TISSUE CULTURE FOR JATROPHA REGENERATION AND PLANT CELL SUSPENSION CULTURE FOR RECOMBINENT PROTEIN PRODUCTION

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กระบวนการเพาะเลี้ยงเนื้อเยื่อสบู่ดำและการเลี้ยงเซลล์พืช ในอาหารเหลวเพื่อผลิตรีคอมบิแนนท์โปรตีน

นางสาวประศมา บุญญานันท์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
สาขาวิชาเทคโนโลยีชีวภาพ
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Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy.

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ประศมา บุญญานันท์ : กระบวนการเพาะเลี้ยงเนื้อเยื่อสบู่ดำและการเลี้ยงเซลล์พืชในอาหารเหลวเพื่อผลิตรีคอมบิแนนท์โปรตีน อาจารย์ที่ปรึกษา : รองศาสตราจารย์ ดร. มารินา เกตุทัต-คาร์นส์, 76 หน้า

เมล็ดต้นสบู่ดำสามารถนำมาสกัดเพื่อใช้ผลิตน้ำมันไบโอดีเซล โดยที่การตัดต่อพันธุกรรมเป็นทางเลือกหนึ่งในการพัฒนาและปรับปรุงสายพันธุ์ การเพาะเลี้ยงเนื้อเยื่อเป็นส่วนหนึ่งของกระบวนตัดต่อพันธุกรรม การล้างชิ้นส่วนใบของต้นสบู่ดำด้วยสารเคมีที่ปลอดภัยต่อผู้ใช้งานและสิ่งแวดล้อมจึงถูกศึกษาโดยเปรียบเทียบการใช้โซเดียมไฮโปคลอไรด์และไฮโดรเจนเพอร์ออกไซด์ที่ความเข้มข้น 0-10% พบว่าการใช้ 5% ไฮโดรเจนเพอร์ออกไซด์สามารถทำให้ชิ้นส่วนพืชปราศจากเชื้อปนเปื้อนได้ 47% จากนั้นได้ทำการหาสูตรอาหารที่เหมาะสมโดยการเปรียบเทียบการใช้ฮอร์โมนพืช BA และ IBA พบว่าอาหารแข็งสูตร MS ที่เติม BA และ IBA ร่วมกัน สามารถกระตุ้นการเกิดชิ้นแคลลัสได้มากถึง 90.5% จากนั้นชิ้นแคลลัสถูกกระตุ้นให้เกิดยอดพบว่าอาหารแข็งสูตร MS ที่เติม BA 2 มิลลิกรัมต่อลิตรสามารถกระตุ้นให้เกิดยอดได้ 43.7% โดยมีจำนวนยอดเฉลี่ย 2 ยอดต่อชิ้นแคลลัส ยอดสมบูรณ์ถูกนำมากระตุ้นบนอาหารแข็งสูตร1/2 MS ที่เติม IBA 0.5 มิลลิกรัมต่อลิตรร่วมกับ phloroglucinol 100 มิลลิกรัมต่อลิตรเพื่อชักนำให้เกิดรากและจึงเกิดเป็นต้นที่สมบูรณ์ในที่สุด

การผลิตโปรตีนมูลค่าสูงสามารถผลิตจากเซลล์พืช ซึ่งมีความปลอดภัยต่อผู้ใช้งาน ต้นทุนการผลิตต่ำและใช้พื้นที่น้อยในการผลิต การทดลองนี้ได้ทำการผลิตโปรตีนเรืองแสงในเซลล์ *Arabidopsis thaliana* เพื่อใช้ทดสอบประสิทธิภาพการบ่งชี้ของเพปไทด์ส่งสัญญาณเพื่อส่งออกนอกเซลล์โดยศึกษาใต้กล้องจุลทรรศน์แบบคอนโฟคอล นอกจากนั้นการเปรียบเทียบผลผลิตโปรตีนเรืองแสงเมื่อใช้โปรโมเตอร์ 35S และโปรโมเตอร์ Rd29a พบว่า โปรโมเตอร์ Rd29a สามารถควบคุมให้มีการผลิตโปรตีนเรืองแสงได้มากกว่าการใช้โปรโมเตอร์ 35S การใช้โปรโมเตอร์ Rd29a ที่มีเพปไทด์ส่งสัญญาณจะให้ผลผลิตสูงในเซลล์แขวนลอยตั้งแต่วันที่ 12 เป็นต้นไป การศึกษาดังกล่าวสามารถทำให้การเก็บเกี่ยวผลผลิตจากอาหารเหลวทำได้ง่ายขึ้น ลดขั้นตอนและสารเคมีจากการสกัดโปรตีนจากเซลล์พืช จากการใช้โปรตีนเรืองแสงเป็นต้นแบบทำให้ทราบว่าอนาคตโปรตีนที่มูลค่าสูงก็จะสามารถนำมาผลิตในเซลล์แขวนลอยของ *Arabidopsis* ได้

