitate the formation of calli.

3.3.4 The embryogenic callus induction

The seeds were then cultivated by 2 mg L-1 2,4-D (2,4-Dichlorophenoxyacetic acid), 30 g L^{-1} sucrose, and 4 g L^{-1} phytagel were used to culture seeds in a sterile medium consisting of MS salts (Upadhyaya *et al*., 2015). Before autoclaving, the pH of the medium was adjusted to 5.8. After that, the seeds were cultured for three weeks at 27°C in dark conditions. The percentage of callus induction and callus size were determined after three weeks of incubation.

$$\textbf{Callus induction frequency} = \frac{(\textbf{The number of seed with callus})}{(\textbf{The number of incubated seed})} \times \textbf{100}\%$$

3.3.5 Plant regeneration

The treatments were utilized to obtain high-quality embryogenic calli, 1.0 mg L^{-1} BAP, 2 g L^{-1} casein hydrolysate, 2 mg L^{-1} kinetin, 0.1 g L^{-1} Myo-Inositol, 1.0 mg L^{-1} NAA, 5.2 g L^{-1} phytagel, and 30 g L^{-1} Sucrose. In this research, the

treatments for media regeneration were Control, T2: 2 mg L^{-1} kinetin + 1 mg L^{-1} NAA (Toki, 1997) and T3: 2 mg L^{-1} kinetin + 1 mg L^{-1} BAP + 1 mg L^{-1} NAA (Khanna and Raina, 1998). Each treatment had three replications with four callus clumps per replication. The regeneration culture was carried out at 27°C on the 14th and 28th days with a photoperiod of 16/8 h (light/dark). The percentage of green spots and the percentage of plant regeneration were determined.

$$\textbf{Green spot} = \frac{(\textbf{No. of callus induced green spot})}{(\textbf{No. of seeds cultured})} \times \textbf{100}\%$$

$$\textbf{Regeneration} = \frac{(\textbf{The number of plantlet producing callus})}{(\textbf{The number of callus piece inoculated})} \times \textbf{100}\%$$

3.3.6 Isolation of RNA purification

The callus samples were taken on the 14th and 28th days of medium regeneration and frozen immediately in liquid nitrogen. Then they isolated total RNA with some modifications to the processes of the manufacturer Gene All Ribospin Plant RNA Mini Kit (GeneAll Biotech, Korea). The 260 nm/280 nm measurement at a level between 1.8 and 2.2 for cDNA synthesis and RT-PCR was obtained using nanodrop (TECAN® Infinite M200 Multi-Detection Microplate Reader Part).

3.3.7 cDNA synthesis and RT-PCR

After verifying RNA quality, the GoTaq® Green Master Mix kit (Promega) was used to enhance the target cDNA. ReverTra Ace® RT Master Mix with gDNA Remover (Toyobo, Osaka, Japan) treats 0.5g of total RNA. This process eliminates and replaces genomic DNA with a single-stranded cDNA. Total RNA was incubated at 37°C, for the DNase reaction for 5 minutes and for the reverse transcription reaction. Finally, THUNDERBIRD SYBR qPCR Mix (Toyobo) and gene-specific primers (Table 4.1) have been used in PCR applications.

Table 4.1 Gene-specific primers sequences

Genes	Primer sequences	
	Forward (5'-3')	Reverse (5'-3')
OsLEC1	CAA GGA GAC GAT CCA GGA GT	GGT AGC GGT GGA GGT AGA CG
OsSERK	TTG CTG GAG GTG TTG CTG	TAC ACC TTT CCA AAG CCA
OsWOX4	CTA GCT TAT CGA TAC CGT CG	CCT ATC TGT TCT TGA GTC GG
OsActin	GGT ATT GTT AGC AAC TGG GAT G	GAT GAA AGA GGG CTG GAAG A

(Neo-Probe, 2018)

3.3.8 PCR analysis

PCR analysis is performed in a total volume of 10 μL containing 5 μL of 2 × GoTaq® Green Master Mix, 1 μL cDNA templates, 2 μL Nuclease-free water and 1 μL Forward (F) and Reverse (R) primer to detect the presence of a specific nucleic acid sequence using the GoTaq® Green Master Mix kit (Promega). The PCR amplification profile consisted of initial denaturation of 95°C for 2 min, followed by 30 cycles of denaturation 95°C for 30 s, annealing at 53°C for 30 s, extension at 72°C for 1 min, and a final extension at 72°C for 5 min. An electrophoresed 2 % agarose gel in 1 X TAE buffer stained with GreenStar™ was used for PCR analysis. In addition, the UV transilluminator was used to visualize the banding patterns.

3.4 Observation variable

3.4.1 Percentage callus induction

Observations were made by observing the time of the onset of callus on the induction media. The number of germinated seeds is calculated based on the total number of seeds grown in a petri dish until the 14th day.

3.4.2 Callus morphology

Observation of the morphology, colour structure, and shape of the callus. Morphological observations were carried out using light microscope.

3.4.3 Somatic embryogenesis formation

Observation of the development of somatic embryogenesis is done by observation of the globular, coleoptilar and scutellar phase. Observation of the development of somatic embryogenesis was conducted using a light microscope.

3.4.4 Green spot

Green spots on explants can be determined by calculating the percentage of green spots produced. Observation of the green spot during the regeneration phase is done through visual observation.

3.4.5 Number of plantlets

Plantlet formation was observed after 45th days in media regeneration. The number of plantlets was observed by counting the plantlets in each treatment.

3.5 Data analysis

Data have been evaluated using variance (ANOVA). If the findings are substantially different, perform further analysis using the Duncan's Multiple Range Test (DMRT) included in the SPSS statistical package version 26.

CHAPTER 4. RESULT AND DISCUSSION

4.1 Rice varieties

This study used local Indonesian rice varieties, Cigeulis, GogoNiti II, Ketan Hitam 1, and TN1, as a control. Through plant tissue culture, according to preliminary experiments, GogoNiti II had higher regeneration rates. In contrast, Cigeulis and Ketan Hitam I had lower regeneration rates. Then, we discovered how different hormones in plant regeneration media affect local Indonesian rice, which performs low and high regeneration. At the same time, we identified the genes that are expressed throughout somatic embryo development.

Figure 4.1 Seed varieties. A: TN1, B: Cigeulis, C: GogoNiti II, and D: Ketan Hitam I

TN1 was used as a control in this study because it was a model in-vitro in the genetic background of rice and had the potential to produce numerous calli after subcultures. In a previous study, TN1 could produce thousands of embryogenic calli from 50 seeds in ten weeks (Sivamani *et al.*, 1996). On the other hand, local Indonesian rice varieties such as Cigeulis, GogoNiti II, and Ketan Hitam I are still limited in the study, especially in vitro.

In the future, we need to use biotechnology, like plant transformation to improve the quality and quantity of local Indonesian rice. However, the problem is that not all the local Indonesian rice can regenerate well into plantlets. Some are easy, and some are difficult. So, we need callus, which is easily viable for plant regeneration. It is often challenging to get the transformants because different plant varieties have different abilities to regenerate callus into plantlets. Process transformation will be successful when the callus can regenerate well after being given agrobacterium. So, we need to identify the problematic factors to regenerate

and find a solution for the best combination of hormones and other ways to increase the callus' ability to regenerate and improve the callus characteristics. Some genes are active, and some are not. After we know that factor, we can activate the gene by inducible use of that hormone or other ways. Then, we can do plant transformation in biotechnology. For success, one must use callus media, which can then be able to regenerate into plants. This study aims to identify the regeneration factor that can cause a callus to regenerate well by using hormones as inducible to activate particular genes. Furthermore, using these varieties, it was hoped to study the different characteristics of the callus of local Indonesian rice. Compared to TN1 regarding callus induction, plant regeneration, and gene expression.

4.2 Callus Induction

The callus is an early indicator of a cell's capacity to regenerate plantlets. Moreover, in this study, the selection of callus cells is required to evaluate the effectiveness of embryogenic callus induction until plant regeneration in rice varieties. Besides, we discovered how vital callus formation is and how it relates to plant regeneration.

4.2.1 Morphological appearance of callus

In order to induce callus, we begin by cultivating the embryos from mature seeds in Murashige and Skoog (MS) medium supplemented with 2.0 mg L^{-1} of 2,4-D at a temperature of 27°C in a dark environment. Then, after three days, we discovered that the initiate calluses of Cigeulis, GogoNiti II, and Ketan Hitam I had appeared. For TN1, initiate calluses appeared after four days. After three weeks of callus induction, we observed the morphological appearance of the callus of TN1, Cigeulis, GogoNiti II, and Ketan Hitam I by using a light microscope (Figure 4.2). Non-embryogenic and embryogenic calluses were observed in the culture.

From observation, the TN1 callus showed a light yellow to whitish color and had a compact cell. However, GogoNiti II and Cigeulis callus turned yellow or cream, with nodular cells and a dissimilar compact cell-like TN1. Also, a hairy thread surrounding the callus appeared in Ketan Hitam I under media callus

induction. Furthermore, the hairy cells become thicker and more whitish during pro-embryo regeneration.

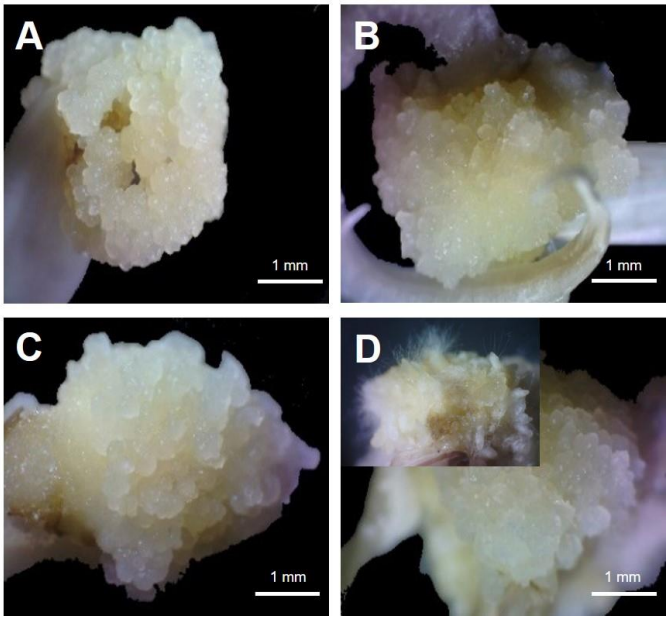


Figure 4.2 The morphology of embryogenic callus of rice varieties on Murashige and Skoog (MS) medium supplemented with 2.0 mg/L of 2,4-D after 3 weeks (Bars = 1 mm). A: TN1, B: Cigeulis, C: GogoNiti II, and D: Ketan Hitam I

Visarada *et al.* (2002) classified callus appearance into four types based on morphology. Type I was a compact, organized callus of white or cream color. In contrast, Type II was a yellow organized callus. Type III was a yellow or brown unorganized callus that turned dark and necrotic. Type IV was a rhizogenic callus.

According to the current study, both TN1 and GogoNiti II were classified as Type I. It showed a light yellow to whitish color with an organized callus, numerous globular in small compact clusters (a pro-embryo stage), embryogenic potential, and the ability to regenerate well. Cigeulis, on the other hand, is classified as Type II (organized yellow callus). In contrast, Ketan Hitam I was classified as a Type IV (rhizogenic callus) with a hairy thread surrounding the callus. The hairy cells become thicker and more whitish during pro-embryo regeneration. Thus, the low embryogenic potential is associated with calluses like these.

4.2.2 Characteristics of callus

Cells will proliferate embryos from mature seeds of rice and form disorganized clumps called callus tissues after applying 2,4-D in media callus induction. They then proliferate and cluster to form pro-embryogenic structures after producing embryogenic cells (Gulzar *et al.*, 2020). Furthermore, genotype had a significant effect on characteristics of callus (Deo *et al*., 2010).

In media callus induction, the standard concentration is 2 mg L^{-1} of 2,4-D. This study used the same concentration in media callus induction to study the formation of callus characters that vary depending on the rice varieties' genetic background in tissue culture. Then, we reported the characteristics of the callus on local Indonesian rice based on the percentage of callus induction and the size of the callus after three weeks under 2 mg L-1 of 2,4-D on MS basal media.