สาขาวิชาเทคโนโลยีชีวภาพ ลายมือชื่อนักศึกษา ประศมา บุญญานันท์

ปีการศึกษา 2562 ลายมือชื่ออาจารย์ที่ปรึกษา

PASAMA BOONYANAN : TISSUE CULTURE FOR JATROPHA REGENERATION AND PLANT CELL SUSPENSION CULTURE FOR RECOMBINENT PROTEIN PRODUCTION. THESIS ADVISOR : ASSOC. PROF. MARIENA KETUDAT-CAIRNS, Ph.D., 76 PP.

TISSUE CULTURE/STERILIZATION/SUSPENSION CULTURE/GREEN FLUORESCENT PROTEIN (GFP)/SIGNAL PEPTIDE

Jatropha seeds can be used for biodiesel production. Genetic modification is one of the ways to develop and improve Jatropha characteristics. Tissue culture is a part of the genetic modification process. Sterilization of Jatropha leaves explants with chemicals that are safe for users and the environment was studied. The use of sodium hypochlorite (NaOCl) and hydrogen peroxide (H_2O_2) were compared. The results indicated that using 5% H_2O_2 can give 47% green explant without contamination. In addition, combination of 6-benzylaminopurine (BA) and Indole-3-butyric acid (IBA) in the medium can induce up to 90.5% calli. The calli transferred to MS medium supplemented with 2 mg/L BA were able to stimulate 43.7% shoot regeneration with an average of 2 shoots/callus. The shoots were transferred to half MS medium supplemented with 0.5 mg/L IBA and 100 mg/L phloroglucinol to promote regeneration of roots and finally whole plants.

The production of high-value proteins can be made from plant cells. The production of Green Fluorescent Protein (GFP) as a model protein was produced in *Arabidopsis thaliana* suspension cell culture. The *GFP* expression was compared between using CaMV 35S promoters and Rd29a promoters. Confocal microscope

showed that GFP can be exported outside the cell by the rice 33 KD protein signal peptide (SS). When compared the production of GFP by each promoter, it was found that the Rd29a promoter can drive GFP production more than using the 35S promoter. Moreover, the GFP production and secretion were the highest at day 12. This result demonstrated that recombinant protein can be produced and secreted outside the cell in suspension culture. Production of other high value recombinant proteins, for example growth factors, can be produced under this system.

School of Biotechnology

Academic Year 2019

Student's Signature ประสมา บุญสูงเนิน

Advisor's Signature

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Pasama Boonyanan

CONTENTS

Page

CONTENTS (Continued)

Page

LIST OF FIGURES

Figure **Page**

LIST OF FIGURES (Continued)

Figure **Page**

LIST OF FIGURES (Continued)

Figure **Page**

LIST OF ABBREVIATIONS

ABA	=	Abscisic acid
AC	=	Activated charcoal
BA	=	6-Benzyladenine
BAP	=	Benzylaminopurine
GA_3	=	Gibberellin
GFP	=	Green fluorescent protein
H_2O_2	=	Hydrogen peroxide
$HgCl_2$	=	Mercury dichloride
IAA	=	Indole-3-acetic acid
IBA	=	Indole-3-butyric acid
Kn	=	Kinetin
MS	=	Murashige and Skoog
NaOCl	=	Sodium hypochlorite
PG	=	Phloroglucinol
PTC	=	Plant tissue culture
ROS	=	Reactive oxygen species
SS	=	Signal peptide
TDZ	=	Thidazuron
Z	=	Zeatin

CHAPTER I

INTRODUCTION

1.1 Simple and Ecologically Friendly Methods for *Jatropha curcas* tissue culture

Jatropha curcas seeds are an alternative source for biodiesel production. However, Jatropha plant can be destroyed by fungi carried by insects. Alternatively, genetically modified crop could improve characteristics of the desire traits, such as fungal resistance. Plant tissue culture is a part of genetically modified plant production. There are two main processes; explant sterilization and plant regeneration. Using optimum sterilization agents indicates successful plant tissue culture. The mercury dichloride ($HgCl_2$) has been used for a long time, but it is toxic to humans. Therefore, the study to find optimum sterilization agents for leaf explants instead of $HgCl_2$ was investigated. Using optimal chemicals can be used to sterilize explants instead of using toxic chemicals. The sodium hypochlorite and the hydrogen peroxide are relatively environmentally friendly sterilizing agents. Those chemicals can eliminate microorganisms from the environment. However, optimum sterilization agents should be determined due to the importance of sterilization to successful plant cell regeneration. Plant regeneration is also a significant process for plant tissue culture. Callus induction can be induced via a combination of auxin and cytokinin, then shoot can be induced from the callus. In addition, use of the plan hormones cytokinin alone

can induce shoot formation. Then, plantlets can be generated via induction medium which contains the necessary nutrients. In this work, the effect of various disinfectants was observed to achieve an optimum disinfection protocol for Jatropha explants. Tissue culture media with appropriate hormones to regenerate Jatropha plantlet was also determined.

1.1.1 Research objectives

The objectives of part 1 of this thesis are:

- To study optimal conditions to sterilize leaf explants by using sodium hypochlorite and hydrogen peroxide.
- To find optimal media for callus induction.
- To find optimal media for shoot induction.

1.2 Green Fluorescent Protein Expression in suspension cell culture

High value products can be produced in plants. It is safe, low cost and can be less allergenic to human. Antibodies, vaccines, cytokines, and growth factors are high-value products that have been generated in plants. Deciding on a promoter to control gene expression is one important part to produce proteins. An optimal promoter can improve productivity. The Cauliflower mosaic virus (CaMV) 35S is a constitutive promoter that has been used to study the gene function and produce high value proteins in plants. The Rd29a is an inducible promoter that needs induction to generate more recombinant protein. Moreover, a signal peptide is the necessary part to direct the target protein to the secretory pathway. The signal peptide from rice 33 KD has been used to produce human growth factor to secrete into the medium in plant suspension culture systems

(Huang et al., 2015). This made the recombinant protein produced from plant cells easier to purify. The floral dip method is a method to transfer the Transfer DNA (T-DNA) into plant cells by using *Agrobacterium tumefaciens*. In this study, the T-DNA contains the promoter, signal peptide and gene of interest (*GFP*). The signal peptide can specify the location to secrete GFP, which in this case the plasma membrane (PM), placing it between the PM and cell wall of the cell. Moreover, the Rd29a inducible promoter and the efficiency of the 35S promoter were compared. Transgenic plants were produced by the floral dip method. The efficiency of the signal peptide from rice protein was observed. Moreover, comparison of promoters in Arabidopsis cells was investigated to produce GFP in suspension culture.

1.2.1 Research objectives

The objectives of part 2 of this thesis are:

- To generate transgenic plants by floral dip method.
- To compare the expression of *GFP* under control of CaMV 35S and Rd29a promoters.
- To observed the localization of GFP tag with Rice 33 KD protein signal peptide under a confocal microscope.

CHAPTER II

LITERATURE REVIEW

2.1 *Jatropha curcas*

2.1.1 Morphology of *J. curcas*

Jatropha is a drought-resistant plant. It is originated from Central America. The seed weighs around 0.53-0.86 grams and contains 22-27% protein and 57-63% lipid (Gübitz et al., 1997). Jatropha is distributed throughout the Caribbean, Portugal, Africa, and Asia. The Jatropha inflorescence has both filaments and anthers (Figure 2.1). They can be pollinated by insect or hand. One fruit contains 2-3 seeds. The color of the fruit changes during development from green to yellow and then black. In the past, Jatropha has been used for erosion control, soap production, and medicine. Jatropha seed contains diterpene esters and curcin, which is poisonous for humans and animals (Heller J., 1996). However, Jatropha cultivars have been improved, some varieties now have less poisonous compounds and can be used as animal feed.