Table 4.2 showed that there was a significant effect ($p \leq 0.05$) of the different rice varieties (TN1, Cigeulis, Gogo Niti II, and Ketan Hitam I) on the percentage of callus induction (%) and the size of callus (mm) that responds to a 2 mg L^{-1} of 2,4-D on MS basal media after three weeks of culture. Ketan Hitam I (88%) showed the best performance same like TN1 (81%) and showed a significant difference with GogoNiti II (70%) and Cigeulis (49%). On the other hand, Cigeulis showed the lowest percentage of callus induction. Moreover, in size of callus, Gogo Niti II (6.76 mm) showed similar size with TN1 (6.60 mm) and had significant difference with Ketan Hitam I (5.06 mm) and Cigeulis (4.93 mm).

Table 4.2 The callus induction (%) and callus size (mm) of 3 weeks calli derived from MS media with 2 mg L^{-1} of 2,4-D

Varieties Name	Percentage of Callus Induction (%)	Callus Size (mm)
TN1	81.00 ± 5.37^{ab}	6.60 ± 0.31^{a}
Cigeulis	49.00 ± 5.22^{c}	4.93 ± 0.29^{b}
GogoNiti II	70.20 ± 5.65^{b}	6.76 ± 0.34^{a}
Ketan Hitam I	87.80 ± 2.31^{a}	5.06 ± 0.19^{b}

Notes : Mean values were taken from an average of three replication (n=3). Means with the same letter in a column are not are significantly different ($p \leq 0.05$) according to Duncan's multiple range test

A concentration of 2 mg L^{-1} of 2,4-D is preferred in mature rice seeds. However, it was widely used by researchers for the primary induction of calluses and the formation of embryogenic calluses (Revathi and Pillai, 2011; Hoque *et al.*, 2013). The present study results confirmed Yinxia and Te-chato (2012), which showed that ninety-two percent callus induction was observed in rice seed explants at 2 mg L^{-1} of 2,4-D in Pusa Basmatil. These similar results were also reported calli produced by dehusked Biris seeds (Libin *et al.*, 2012) and Indian rice seed (Upadhyaya *et al.*, 2015) in MS media supplemented with 2 mg L^{-1} of 2,4-D. Thus, this hormone triggers most embryogenic callus growth and tissue culture systems to revert cells in the explant during the early developmental stages of somatic embryos (Mahdavi-Darvari *et al.*, 2015; Ahmad *et al.*, 2016).

Interestingly, despite using the same concentration, different varieties responded differently. These findings indicate that when treated with the same concentration of 2,4-D, 2 mg L-1, the different characters of callus will respond due to the genetic background in tissue culture. The analysis revealed that genotypic variation exists, and this genetic background can affect callus' morphological appearance and characteristics. Furthermore, different varieties showed different responses to callus induction (Suraiya and Alina 2018). This conclusion is consistent with a previous report by Upadhyaya *et al.* (2015) and Wani *et al.* (2010), which state that cultivars have varying genotypic efficiency for inducing callus. Additionally, several genotypes, including Pajam, Lucky, and Kalizira, showed significantly affected callus induction (Khaleda and Al-Forkan, 2006). Thus, we explored the existing genetic background to obtain precise information on callus induction and plant regeneration response in Javanica rice.

4.3 Plant Regeneration

Next, after three weeks of media callus induction, the embryogenic calli were transferred to plant regeneration media. This plant regeneration medium had three different treatments, which were Control (no hormone), T2: 2 mg L-1 kinetin + 1 mg L-1 NAA (Toki, 1997), and T3: 2 mg L-1 kinetin + 1 mg L-1 BAP + 1 mg

L-1 NAA (Khanna and Raina, 1998). Then, these cultures grow at a temperature of 27°C with a photoperiod of 16/8 h (light/dark).

4.3.1 Developmental of somatic embryogenesis

During somatic embryogenesis, monocotyledonous plants, like rice, have four embryogenesis stages: pro-embryo, globular, scutellar, and coleoptilar. Each stage had a unique surface structure (Debbarma *et al.*, 2019). Somatic embryogenesis in rice was studied in this research. From observation, first, the pro-embryos formed with the masses on the surface of white translucent calluses appeared smooth. It is known as an embryogenic callus. It was then transferred to plant media regeneration. After that, it changed to a globular stage with a mesh-like structure that replaced the fibrillary material. The resulting development of the globular embryo showed that the apical scutellum region flattened, and the ridge's mesh-like structure became low. Then a scutellar notch is formed and developed by the emergence of the coleoptilar. Later, elongated cells on the surface of the coleoptile were identified. Finally, the coleoptile and root development appeared (Mariani, 1998).

Then, we discovered the duration of developing somatic embryogenesis to form globular (A), scutellar (B), and coleoptilar (C) stages by applying different hormones of media regeneration (Figure 4.3). The result showed a significant effect ($p \leq 0.05$) in different hormones of media regeneration to form globular (A). In GogoNiti II and TN1, the combination hormones of T2 and T3 showed the significantly best performance in the rapid growth that took only one day to form globular compared to control (no hormones). However, in Cigeulis, the combination hormone of T2 (1 day) showed significantly better performance than the combination hormones of T3 (2 days) and control (no hormone) (3 days). In Ketan Hitam I, the results showed no significant effect of different hormones on media regeneration. In short, these varieties grew faster when using both combinations of hormones than the control (no hormone) at the globular stage.

Besides, the result showed a significant effect ($p \leq 0.05$) in different hormones of media regeneration to form scutellar (B), especially in TN1 and

GogoNiti II. The combination hormones of T3 and T2 showed the significantly best performance rapid growth to form scutellar compared to control (no hormone). Nonetheless, Ketan Hitam I and Cigeulis demonstrated that both combination hormones had no significant effect on the formation of scutellar compare to control (no hormone).

Lastly, the result showed a significant effect ($p \leq 0.05$) in different hormones of media regeneration to form coleoptilar. In TN1 (9 days) and GogoNiti II (7 days) demonstrated rapid growth to transition from globular to coleoptilar stages with the best combination hormone T3, whereas the combination hormone T2 demonstrated second-best performance in comparison to control. Nevertheless, Ciguelis showed no significant different hormones of media regeneration in developing coleoptilar. While, in Ketan Hitam I, T3 showed the best performance, which only took ten days to form coleoptilar compared to T2 (13 days) and control (13 days). In this study, the development of coleoptilar from globular phases took 1–2 weeks, depending on the variety and hormone used in media regeneration. According to hormones and genotype, GogoNiti II showed the best performance on T3 and the best genotype that can produce coleoptilar in a short period.

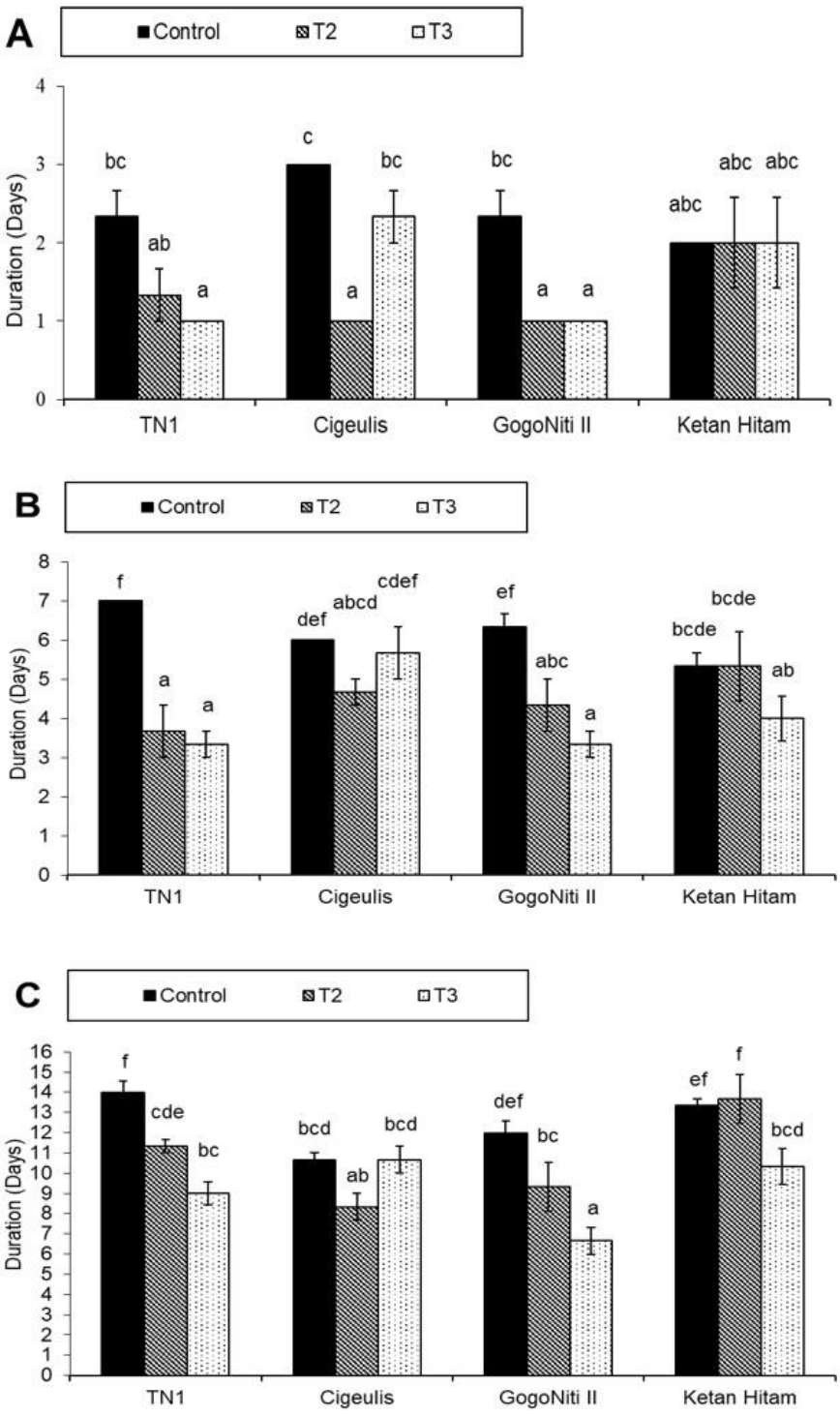


Figure 4.3 The duration for development of somatic embryogenesis stages in different media regeneration hormones. Means with different letters indicate significant differences according to Duncan's multiple range test ($p \leq 0.05$) (n = 3). A: Duration to form globular stage, B: Duration to form scutellar stage, and C: Duration to form coleoptilar stage. Control: no hormone, T2: 2 mg L^{-1} kinetin + 1 mg L^{-1} NAA, and T3: 2 mg L^{-1} kinetin + 1 mg L^{-1} BAP + 1 mg L^{-1} NAA

4.3.2 Percentage of green spots and plant regeneration

Plant regeneration processing starts from the callus formation to pro-embryo, globular, scutellar, and coleoptile. The more potential character of morphogenesis happens, the higher the percentage of green spots or character of somatic embryogenesis development is shown. The differential formation characteristics of morphogenesis were observed during 14 and 28 days using different combinations of hormones and different varieties (Figure 4.4). Plants on 14 and 28 showed morphogenesis into plant regeneration. However, on 28, specific characteristics of morphogenesis were formed towards plant regeneration into plantlet.

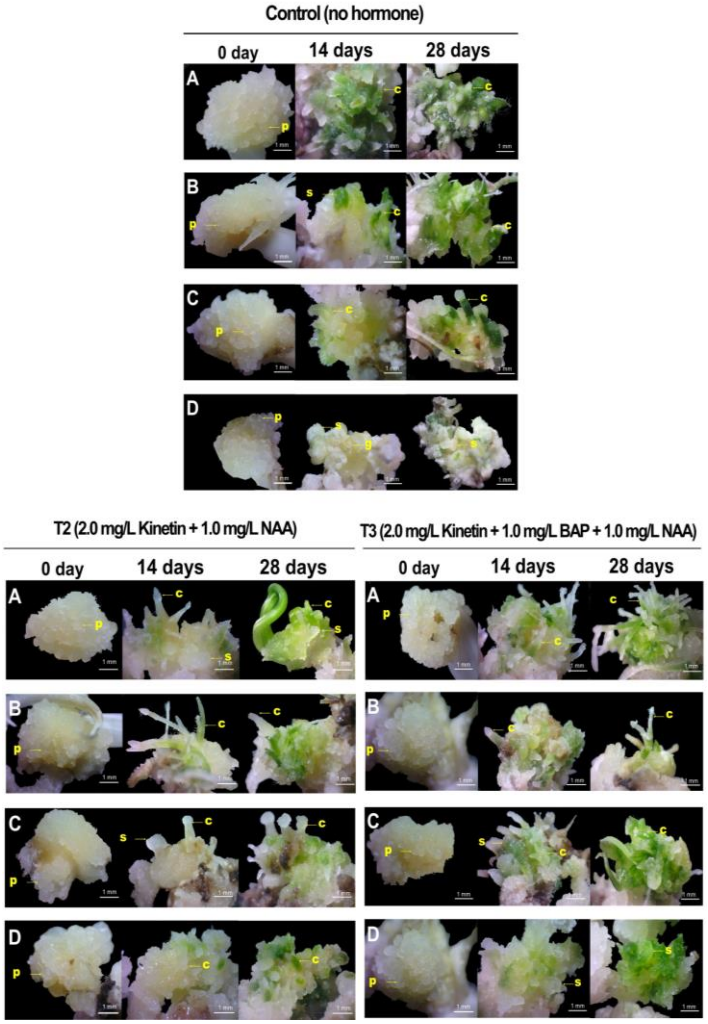


Figure 4.4 The influence of a combination hormone with different varieties on morphological changes in somatic embryos during 14 days and 28 days. A: TN1, B: Cigeulis, C: GogoNiti II, and D: Ketan Hitam I. (p) Pro-embryo. (g) Globular. (s) Scutellar. (c) Coleoptilar. (Bar = 1mm).

Green spot formation is a vital phenomenon to observe because it is a precursor for plant regeneration. It demonstrated a greenish callus area that will differentiate into a shoot in the following days. In this study, the timing of callus greening depends on different combination hormones and different genotypes.

The green spots indicate that the final step, which involves converting divinyl-protochlorophyllide-a (P-chlide) to divinyl-chlorophyllide-a (chlide), is then converted to chlorophyll in chlorophyll biosynthesis. In higher plants, chlorophyll was produced in chloroplasts, which develop from plastids after chloroplast formation (Sinha *et al.,* 2019). Next, most of the green spots will grow into multiple shoots then form plantlets. Researchers also found a correlation between green spot production in mature embryo culture and plant regeneration (Mendoza and Kaeppler, 2002).

The percentage of green spots ($p \geq 0.05$) and plant regeneration ($p \geq 0.05$) between each treatment (Control, T2, and T3) on the different varieties (TN1, Cigeulis, GogoNiti II, and Ketan Hitam I) showed in Table 4.3. Based on the percentage of green spots, T2 (2 mg L-1 kinetin + 1 mg L-1 NAA) showed a high percentage of green spots in all varieties, including TN1, GogoNiti II, Cigeulis, and Ketan Hitam (100%, 92%, 89%, and 82%). On the other hand, T3 also showed a high percentage of green spots only for Cigeulis and GogoNiti II (93% and 83%). Therefore, only Cigeulis (80%) had a high percentage of green spots in control.

Based on the percentage of plant regeneration, GogoNiti II showed the best T2 (100%) performance compared to other varieties and other treatments. In contrast, the second-highest performance was T3 that showed in TN1, Cigeulis, and GogoNitiI (84%, 71%, and 84%), and T2 that showed only in TN1 (88%).

Table 4.3 Percentage of green spots (3 weeks) and plant regeneration (6 weeks) of calli derived from MS media transferred to the regeneration medium. That supplemented with Control (no hormone), T2 (Kinetin 2 mg L^{-1} and NAA 1 mg L^{-1}), and T3 (BAP 1 mg L^{-1}, Kinetin 2 mg L^{-1}, and NAA 1 mg L^{-1}) on different genotypes.

Varieties Name	Percentage of green spots (%)			Percentage of plant regeneration (%)		
	Control	T2	T3	Control	T2	T3
TN1	78 ± 22^{bc}	100 ± 0^{a}	61 ± 6^{bc}	0 ± 0^{d}	78 ± 4^{bc}	71 ± 6^{bc}
Cigeulis	80 ± 20^{bc}	56 ± 29^{bc}	83 ± 8^{bc}	0 ± 0^{d}	67 ± 0^{bc}	50 ± 24^{c}
GogoNiti II	72 ± 17^{bc}	92 ± 8^{a}	93 ± 7^{a}	17 ± 23^{d}	$^{*}100\pm0^{a}$	74 ± 9^{bc}
Ketan Hitam I	37 ± 9^{c}	82 ± 10^{bc}	71 ± 4^{bc}	0 ± 0^{d}	0 ± 0^{d}	0 ± 0^{d}

Notes : Mean values for percentage of green spot (n=3) and percentage of plant generation (n=2) were taken from an average of its replications. According to Duncan's multiple range test, mean values ±SD followed by the same letters in each column are not significantly different at ($p\leq0.05$). However, values in rows or columns with different letters differ significantly. * The highest percentage of plant regeneration.

The results indicate that although Ketan Hitam I had a high percentage of green spots, it had a low percentage of plant regeneration. This happens because non-embryogenic callus with elongated regions produces green spots that look like root apexes but are different structures from an existing green spot. These green spots are formed adjacent to but not in direct contact with existing green spots (Heyser and Nabors, 1982). This maybe prolonged exposure to light causes a high percentage number the growth of green spots forms in non-somatic areas. However, many green spots are grown in somatic embryos, unable to develop into multiple shoots to form plantlets. This is because it may lose its capacity to regenerate by performing a dedifferentiation process maybe because of it genetic backgrund. According to Visarada *et al.* (2002), because the callus characteristics were classified as rhizogenic callus, it was shown to turn green spots and shoots faster but could not regenerate into plantlets. Therefore, green spot forms in non-somatic embryos had significantly lower regeneration potential in both short and long-term cultures (Ben and Amer, 1997).

Factors influencing the success of plant regeneration and the number of plantlet include genotype, degree of callus expansion, and the composition of

growth regulators in plant regeneration media (Heyser and Nabors, 1982; Saharan *et al.*, 2004). T2 and T3 showed significantly different compared to controls (without hormones) because auxin and cytokinins induce the frequency of plant regeneration (Ahmad *et al.* 2016). This study used Kinetin as cytokinin and BAP and NAA at different hormone ratios. Kinetin plays a role in cell morphogenesis and somatic embryogenesis. This can occur when sufficient nutrients support cell growth (G1 cell growth phase). In addition, Kinetin can activate the process of RNA transcription and translation, leading to cell proliferation (G2) and cell division (Umar *et al*. 2017). For T2 and T3 treatments, 2 mg L^{-1} Kinetin was used because cytokinins were required for basal auxin biosynthesis in root and shoot tissues. In addition, biosynthesis should be regulated interactively because developmental or environmental changes can affect auxin (Jones *et al.*, 2010). However, auxin-cytokinin interactions are essential for plant growth and development because they produce and control the growth of plant tissues and organs (Yamada *et al*., 1986; Saharan *et al.*, 2004).

4.3.3 Morphology analysis of plantlet

After 45 days, the callus can produce shoots, roots, or entire plantlets from embryogenic calli. Non-embryogenic calli, on the other hand, can only produce shoots or roots and can not regenerate plantlets. Further, all varieties analyzed the plantlet's morphology after 45 days on media regeneration. GogoNiti II that treated with T2 showed the best performance compare other treatments relative that responded positively to the percentage of plant regeneration. As shown in Table 4.4, T2 in GogoNiti II generated a more significant number of plantlets (9), with morphology analysis revealing plant height (41.87 mm), number of leaves (4), leaf length (15.16 mm), leaf width (0.95 mm), and root length (26.71 mm). In contrast, T3 generated only five plantlets with morphology analysis revealing plant height (54.56 mm), the number of leaves (4), leaf length (50.83 mm), leaf width (0.88 mm), and root length (3.74 mm), and Control generated two plantlets with morphology analysis revealing plant height (23.19 mm), the number of leaves (5), leaf length (23.19 mm) and leaf width (0.94 mm).

On the other hand, T3 in TN1 had the second-highest number of plantlets (3), with morphology analysis revealing plant height (28.67 mm), the number of leaves (5), leaf length (22.26 mm), leaf width (1.06 mm), and root length (6.41 mm). In comparison to Control, which did not produce a plantlet or a slow response, T2 generated four plantlets with morphology analysis revealing plant height (116.49 mm), the number of leaves (8), leaf length (105.59 mm), leaf width (1.48 mm), and root length (10.90 mm).

Cigeulis showed the low number of plantlets. Within treatements, T3 which was analysis revealing 2 plantlets with morphology plant height (67.86 mm), the number of leaves (5), leaf length (36.95 mm), leaf width (1.01 mm), and root length (30.91 mm) compared to T2 show the number of plantlet which was 1 plantlets with morphology analysis revealing plant height (139.49 mm), the number of leaves (7), leaf length (133.69 mm), leaf width (1.05 mm), and root length (5.80 mm). However, Ketan Hitam I showed not produce plantlet or a slow response in all treatment.

Table 4.4 Morphology analysis after 45 days become plantlet for each variety.

Variety	**Hormone**	**NP**	**PH (mm)**	**NL**	**LL (mm)**	**LW (mm)**	**RL (mm)**
TN1	Control	0	0	0	0	0	0
	T2	4	116.49	8	105.59	1.48	10.90
	T3	3	28.67	5	22.26	1.06	6.41
Cigeulis	Control	1	8.27	4	4.29	1.35	3.98
	T2	1	139.49	7	133.69	1.05	5.80
	T3	2	67.86	5	36.95	1.01	30.91
Gogo Niti II	Control	2	23.19	5	23.19	0.94	-
	T2	*9	41.87	4	15.16	0.95	26.71
	T3	5	54.56	4	50.83	0.88	3.74
Ketan Hitam I	Control	0	0	0	0	0	0
	T2	0	0	0	0	0	0
	T3	0	0	0	0	0	0

Notes : NP number of plantlets, PH plant height, NL number of leaves, LL leaf length, LW leaf width, RL root length.

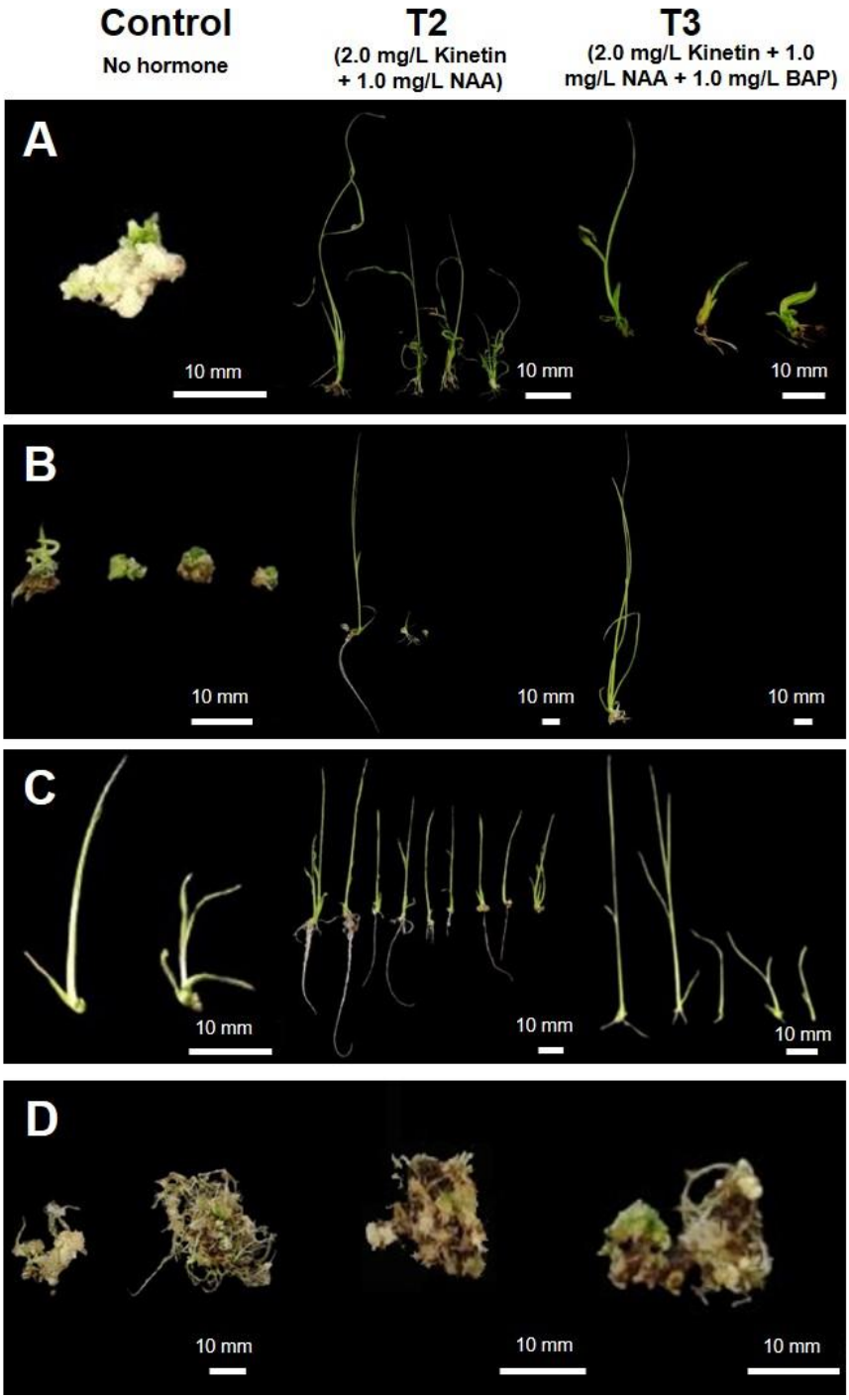


Figure 4.5 Plantlet on the 45th days after apply hormone in media regeneration. (Bars: 10 mm). A: TN1, B: Cigeulis, C: GogoNiti II, and D: Ketan Hitam I. T2: 2 mg L^{-1} kinetin + 1 mg L^{-1} NAA, and T3: 2 mg L^{-1} kinetin + 1 mg L^{-1} BAP + 1 mg L^{-1} NAA