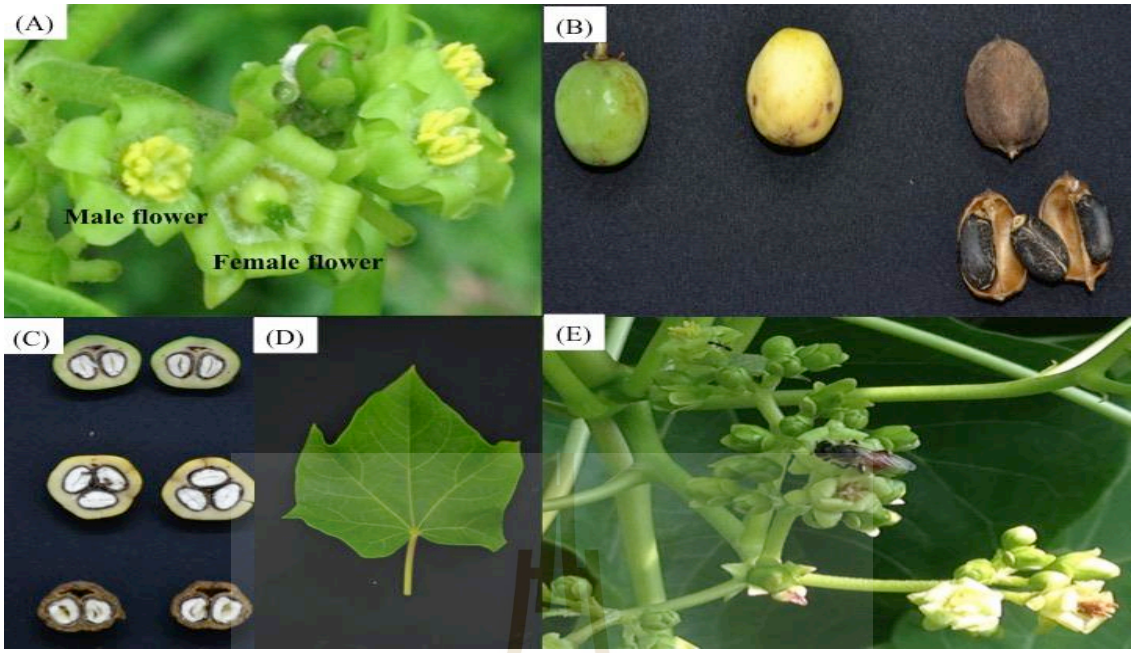

Figure 2.1 The components of Jatropha. (A) Jatropha male and female flowers; (B-C) seed development; (D) Jatropha leaf; (E) flowers pollinated by insect.

The Jatropha seed contains high levels of unsaturated fatty acids, which comprise 44.7% oleic acid (18:1) and 32.8% linoleic acid (18:2). The oil from Jatropha seed has excellent characteristics to be used as substrate for biodiesel production. The excellent quality of biodiesel substrates is high monounsaturated fatty acid and low polyunsaturated fatty acid. When the compositions of vegetable oils, such as Jatropha, sunflower, soybean, and palm are compared, Jatropha oil has the highest monounsaturated fatty acid of 45.4% (Akbar et al., 2009). Unfortunately, up to 65% of its productivities are lost due to insects and fungi (Figure 2.2). Blue physic nut bug (*Chrysocoris nobilis* Fabricius) and striped mealy bug (*Ferrisia virgata* (Cockerell)) are the main insects that destroy and attack Jatropha inflorescences, leaf, and stem by digging, sucking and rolling.

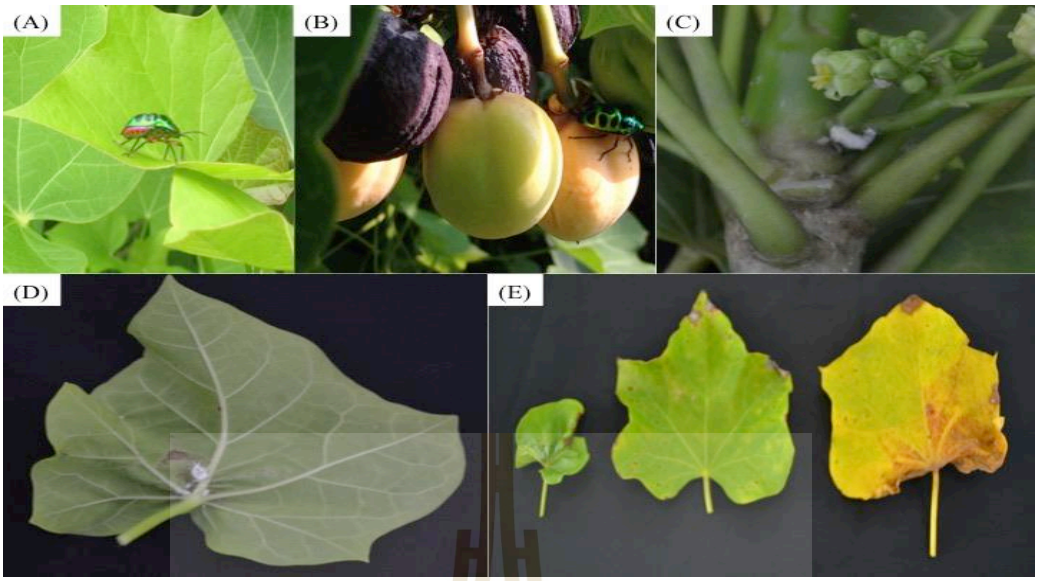

Figure 2.2 Destruction of Jatropha. (A-B) leaf and seed are attacked by blue physic nut bug; (C-D) petioles attacked by striped mealy bug; (E) suction and rolling of Jatropha leaf from plant pathogens.

2.1.2 Plant tissue culture

The process of plant tissue culture is presented in Figure 2.3. Firstly, explants are harvested. Then, those explants are cleaned and sterilized with tap water and several sterilization agents. Fungi, bacteria, and other microorganisms must be removed to prevent contaminations. Secondly, media contained macronutrients, micronutrients. The medium is important for callus, shoot, and root regeneration. Thirdly, callus induction a method by which many plant cells could be induced with the balancing of auxin and cytokinin. The calli may be compact or friable. Fourthly, shoot induction a method that regenerates shoot with the balancing of high cytokinin. Fifth, root regeneration a method to induce root with a high concentration of auxin. Lastly, the acclimatization of plantlets by transferred to soil to prepare plantlets to grow outside the controlled environment.

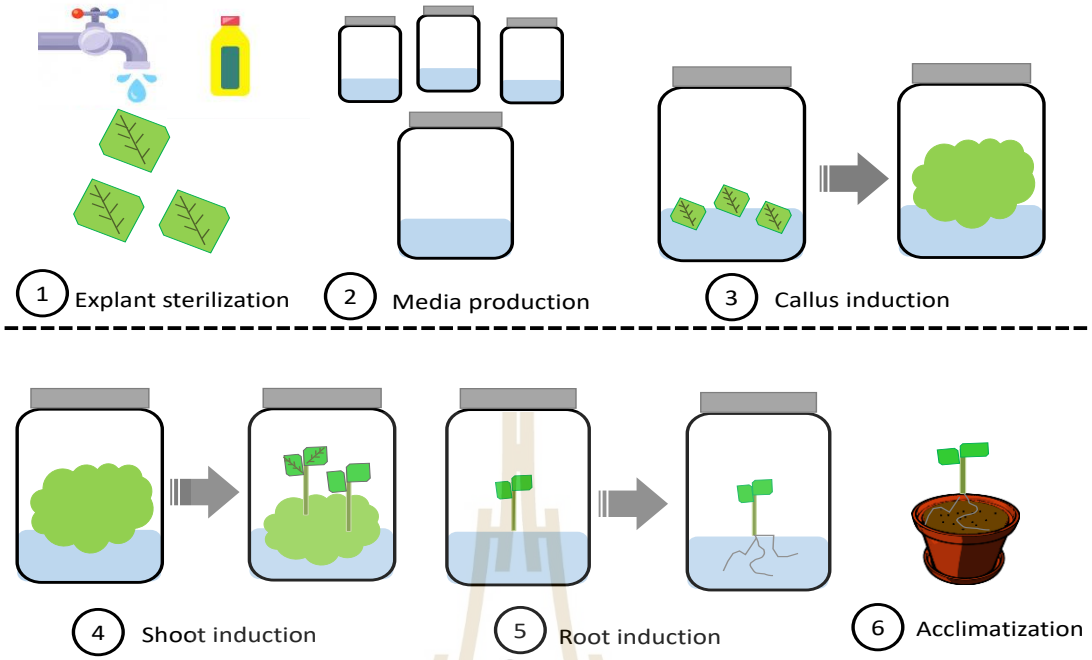

Figure 2.3 Plant tissue culture process.