In mass propagation, the total number of plantlet regenerations was the most critical factor. A previous study shows that increasing NAA concentration improves plantlet number (Tsukahara and Hirosawa, 1992). However, the concentration of NAA in T2 and T3 concentration was the same in this research. Thus, it may cause the genotype and different hormone combinations related to this study's expression genes.

Aside from that, the effects of different exogenous hormone combinations and different genotypes responded significantly on somatic embryogenesis development, the percentage of plant regeneration, and morphology analysis of plantlets. Furthermore, according to Yang and Zhang (2010), transcriptional and epigenetic factors regulate hormone activity through biosynthesis and signaling effects, generating feedback networks. In addition, an increasing number of studies have revealed a strong link between plant hormone signaling and epigenetic pathways, with epigenetic mechanisms regulating it simultaneously.

4.4 Gene Expression

Somatic embryogenesis is a complete model of totipotency that requires the activation of a complex signalling network and the reprogramming of gene expression patterns that are regulated in a specific way. Typically, this gene regulation occurs in response to external stimuli such as the usage of hormones or specific stress conditions such as low or high temperature, heavy metals, osmotic shock, or drought (Méndez-Hernández *et al.*, 2019). Somatic embryogenesis of a complex network of interactions regulates the expression of numerous genes, and several gene sets are activated or suppressed via different signal transduction pathways (Nolan *et al*, 2003; Mahdavi-Darvari *et al.*, 2015).

In this study, during somatic embryo development, the relative expression levels of the *OsSERK*, *OsLEC1* and *OsWOX4* genes were analysed via thickness of band. A gene expression analysis was performed after the callus had been differentiated in the second subculture (14 and 28 days) in media regeneration. Analysis of gene expression reveals no gene expression related to somatic embryogenesis during the first weeks of media regeneration. That is why, in this

study, we focus on somatic embryo development at 14 and 28 days in media regeneration. Based on Figure 4.6, *OsSERK*, *OsWOX4*, and *OsLEC1* showed different expression patterns.

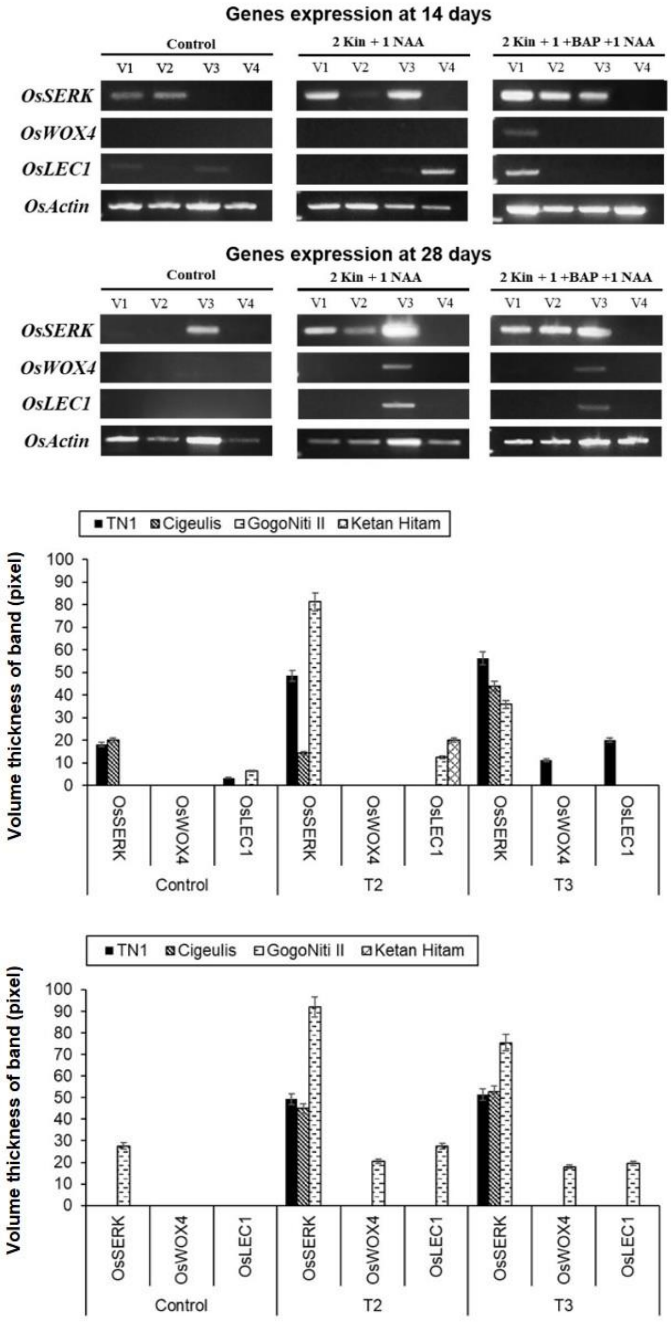


Figure 4.6 Expression of *OsSERK*, *OsWOX4* and *OsLEC1* were performed from total RNA samples by using thickness of band from callus under media regeneration with different hormones at 14 and 28 days. *OsActin* was used as a reference gene. (V1) TN1. (V2) Cigeulis. (V3) GogoNiti II. (V4) Ketan Hitam I. Control (no hormone). T2 (2.0 mg/L Kinetin, 1.0 mg/L NAA). T3 (2.0 mg/L Kinetin, 1.0 mg/L BAP, 1.0 mg/L NAA).

This may be because of the hormones and genotypes that also influence gene expression that has been identified. Based on this result, we can conclude that the process epigenetic happen during somatic embryo development because there is a relationship between gene expression and morphological changes in plant regeneration by using different varieties and combinations of hormones.

Only the *OsSERK* gene was significantly expressed at 14 and 28 days during somatic embryo development. Based on gene expression during day 28, it is clear from the data that expressing *OsSERK* can be related to the morphology of plant regeneration, which can regenerate well into plantlets. Because *OsSERK* can function well, especially in morphogenesis and other developmental processes (Singla *et al*. 2009). We can conclude that *OsSERK* accumulation responds to the morphogenetic capacity of callus tissues in *Oryza sativa* on day 28 during somatic embryo development.

Another monocot, the *CnSERK* gene, was expressed in coconut during embryogenic callus formation before embryo development and in somatic embryos. Besides, it was not detected or expressed at lower levels in non-embryogenic tissues. Similar findings have been obtained in cacao. *TcSERK* expression was detected in somatic and zygotically mature *Theobroma cacao* embryos, indicating a function in embryo development (de Oliveira Santos *et al*. 2005). In dicot species, in carrot, it has been reported that *DcSERK* expression begins during the early globular stage and ceases once the globular stage has reached completion of development (Schmidt *et al.*, 1997). Until the heart stage, Arabidopsis, *AtSERK1* increases the efficiency of somatic embryogenesis initiation by three to fourfold (Hecht *et al.*, 2002). Thus, in most cases, *SERK* genes have played a role in somatic embryogenesis, specifically in the early stages. However, these findings demonstrate the novelty. The *OsSERK* gene expressed during somatic embryo development was discovered in rice.

Besides, this study showed that different combinations of hormones have a differential effect on gene expression. On days 14 and 28, the data showed that T2 and T3 had high expression levels of *OsSERK* compared to control. The pattern of *OsSERK* gene expression increased with the addition of a combination hormone

compared to the control. Results show that exogenous auxin levels may stimulate *SERK* gene regulation and that auxin exposure could impact endogenous or exogenous *SERK* signals. Also, adding auxin to the culture media causes high *SERK* expression in certain species, including carrots and Arabidopsis. Identical findings have been made in *M. truncatula*. In the presence of both NAA and BAP, *MtSERK1* expression is significantly increased. Also found in cacao. In *Cocos nucifera* (L.), cells detect the accumulation of 2,4-D and activate a signalling pathway that induces the expression of *SERK* involved in somatic embryogenesis (Pérez-Núñez *et al.*, 2009). Additionally, BAP promotes NAA induction because BAP does not induce *MtSERK1* expression on its own (Nolan *et al.* 2003). In this study, the *SERK* family's function is related to interactions within the complex network of somatic embryogenesis.

On day 28, only GogoNiti II expressed *OsSERK, OsWOX4,* and *OsLEC1* when treated with T2 and T3 compared to control (no hormone). Thus, *OsSERK* accumulation with the presence of *OsWOX4* and *OsLEC1* may also affect the process of embryogenesis, which enhances their ability to respond positively to morphology after 45 days in callus tissue in GogoNiti II (Figure 4.5). Still, it needs to be studied further because there is still no evidence that *SERKs* are involved in a *WUS* signalling pathway (Méndez-Hernández *et al.*, 2019). However, the expression of *OsWOX4* can be related to *OsLEC1*. For example, in *Arabidopsis thaliana*, *WUSCHEL* (*WUS*) is responsible for activating *LEC* genes. In *Gossypium hirsutum*, *WUS* activates *GhLEC1*, *GhLEC2*, and *GhFUS3* genes to develop somatic embryogenesis and induce cell differentiation (Kumar and Van Staden, 2017). Therefore, in this study, *OsSERK* accumulation was found together with *OsWOX4* and *OsLEC1* during somatic embryo development to cause a positive response to morphogenetic potential by generating many plantlets from callus cells.

In contrast, *OsWOX4* and *OsLEC1* were absent or expressed at low levels (Figure 4.6). Sometimes, the total number of genes turned off in somatic cells is higher than the number of genes turned on to facilitate the transition from somatic to embryogenic development. This is because differential gene expression can influence a cell's embryogenic ability (Quiroz-Figueroa *et al.*, 2006). There are

several other studies that have found *ScLEC1* has not been detected in *Secale* L. after four weeks on regenerating medium (Gruszczyńska and Rakoczy-Trojanowska, 2011b). That is, the previous study discovered that *LEC1* was highly expressed in somatic embryogenesis in *Coffea canephora* at day 7 compared to days 14 and 21, which were low or almost undetectable. In addition, on days 14 and 28, *WOX4* was completely absent or low in expression. This is because *WOX4* expression decreases during embryo maturation (Nic-Can *et al.*, 2013).

CHAPTER 5. CONCLUSION AND RECOMMENDATION

5.1 Conclusion

In conclusion, *OsSERK* was the only gene expressed at very high levels on days 14 and 28 during somatic embryogenesis in all rice varieties in this study. In contrast, the *OsWOX4* and *OsLEC1* genes were either not expressed or expressed at low levels. Therefore, *SERK* accumulation may be necessary for somatic embryo development. However, three genes that can be expressed may indicate positive results in plant regeneration morphology, like GogoNiti II. Furthermore, T2 and T3 also affect somatic embryogenesis, plant regeneration, plantlets, and gene expression differently. Additionally, plant hormone signalling is related to epigenetic changes. Epigenetic processes have been discovered to alter rice cells during somatic embryonic development efficiently. These biological variables may offer future knowledge for rice breeding and understanding rice biology.

5.2 Recommendation

Some further research on epigenetic regulation that can provide very valuable detailed information for the field of biotechnology in gene transformation in plants in the future, with the goal of increasing the quality and quantity of local Indonesian rice, hope can give benefit to this country in the future in order to meet population and demand in 2050.

BIBLIOGRAPHY

Abe, T., and Y. Futsuhara. 1986. Genotypic variability for callus formation and plant regeneration in rice (*Oryza sativa* L.). *Theoretical and Applied Genetics*. 72: 3-10.

Abrahamian, P., and A. Kantharajah. 2011. Effect of vitamins on in vitro organogenesis of plant. *American Journal of Plant Sciences*. 2: 669.

Ahmad, F. I., A. Wagiran, A. Abd Samad, Z. Rahmat, and M. R Sarmidi. 2016. Improvement of efficient in vitro regeneration potential of mature callus induced from Malaysian upland rice seed (*Oryza sativa cv. Panderas*). *Saudi Journal of Biological Sciences*. 23: S69-S77.

Badawi, T. A. 2004. Rice-based production systems for food security and poverty alleviation in the Near East and North Africa: new challenges and technological opportunities. *Proceedings.* February 2004. *FAO Rice Conference Rome, Italy*: 12-13.

Ben Amer, I. M., and A. Borner. 1997. Relationship between green spot initiation and plantlet regeneration of wheat callus grown under short-term conditions. *Plant cell, tissue and organ culture*. 50: 67-69

Campos-Rivero, G., P. Osorio-Montalvo, R. Sánchez-Borges, R. Us-Camas, F. Duarte-Aké and C. De-la-Peña. 2017. Plant hormone signaling in flowering: an epigenetic point of view. *Journal of plant physiology*. 214: 16-27.

Debbarma, R., D. Sudhakar, K. K. Kumar, and K. Soorianathasundaram. 2019. Morphological and Ultrastructure of Developmental Stages of Somatic Embryos of Popular Banana Cultivars. *International Journal of Current Microbiology and Applied Science*. 8: 1676-1683.

Deshmukh, D. B. 2015. Molecular and phenotypic evaluation of rice near isogenic lines (Nils) with pi2 and pi5 genes in bpt5204 background for blast resistance (Doctoral dissertation, University of Agricultural Sciences Dharwad).

de Oliveira Santos, M., E. Romano, K. S. C. Yotoko, M. L. P. Tinoco, B. B. A. Dias, and F. J. L. Aragao. 2005. Characterisation of the cacao somatic embryogenesis receptor-like kinase (*SERK*) gene expressed during somatic embryogenesis. *Plant Science*. 168: 723-729.

Deo, P. C., M. Taylor, R. M. Harding, A. P. Tyagi, and D. K. Becker. 2010. Initiation of embryogenic cell suspensions of taro (*Colocasia esculenta var.*

esculenta) and plant regeneration. *Plant Cell, Tissue and Organ Culture (PCTOC)*. 100: 283-291.

Dolzblasz, A., J. Nardmann, E. Clerici, B. Causier, E. van der Graaff, J. Chen, and T. Laux. 2016. Stem cell regulation by Arabidopsis *WOX* genes. *Molecular plant*. 9: 1028-1039.

Dudley, J. W. 2002. Integrating molecular techniques into quantitative genetics and plant breeding. *Quantitative Genetics, Genomics and Plant Breeding. CAB International, Wallingford, UK*. 69-83.

Escobar-Guzmán, R. E., F. Hernández-Godínez, O. M. De La Vega, and N. Ochoa-Alejo. 2009. In vitro embryo formation and plant regeneration from anther culture of different cultivars of Mexican husk tomato (*Physalis ixocarpa* Brot.). *Plant Cell, Tissue and Organ Culture (PCTOC)*. 96: 181-189.

Gambino, G., M. Minuto, P. Boccacci, I. Perrone, R. Vallania, and I. Gribaudo. 2011. Characterization of expression dynamics of *WOX* homeodomain transcription factors during somatic embryogenesis in *Vitis vinifera*. *Journal of experimental botany*. 62: 1089-1101.

Ganeshan, S., M. Båga, B. L. Harvey, B. G. Rossnagel, G. J. Scoles, and R. N. Chibbar. 2003. Production of multiple shoots from thidiazuron-treated mature embryos and leaf-base/apical meristems of barley (*Hordeum vulgare*). *Plant Cell, Tissue and Organ Culture*. 73: 57-64.

Gelvin SB. 2003. Agrobacterium-mediated plant transformation: the biology behind the "gene-jockeying" tool. Microbiology and molecular biology reviews. 67: 16-37.

Gruszczyńska, A., and M. Rakoczy-Trojanowska. 2011a. Expression analysis of somatic embryogenesis-related SERK, LEC1, VP1 and NiR ortologues in rye (*Secale cereale* L.). *Journal of applied genetics*. 52: 1-8.

Guo, F., C. Liu, H. Xia, Y. Bi, C. Zhao, Zhao S., and X. Wang. 2013. Induced expression of *AtLEC1* and *AtLEC2* differentially promotes somatic embryogenesis in transgenic tobacco plants. *PLoS One*. *8*: e71714.

Gulzar, B., A. Mujib, M. Q. Malik, R. Sayeed, J. Mamgain, and B. Ejaz. 2020. Genes, proteins and other networks regulating somatic embryogenesis in plants. *Journal of Genetic Engineering and Biotechnology*. 18: 1-15.

Hecht, V., J. P. Vielle-Calzada, M. V. Hartog, E. D. Schmidt, K. Boutilier, U. Grossniklaus, and S. C. de Vries. 2001. The *Arabidopsis SOMATIC EMBRYOGENESIS RECEPTOR KINASE 1* gene is expressed in developing

ovules and embryos and enhances embryogenic competence in culture. *Plant Physiology*. 127: 803-816.

Heyser, J. W., and M. W. Nabors. 1982. Long term plant regeneration, somatic embryogenesis and green spot formation in secondary oat (*Avena sativa*) callus. *Zeitschrift für Pflanzenphysiologie*. 107: 153-160.

Horstman, A., M. Li, I. Heidmann, M. Weemen, B. Chen, J. M. Muino, and K. Boutilier. 2017. The BABY BOOM transcription factor activates the *LEC1-ABI3-FUS3-LEC2* network to induce somatic embryogenesis. *Plant physiology*. 175: 848-857.

Hoque, K. M. A., Z. A. Azdi, and S. H. Prodhan, 2013. Development of callus initiation and regeneration system of different indigenous indica rice varieties. *J. Biol*. 1: 46-51.

Jones, B., S. A. Gunnerås, S. V. Petersson, P. Tarkowski, N. Graham, S. May, and K. L jung. 2010. Cytokinin regulation of auxin synthesis in Arabidopsis involves a homeostatic feedback loop regulated via auxin and cytokinin signal transduction. *The Plant Cell*. 22: 2956-2969.

Kadhimi, A. A., C. R. C. M. Zain, A. N. Alhasnawi, A. Isahak, M. F. Ashraf, A. Mohamad, and W. M. W. Yusoff. 2016. Impact of gamma rays exposure and growth regulators on *Oryza sativa* L. cv MR269 callus induction. In *AIP Conference Proceedings* 1784(1). November 2016. *AIP Publishing LLC:* 020006.

Khaleda, L., and M. Al-Forkan. 2006. Genotypic variability in callus induction and plant regeneration through somatic embryogenesis of five deepwater rice (*Oryza sativa* L.) cultivars of Bangladesh. *African Journal of Biotechnology*. 5: 16.

Khanna, H. K., and S. K. Raina. 1998. Genotype x culture media interaction effects on regeneration response of three indica rice cultivars. *Plant Cell, Tissue and Organ Culture*. 52: 145-153.

Khush, G. S. (1987) Rice breeding: past, present and future. *Journal of Genetics*. 66: 195-216.

Kumar, V., and J. Van Staden. 2017. New insights into plant somatic embryogenesis: an epigenetic view. *Acta Physiologiae Plantarum*. 39: 1-17.

Libin, A., P. J. H. King, K. H. Ong, J. K. Chubo, and P. Sipen. 2012. Callus induction and plant regeneration of Sarawak rice (*Oryza sativa* L.) variety Biris. *African Journal of Agricultural Research*. 7: 4260-4265.

Mahdavi-Darvari, F., N. M. Noor, and I. Ismanizan. 2015. Epigenetic regulation and gene markers as signals of early somatic embryogenesis. *Plant Cell, Tissue and Organ Culture (PCTOC)*. 120: 407-422.

Mariani, T. S., H. Miyake and Y. Takeoka. 1998. Changes in surface structure during direct somatic embryogenesis in rice scutellum observed by scanning electron microscopy. *Plant Production Science*. 1: 223-231.

Méndez-Hernández, H. A., M. Ledezma-Rodríguez, R. N. Avilez-Montalvo, Y. L.Juárez-Gómez, A. Skeete, J. Avilez-Montalvo, and V. M. Loyola-Vargas. 2019. Signaling overview of plant somatic embryogenesis. *Frontiers in plant science*. 10: 77.

Mendoza, M. G., and Kaeppler, H. F. 2002. Auxin and sugar effects on callus induction and plant regeneration frequencies from mature embryos of wheat (*Triticum aestivum* L.). *Vitr. Cell. Dev. Bio. - Plant.* 38: 39-45.

Mirsam, H., and Sudartik, E. 2017. Resistance of some rice varieties against rice seed bug (*Leptocorisaacuta*). In Proceeding International Conference on Natural and Social Science (ICONSS). 1:1.

Neo-Probe. 2018. Bioneer Oligo Synthesis Report. www.neoprobe.co.kr. 2018.

Nic-Can, G. I., A. López-Torres, F. Barredo-Pool, K. Wrobel, V. M. Loyola-Vargas, R. Rojas-Herrera, and C. De-la-Peña, 2013. New insights into somatic embryogenesis: *LEAFY COTYLEDON1*, *BABY BOOM1* and *WUSCHEL-RELATED HOMEOBOX4* are epigenetically regulated in Coffea canephora. *PLoS One*. 8: e72160.

Nolan, K. E., R. R. Irwanto, and R. J. Rose. 2003. Auxin up-regulates *MtSERK1* expression in both Medicago truncatula root-forming and embryogenic cultures. *Plant Physiology*. 133: 218-230.

Osorio-Montalvo, P., L. Sáenz-Carbonell, and C. De-la-Peña. 2018. 5-azacytidine: A promoter of epigenetic changes in the quest to improve plant somatic embryogenesis. *International journal of molecular sciences*, *19*: 3182.

Paudel, Y. P., S. Pradhan, B. Pant, and B. N. Prasad. 2012. Role of blue green algae in rice productivity. *Agriculture and Biology Journal of North America*. 3: 332-335.

Pérez-Núñez, M. T., R. Souza, L. Sáenz, J. L. Chan, J. J. Zuniga-Aguilar, and C. Oropeza, 2009. Detection of a SERK-like gene in coconut and analysis of its expression during the formation of embryogenic callus and somatic embryos. *Plant cell reports*. 28: 11-19.

Quiroz-Figueroa, F. R., R. Rojas-Herrera, R. M. Galaz-Avalos, and V. M. Loyola-Vargas, 2006. Embryo production through somatic embryogenesis can be used to study cell differentiation in plants. *Plant Cell, Tissue and Organ Culture*. 86: 285-301.

Revathi, S., and M. A. Pillai. 2011. In vitro callus induction in rice (*Oryza sativa* L.). *Research in Plant Biology*. 1: 13-15.

Rueb, S., M. Leneman, R. A. Schilperoort, and L. A. M. Hensgens. 1994. Efficient plant regeneration through somatic embryogenesis from callus induced on mature rice embryos (*Oryza sativa* L.). *Plant Cell, Tissue and Organ Culture*. 36: 259-264.

Salaün, C., Lepiniec, L., and Dubreucq, B. 2021. Genetic and Molecular Control of Somatic Embryogenesis. *Plants*. 10: 1467.

Saharan, V., R. C. Yadav, N. R. Yadav, and B. P. Chapagain. 2004. High frequency plant regeneration from desiccated calli of indica rice (*Oryza sativa* L.). *African Journal of Biotechnology*. 3: 256-259.