The sterilization process is an essential process for plant tissue culture. It is a process of elimination of microorganisms including bacteria and fungi, as well as reproductive material as spores. The procedure can be done by both physical and chemical methods.

1) Physical method

Heat can be used by flaming for strong explant tissues, such as seed, stem, and bud, but must be very rapid. Microorganisms could be damaged by heat due to loss of water or denaturation of enzymes, other proteins, and nucleic acids (Purohit., 2012). Moreover, vibration or sonication is regularly used. Using sterilizing agents with stirring have been combined and used in some sterilization methods.

2) Chemical method

The efficiency of decontamination depends on the sterilizing agents. Several sterilizing agents have been used in the laboratory.

2.1) Alcohol solution is a broad-spectrum sterilizing agent that can damage vegetative bacteria, viruses, and fungi, but it could not destroy spores. However, it is extremely toxic to plant. Therefore, if 70% EtOH is used it will be used in a very short time for only a few seconds or min. Dormant buds, seeds, and unopened flower buds can be sterilized for an extended periods of time. 70% alcohol solution has more efficient than absolute alcohol. The mode of action of 70% alcohol is the presence of water. Alcohol denatures proteins of the cell membrane, then alcohol solution penetrates the cell wall and permeates the entire cell (McDonnell and Russell,. 1999). Finally, cells died.

2.2) Phenol has the activities to be a bactericidal, fungicidal and also mycobactericidal. It can inactive spores and viruses. The chloroxylenol (Dettol) can be used as a disinfectant at a high concentration and can be used as antiseptic at a low concentration. The mode of action of phenol is to disrupt the membranes, precipitate proteins and inactivate enzymes.

2.3) Hydrogen peroxide which is widely used due to it being relatively friendly to the environment. However, the high concentration can be sporicidal. The mode of action of hydrogen peroxide is hydroxyl free radicals attack to cell wall component which is the lipid bilayer. Moreover, hydroxyl free radicals can denature proteins and DNAs.

2.4) Heavy metals are the most dangerous chemicals that have been used for explant sterilization. The mode of action of mercuric chloride is to precipitate proteins and oxidize the sulfhydryl groups of proteins.

2.5) Surface active agents can act as wetting agents, emulsifiers, foaming agents, and dispersants. The mode of action is to concentrate the interfaces between the lipid membranes of the microorganisms. Then, the membrane can be disrupted and the cell components are leaked out. Soap and other detergents can be used on vegetative cells. They are mostly used to clean explants before thoroughly washing with tap water (McDonnell and Russell,. 1999).

2.6) Halogens are referred to member of chemical elements including fluorine, chlorine, bromine, iodine and astatine. The mode of action is to damage cell membranes and DNAs, as well as inhibition of proteins. Chlorine, bleach, and hypochlorite are chlorinated compounds that could react with water to form hypochlorous acid, which can be a microbicidal agent (Fukuzaki, 2006).

Different plants, different tissues required different sterilization chemicals and techniques. Therefore, in this study the sterilization optimization for Jatropha leaf explants was investigated.

Review of sterilization techniques for different plants were summarized below. In the study of Kuppusamy 2019, five species of *Eucalyptus* hybrid were tested for optimum conditions for sterilization and they found that 0.1% $HgCl_2$ in combination with 70% ethanol, 1% sodium hypochlorite, and 1 mg/mL rifampicin gave the highest of survival rate (Kuppusamy et al., 2019). Moreover, $HgCl_2$ has been used in several explants, for examples: cotyledon, nodal, sword sucker and internode (Maina et al., 2010; Debbarma et a., 2016; Mahmoud and Al-Ani., 2016; Shukla et al., 2019; Azmi et al., 2019). Non-toxic-chemicals have been investigated. Using 10 ppm hydrogen peroxide for 3 min to sterilize palms gave 100% survival rate (Metwaly et al., 2018). Moreover, the contamination rate was reduced when 5% hydrogen peroxide was used

for 5 min, followed by 1% sodium hypochlorite for 15 min to sterilized rhizomes of *Zantedeschia aethiopica* (Chen et al., 2017). Calcium hypochlorite and sodium hypochlorite were compared to be the agent to sterile grandifloras explants. However, using 10-20% sodium hypochlorite for 30 min was the best condition to sterilize *Elaeocarpus grandiflorus* explants, which gave the lowest contamination rate (37.5 %) and dead explant (27.5%) (Rahayu et al., 2019).