Sakulsingharoj, C., P. Inta, R. Sukkasem, S. Pongjaroenkit, S. Chowpongpang, and V. Sangtong. 2014. Overexpression of *OSB2* gene in transgenic rice up-regulated expression of structural genes in anthocyanin biosynthesis pathway. *Genomics and Genetics. 7*: 73-182.

Santos, M. O., and F. J. Aragão. 2009. Role of SERK genes in plant environmental response. *Plant signaling & behavior*. 4: 1111-1113.

Schmidt, E. D., F. Guzzo, M. A. Toonen, and S. C. De Vries. 1997. A leucine-rich repeat containing receptor-like kinase marks somatic plant cells competent to form embryos. *Development*. 124: 2049-2062.

Searchinger, T., R. Waite, C. Hanson, J. Ranganathan, P. Dumas, E. Matthews, and C. Klirs. 2019. *Creating a sustainable food future: a menu of solutions to feed nearly 10 billion people by 2050.* Washington: *Final report* WRI.

Siddique, A. B., I. Ara, S. S. Islam, and N. Tuteja. 2014. Effect of air desiccation and salt stress factors on in vitro regeneration of rice (*Oryza sativa* L.). *Plant signaling & behavior*. 9: e977209.

Singla, B., J. P. Khurana, and P. Khurana. 2009. Structural characterization and expression analysis of the *SERK/SERL* gene family in rice (*Oryza sativa*). *International journal of plant genomics*. 2009: 1-8.

Sinha, A., M. Solanki and L. I. Shukla. 2019. Evidences for differential expression of miR167d-5p, target, positional nucleotide preference, and its role in

somatic and different stages of regenerating calli of *Oryza sativa*. *Plant Cell, Tissue and Organ Culture (PCTOC)*. 136: 537-548.

Sivamani, E., P. Shen, N. Opalka, R. N. Beachy, and C. M. Fauquet. 1996. Selection of large quantities of embryogenic calli from indica rice seeds for production of fertile transgenic plants using the biolistic method. *Plant Cell Reports*. 15: 322-327.

Springer, N. M., and R. J. Schmitz. 2017. Exploiting induced and natural epigenetic variation for crop improvement. *Nature reviews genetics*. 18: 563-575.

Suer, S., J. Agusti, P. Sanchez, M. Schwarz, and T Greb. 2011. *WOX4 i*mparts auxin responsiveness to cambium cells in Arabidopsis. *The Plant Cell*. 23: 3247-3259.

Suraiya, M., and W. Alina. 2018. Efficient callus induction and regeneration in selected indica rice. *Agronomy*. 8.

Tiwari, S., M. C. Yadav, N. Dikshit, V. K. Yadav, D. R. Pani, and M. Latha. 2020. Morphological characterization and genetic identity of crop wild relatives of rice (*Oryza sativa* L.) collected from different ecological niches of India. *Genetic Resources and Crop Evolution*. 67: 2037-2055.

Toki, S., 1997. Rapid and efficientAgrobacterium-mediated transformation in rice. *Plant Molecular Biology Reporter*. 15: 16-21.

Tsukahara, M., and T. Hirosawa. 1992. Characterization of factors affecting plantlet regeneration from rice (*Oryza sativa* L.) callus. *The botanical magazine= Shokubutsu-gaku-zasshi*. 105: 227-233.

Umar, R., Y. Wibisono, and Ermawati, N. 2017. Effect of Medium Compositions on The Growth of Rice (*Oryza sativa* L. cv. Ciherang) Callus. *UNEJ e-Proceeding*: 97-100.

Utami, I., R. Rusmana, F. R. Eris, and S. Sjaifuddin. 2019. Physical properties on Indonesian local rice varieties. In *IOP Conference Series: Earth and Environmental Science* 383 (1). November 2019. *IOP Publishing: 012026.*

Upadhyaya, G., M. Sen and A. Roy. 2015. In vitro callus induction and plant regeneration of rice (*Oryza sativa* L.) var. 'Sita', 'Rupali' and 'Swarna Masuri'. *Asian Journal of Plant Science and Research*. 5: 24-27.

Visarada, K. B. R. S., M. Sailaja, and N. P. Sarma. 2002. Effect of callus induction media on morphology of embryogenic calli in rice genotypes. Biologia Plantarum. 45: 495-502.

Von Arnold, S., I. Sabala, P. Bozhkov, J. Dyachok, and L. Filonova. 2002. Developmental pathways of somatic embryogenesis. *Plant cell, Tissue and Organ culture*. 69: 233-249.

Wani, S. H., P. A. Sofi, S. S. Gosal, and N. B. Singh. 2010. In vitro screening of rice (*Oryza sativa* L) callus for drought tolerance. *Communications in Biometry and Crop Science*. 5: 108-115.

Yamada, Y., Z. Q. Yang, and D. T. Tang. 1986. Plant regeneration from protoplast-derived callus of rice (*Oryza sativa* L.). *Plant cell reports*. 5: 85-88.

Yamamuro, C., J K Zhu, and Yang, Z. 2016. Epigenetic modifications and plant hormone action. *Molecular plant*. 9: 57-70.

Yang, X., and X. Zhang. 2010. Regulation of somatic embryogenesis in higher plants. *Critical Reviews in Plant Science*. *29*: 36-57.

Ye, N., G. Zhu, Y. Liu, A. Zhang, Y. Li, R. Liu, and J. Zhang, 2012. Ascorbic acid and reactive oxygen species are involved in the inhibition of seed germination by abscisic acid in rice seeds. *Journal of experimental botany*. 63: 1809-1822.

Yinxia, Z., and S. Te-Chato. 2012. Callus induction and plantlet regeneration from mature embryos of indica rice (*Oryza Sativa* L.) cultivar Kra Dang Ngah. *J AgricTech*. 8: 2423-2433.

Zhao, P., K. Begcy, T. Dresselhaus, and M. X. Sun, 2017. Does early embryogenesis in eudicots and monocots involve the same mechanism and molecular players?. *Plant Physiology*. *173*: 130-142.

Zhu, Y. 2010. The epigenetic involvement in plant hormone signaling. *Chinese Science Bulletin*. 55: 2198-2203.

APPENDICES

Appendix 1. Documentation of research activities

a. Making Media

b. Autoclave

c. Using Laminar Air Flow

d. Using light microscope

e. Process of RNA extraction

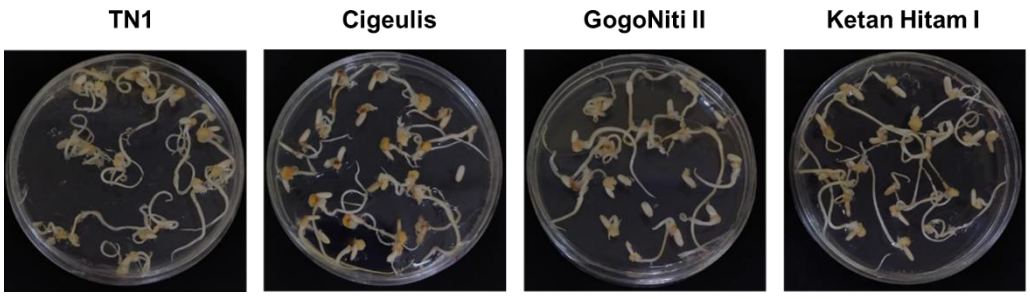
f. Observation of culture

Appendix 2. Callus Induction

Analysis data by SPSS 16 V

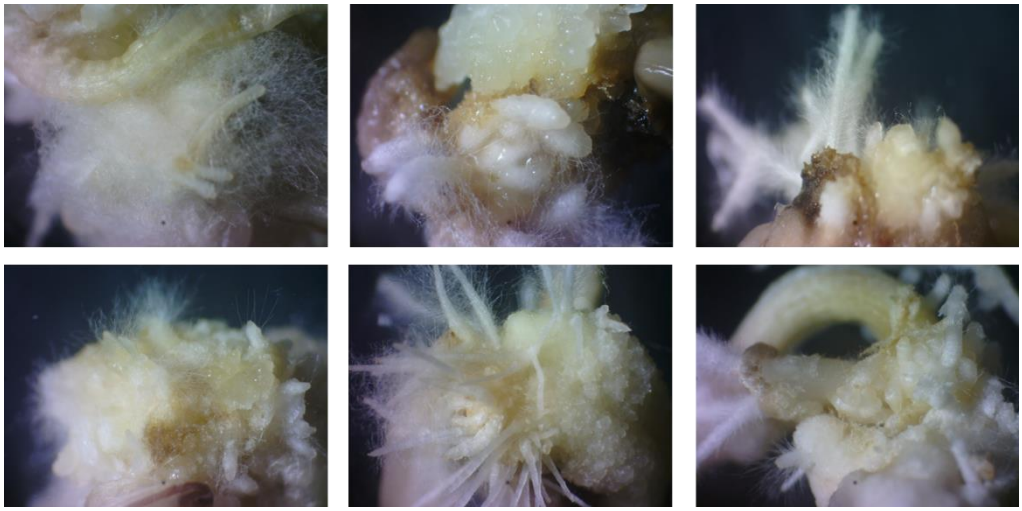
Descriptives								
Varieties name		**R1**	**R2**	**R3**	**R4**	**R5**	**Statistic**	**Std. Error**
Callus induction frequency	**TN1**	93	87	75	63	87	81.00	5.37
	Cigeulis	47	38	53	67	40	49.00	5.22
	Gogo Niti II	80	73	81	50	67	70.20	5.65
	Ketan Hitam I	87	92	87	80	93	87.80	2.31

Callus induction frequency				
Duncan[a,b]				
		Subset		
Varieties name	N	c	b	a
Cigeulis	5	49.00		
Gogo Niti II	5		70.20	
TN1	5		81.00	81.00
Ketan Hitam	5			87.80
Sig.		1.00	0.13	0.33



Callus induction of cultured on MS media with 2 mg/l of 2,4-D after 2 week

Appendix 3. Characteristics of Callus



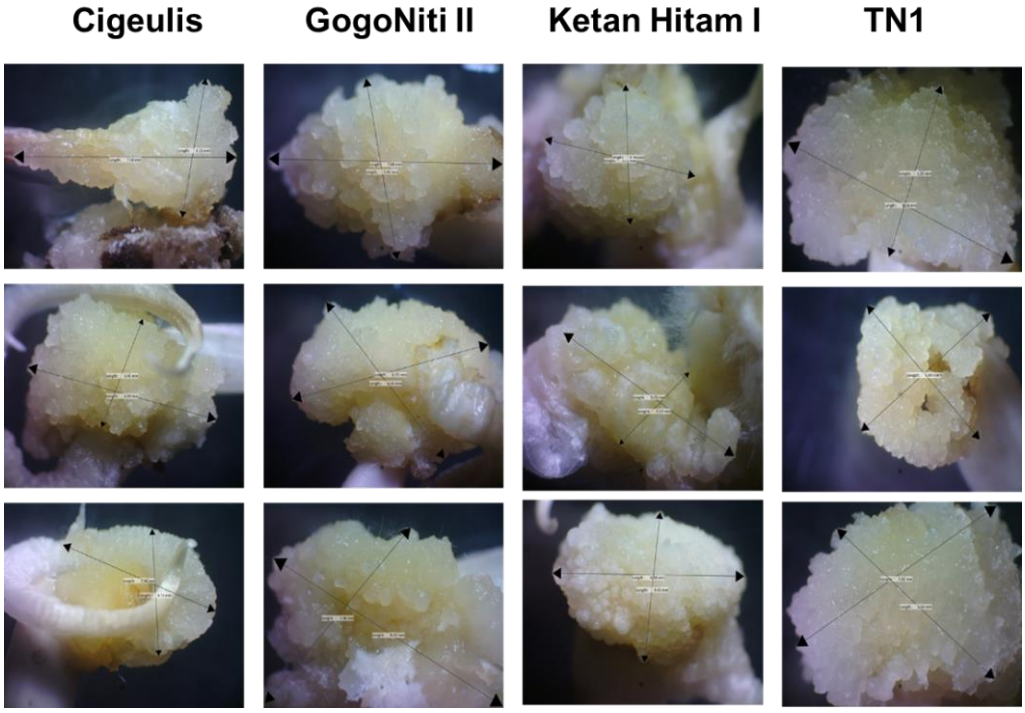
Specialized characteristics of callus in media callus induction in Local Indonesia rice.

Appendix 4. Size of callus

Analysis data by SPSS 16 V

Descriptives								
Varieties name		**R1**	**R2**	**R3**	**R4**	**R5**	**Statistic**	**Std. Error**
Size of callus	**TN1**	7	5	7	7	7	6.60	0.31
	Cigeulis	5	6	5	5	4	4.93	0.29
	Gogo Niti II	6	7	8	7	6	6.76	0.34
	Ketan Hitam I	5	5	5	6	5	5.06	0.19

Size of callus			
Duncan[a,b]			
		Subset	
Varieties name	N	b	a
Cigeulis	5	4.93	
Ketan Hitam	5	5.06	
TN1	5		6.60
Gogo Niti II	5		6.76
Sig.		0.74	0.71



Measure size of allus using light microscope by for each variety of rice Image Raster 3.

Appendix 5. Duration of somatic embryogenesis

Analysis data by SPSS 16 V

Globular

Descriptives							
Varieties name		**Treatment**	**R1**	**R2**	**R3**	**Statistic**	**Std. Error**
Globular	**TN1**	V1T1	3	2	2	2.33	0.58
		V1T2	1	2	1	1.33	0.58
		V1T3	1	1	1	1.00	0.00
	Cigeulis	V2T1	3	3	3	3.00	0.00
		V2T2	1	1	1	1.00	0.00
		V2T3	3	2	2	2.33	0.58
	Gogo Niti II	V3T1	3	2	2	2.33	0.58
		V3T2	1	1	1	1.00	0.00
		V3T3	1	1	1	1.00	0.00
	Ketan Hitam I	V4T1	2	2	2	2.00	0.00
		V4T2	2	2	1	1.67	0.58
		V4T3	3	3	1	2.33	1.15

Globular					
Duncan[a,b]					
		Subset			
Treatment	N	a	b	c	d
V1T3	3	1.00			
V2T2	3	1.00			
V3T2	3	1.00			
V3T3	3	1.00			
V1T2	3	1.33	1.33		
V4T2	3	1.67	1.67	1.67	
V4T1	3		2.00	2.00	
V1T1	3			2.33	2.33
V2T3	3			2.33	2.33
V3T1	3			2.33	2.33
V4T3	3			2.33	2.33
V2T1					3.00
Sig.	3	0.16	0.13	0.16	0.16

Scutellar

Descriptives							
Varieties name		**Treatment**	**R1**	**R2**	**R3**	**Statistic**	**Std. Error**
Scutellar	**TN1**	V1T1	7	7	7	7.00	0.00
		V1T2	3	5	3	3.67	1.15
		V1T3	4	3	3	3.33	0.58
	Cigeulis	V2T1	6	6	6	6.00	0.00
		V2T2	5	4	5	4.67	0.58
		V2T3	7	5	5	5.67	1.15
	Gogo Niti II	V3T1	7	6	6	6.33	0.58
		V3T2	5	3	5	4.33	1.15
		V3T3	4	3	3	3.33	0.58
	Ketan Hitam I	V4T1	5	6	5	5.33	0.58
		V4T2	5	7	4	5.33	1.53
		V4T3	5	4	3	4.00	1.00

Scutellar							
Duncan[a,b]							
		Subset					
Treatment	N	a	b	c	d	e	f
V1T3	3	3.33					
V3T3	3	3.33					
V1T2	3	3.67					
V4T3	3	4.00	4.00				
V3T2	3	4.33	4.33	4.33			
V2T2	3	4.67	4.67	4.67	4.67		
V4T1	3		5.33	5.33	5.33	5.33	
V4T2	3		5.33	5.33	5.33	5.33	
V2T3	3			5.67	5.67	5.67	5.67
V2T1	3				6.00	6.00	6.00
V3T1	3					6.33	6.33
V1T1	3						7.00
Sig.	3	0.11	0.10	0.10	0.10	0.22	0.10

Coleoptilar

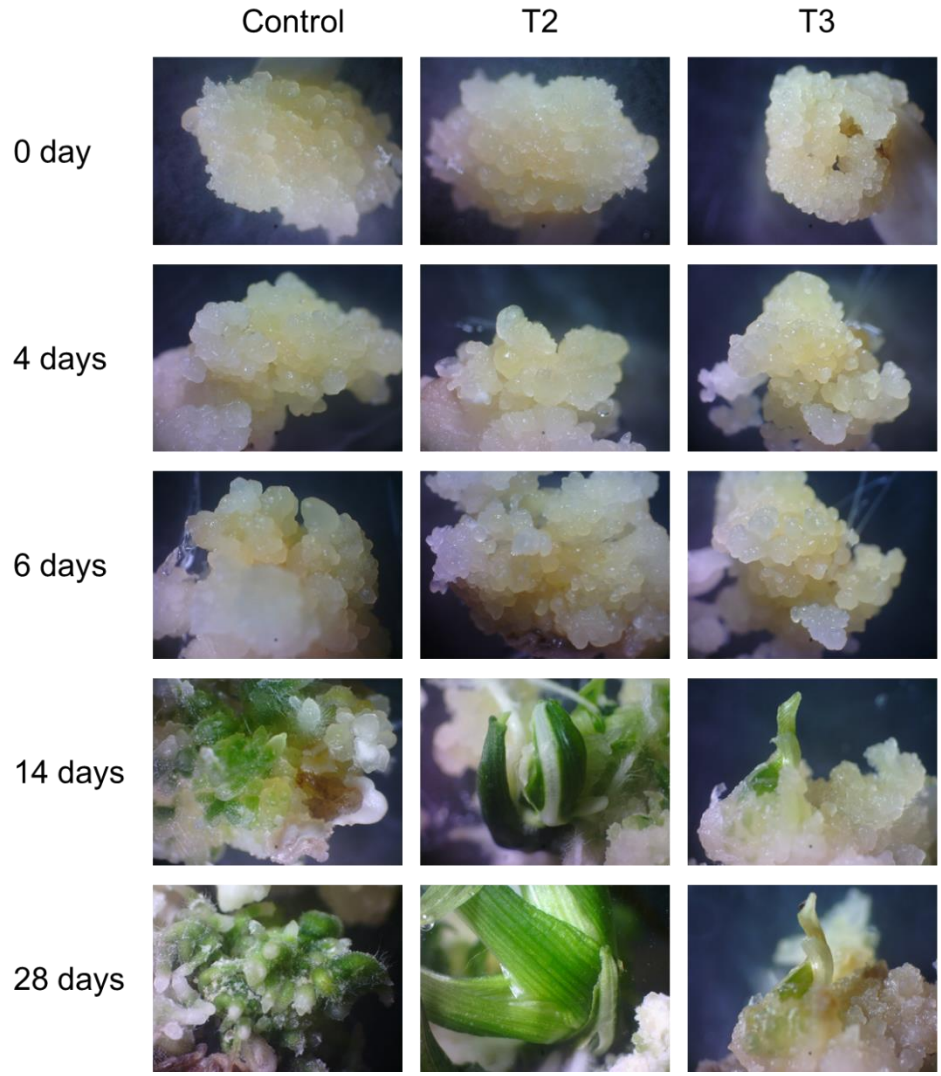
Descriptives							
Varieties name		**Treatment**	**R1**	**R2**	**R3**	**Statistic**	**Std. Error**
Coleoptilar	**TN1**	V1T1	15	14	13	14.00	1.00
		V1T2	11	12	11	11.33	0.58
		V1T3	10	8	9	9.00	1.00
	Cigeulis	V2T1	11	11	10	10.67	0.58
		V2T2	9	7	9	8.33	1.15
		V2T3	12	10	10	10.67	1.15
	Gogo Niti II	V3T1	13	12	11	12.00	1.00
		V3T2	10	7	11	9.33	2.08
		V3T3	8	6	6	6.67	1.15
	Ketan Hitam I	V4T1	13	14	13	13.33	0.58
		V4T2	13	16	12	13.67	2.08
		V4T3	12	10	9	10.33	1.53

Coleoptilar							
Duncan[a,b]							
		Subset					
Treatment	N	a	b	c	d	e	f
V3T3	3	6.67					
V2T2	3	8.33	8.33				
V1T3	3		9.00	9.00			
V3T2	3		9.33	9.33			
V4T3	3		10.33	10.33	10.33		
V2T1	3		10.67	10.67	10.67		
V2T3	3		10.67	10.67	10.67		
V1T2	3			11.33	11.33	11.33	
V3T1	3				12.00	12.00	12.00
V4T1	3					13.33	13.33
V4T2	3						13.67
V1T1	3						14.00
Sig.	3	0.12	0.05	0.05	0.16	0.08	0.09

Appendix 6. Morphology of somatic embryogenesis

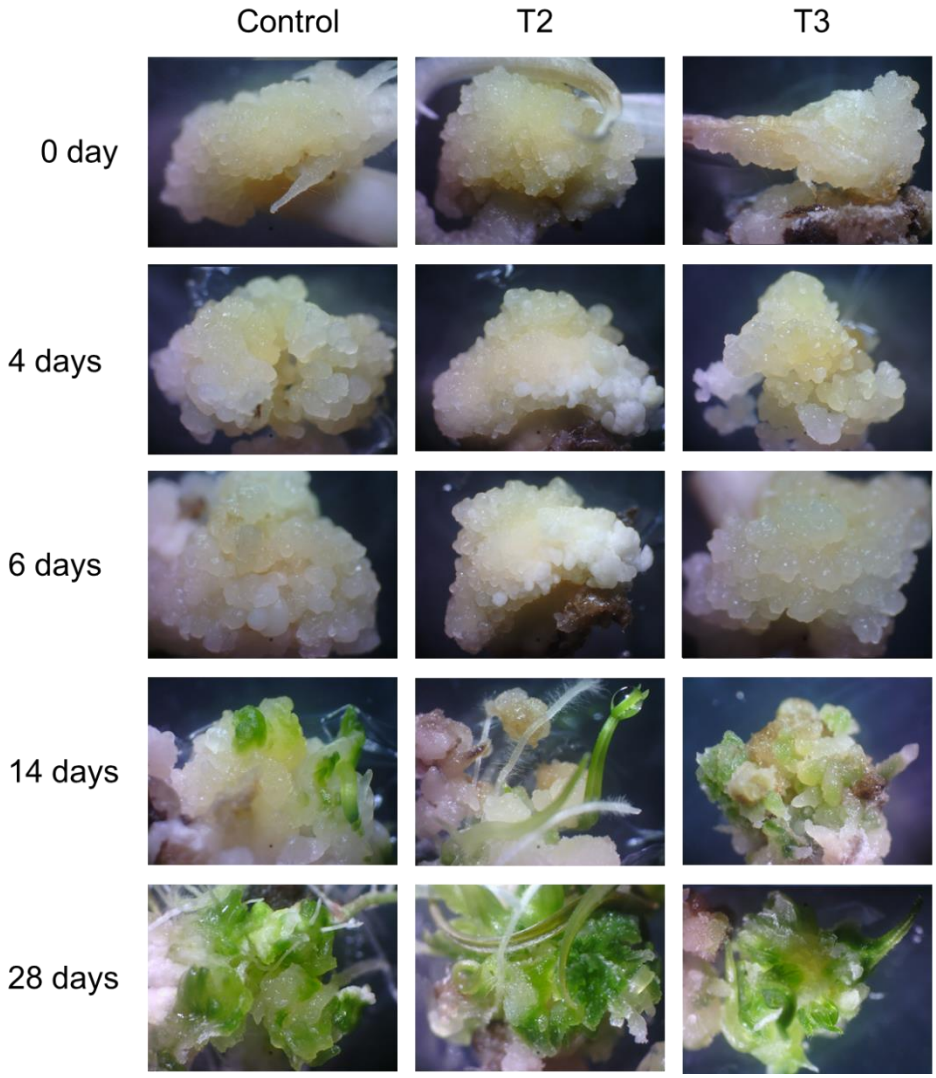
Analysis by using light microscope

TN1



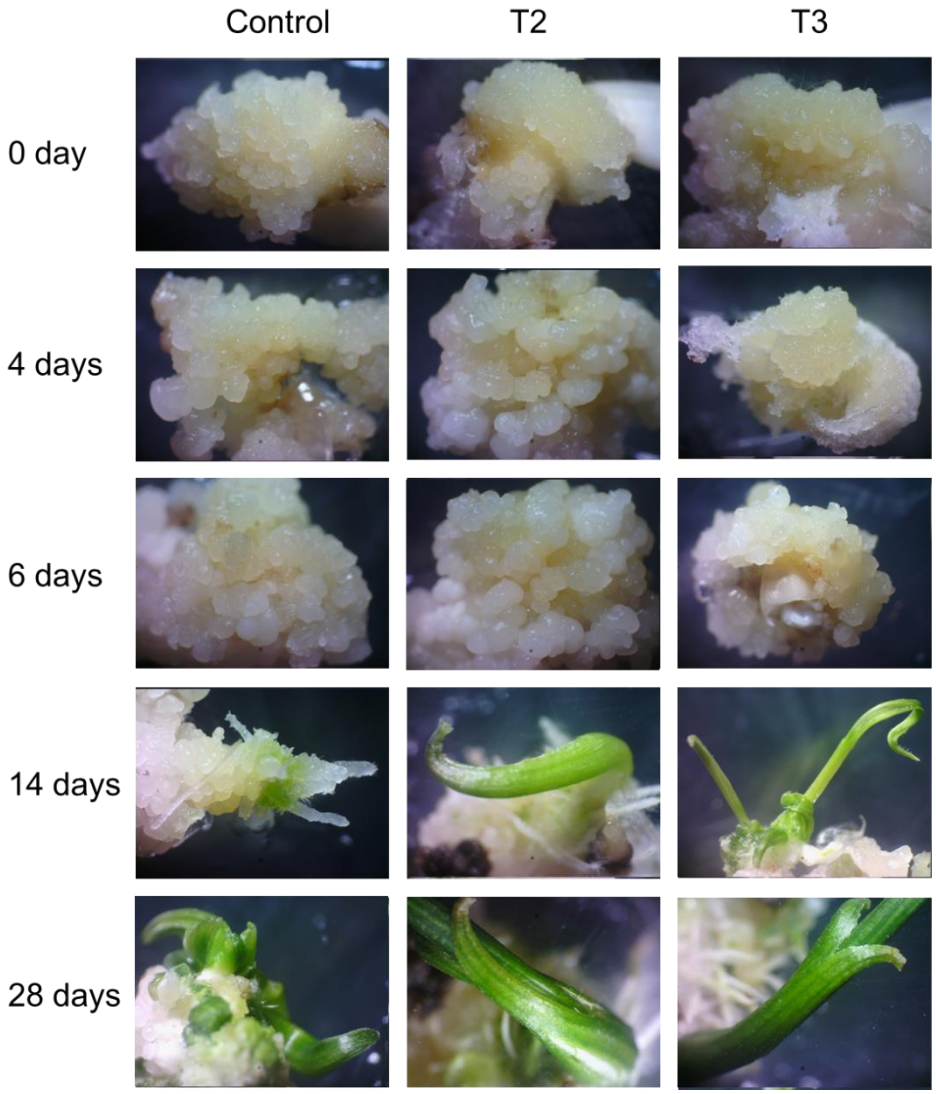
Development of somatic embryogenesis using light microscope of TN1.

Ciguelis



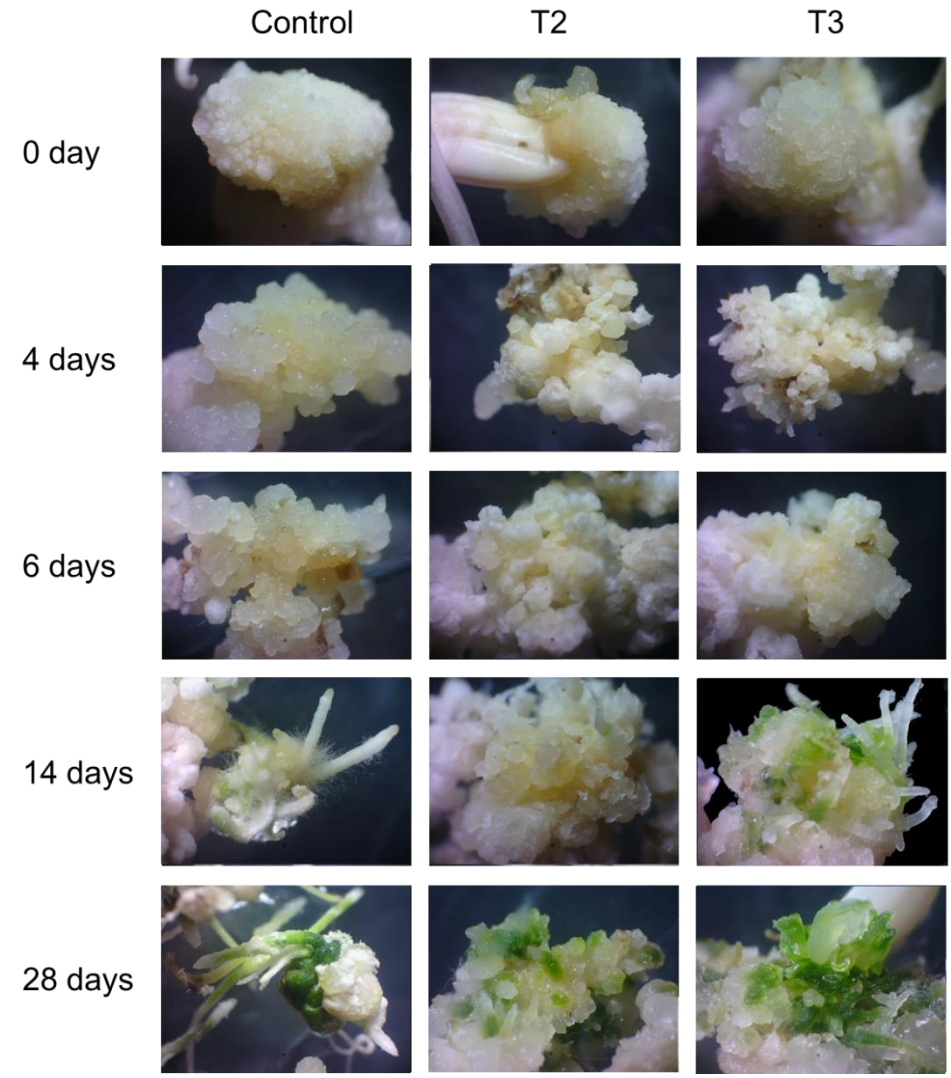
Development of somatic embryogenesis using light microscope of Cigeulis.

GogoNiti II



Development of somatic embryogenesis using light microscope of GogoNiti II.

Ketan Hitam I



Development of somatic embryogenesis using light microscope of Ketan Hitam I.

Appendix 7. Green spots

Analysis data by SPSS 16 V

Descriptives							
Varieties name		**Treatment**	**R1**	**R2**	**R3**	**Statistic**	**Std. Error**
Percentage of green spots	**TN1**	V1T1	33	100	100	77.78	38.49
		V1T2	100	100	100	100.00	0.00
		V1T3	50	67	67	61.11	9.62
	Cigeulis	V2T1	100	40	100	80.00	34.64
		V2T2	67	100	67	83.50	23.33
		V2T3	75	75	100	83.33	14.43
	Gogo Niti II	V3T1	75	100	40	71.67	30.14
		V3T2	100	100	75	91.67	14.43
		V3T3	100	100	80	93.33	11.55
	Ketan Hitam I	V4T1	40	20	50	36.67	15.28
		V4T2	80	67	100	82.22	16.78
		V4T3	80	67	67	71.11	7.70

Percentage of green spots			
Duncan[a,b]			
		Subset	
Treatment	N	a	b
V1T3	3	36.67	
V2T2	3	61.11	61.11
V3T2	3	71.11	71.11
V3T3	3	71.67	71.67
V1T2	3		77.78
V4T2	3		80.00
V4T1	3		82.22
V1T1	3		83.33
V2T3	3		83.50
V3T1	3		91.67
V4T3	3		93.33
V2T1	3		100.00
Sig.	3	0.08	0.07

Appendix 8. Plant regeneration

Analysis data by SPSS 16 V

Descriptives							
Varieties name		**Treatment**	**R1**	**R2**	**R3**	**Statistic**	**Std. Error**
Percentage of plant regeneration	**TN1**	V1T1	0	0	0	0.00	0.0
		V1T2	100	75	75	83.33	8.3
		V1T3	100	67	67	78.00	11.0
	Cigeulis	V2T1	0	0	0	0.00	0.0
		V2T2	50	75	50	58.33	8.3
		V2T3	67	75	75	72.33	2.7
	Gogo Niti II	V3T1	67	75	67	69.67	2.7
		V3T2	100	100	100	100.00	0.0
		V3T3	67	100	67	78.00	11.0
	Ketan Hitam I	V4T1	0	0	0	0.00	0.0
		V4T2	0	0	0	0.00	0.0
		V4T3	0	0	0	0.00	0.0

Percentage of plant regeneration					
Duncan[a,b]					
		Subset			
Treatment	N	a	b	c	d
V3T2	3	100.00			
V1T2	3	83.33	16.67		
V1T3	3	78.00	22.00	5.33	
V3T3	3	72.33	5.33	0.00	
V2T3	3	69.67	5.33	0.00	
V3T1	3		11.00	5.67	
V2T2	3		13.67	8.33	0.00
V1T1	3			19.67	0.00
V2T1	3				0.00
V4T1	3				0.00
V4T2	3				0.00
V4T3	3				0.00
Sig.	3	20.05	20.03	19.99	17.68

Appendix 9. Gene Expression

Analysis data by Gel Analyzer

14 days of gene expression

Varieties	Genes expression	Treatments	Band	Rf	Raw Volume
TN1	*OsSERK*	Control	1	0.841	2179
		T2	1	0.797	928
		T3	1	0.84	1913
	OsWOX4	Control			
		T2			
		T3	1	0.712	1286
	OsLEC1	TN1	1	0.804	1051
		T2			
		T3	1	0.699	757
	OsActin	Control	1	0.714	967
		T2	1	0.65	679
		T3	1	0.753	508
Cigeulis	*OsSERK*	Control	1	0.866	1445
		T2	1	0.783	1295
		T3	1	0.838	1507
	OsWOX4	Control			
		T2			
		T3			
	OsLEC1	Control			
		T2			
		T3			
	OsActin	Control	1	0.652	1090
		T2	1	0.585	590
		T3	1	0.698	413
Gogo Niti II	*OsSERK*	Control	1	0.879	3125
		T2			
		T3			

	OsWOX4	Control			
		T2			
		T3			
	OsLEC1	Control	1	0.825	1254
		T2	1	0.554	683
		T3			
	OsActin	Control	1	0.674	1096
		T2	1	0.599	716
		T3	1	0.661	449
Ketan Hitam I	*OsSERK*	Control			
		T2			
		T3	1	0.851	1041
	OsWOX4	Control			
		T2			
		T3			
	OsLEC1	Control			
		T2	1	0.639	933
		T3			
	OsActin	Control	1	0.652	661
		T2	1	0.603	694
		T3	1	0.665	397

28 days of gene expression

Varieties	Genes expression	Treatments	Band	Rf	Raw Volume
TN1	*OsSERK*	Control	1	0.766	1008
		T2	1	0.863	1496
		T3	1	0.638	1535
	OsWOX4	Control			
		T2			
		T3			
	OsLEC1	TN1			
		T2			
		T3			
	OsActin	Control	1	0.696	495
		T2	1	0.597	805
		T3	1	0.517	501
Cigeulis	*OsSERK*	Control	1	0.755	1028
		T2	1	0.84	1892
		T3	1	0.61	1187
	OsWOX4	Control			
		T2			
		T3			
	OsLEC1	Control			
		T2			
		T3			
	OsActin	Control	1	0.623	537
		T2	1	0.552	909
		T3	1	0.517	501
Gogo Niti II	*OsSERK*	Control	1	0.701	1208
		T2	1	0.853	3301
		T3	1	0.594	1563
	OsWOX4	Control			
		T2	1	0.626	1202
		T3	1	0.815	1757

	OsLEC1	Control			
		T2	1	0.737	2072
		T3	1	0.789	1179
	OsActin	Control	1	0.616	702
		T2	1	0.512	1003
		T3	1	0.49	688
Ketan Hitam I	*OsSERK*	Control			
		T2	1	0.841	2723
		T3	1	0.6	971
	OsWOX4	Control			
		T2			
		T3			
	OsLEC1	Control			
		T2			
		T3			
	OsActin	Control	1	0.517	501
		T2	1	0.495	836
		T3	1	0.46	544