$HgCl_2$ has been used to sterilization Jatropha axillary bud. The use of 0.1% $HgCl_2$ for 5 min was the optimum condition which gave 15% contamination and 8% death. Moreover, beneficial sterilization processes could give a high percentage of 82% establishment rate (Behera et al., 2014). In addition, $HgCl_2$ has been used to sterilize other explants of Jatropha such as nodal segment, apical shoot, leaf, and cotyledonary petiole (Datta et al., 2007; Kumar et al., 2010; Garg et al., 2014; El-Sayed et al., 2020). Using a less toxic chemical, such as sodium hypochlorite, for anther and hypocotyl has been done (Sujatha et al., 2000; Madhaiyan et al., 2015; Madan et al., 2019), while hydrogen peroxide for Jatropha leaf sterilization has not been investigated.

Tissue culture medium is also important for plant cell regeneration. Macronutrients and micronutrients are essential for plant cell and plant growth, and the optimum concentration of each nutrient can increase the growth rate of plants. Carbon and energy sources are provided as carbohydrates, of which glucose and fructose are frequently used in the laboratory. Vitamins are required for various metabolic processes. Although, plants can produce these by themselves, they still need more from the culture medium. Amino acids or other nitrogen supplements provide an immediately available nitrogen source that can be uptaken by plant cell is more rapidly than inorganic nitrogen. Undefined organic supplements are referred to activated charcoal (AC). AC can absorb

inhibitors and growth regulators. Therefore, using an optimum concentration of AC should be considered. Plant hormones are natural organic substances involved in the physiological process. Moreover, these natural plant molecules influence growth, differentiation, and development, as well as stomatal movement. Two main plant hormones are influential for plant tissue culture. There are auxin and cytokinin.

1) Auxin

Auxin promotes both cell division and cell growth. Moreover, it is involved in stem internodes elongation, tropism, apical dominance, abscission, and rooting. Indole-3-acetic acid (IAA) is a plant hormone that occurs in nature. There are several chemical forms of auxin that have similar effect to IAA. The 4-chloroindoleacetic acid (4-chlorol IAA) can be found in young leaf of legumes. The phenylacetic acid (PAA) can be found in a more abundant amount than IAA but has less activity. IBA, NAA, and 2,4-D are in auxins plant hormone. But indole-3-butyric acid (IBA) is mostly used in plant tissue culture because it is cheap price and more convenient to handle.

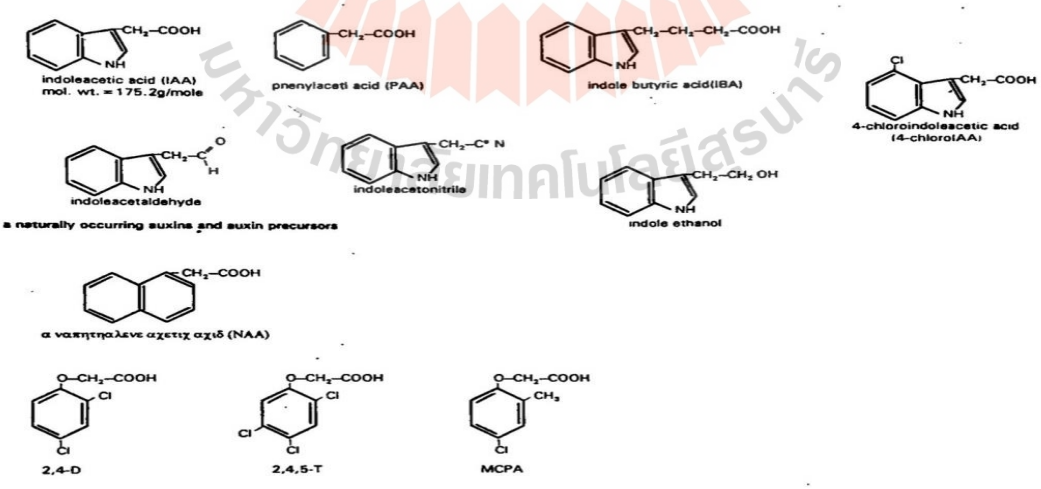

Figure 2.4 Structures of naturally and synthetic compounds having auxin activity (Malik, 1999).

2) Cytokinin

Cytokinin is a group of plant hormones. The naturally occurred cytokinins are zeatin L (Z) and isopentyl adenine (iP). Which these plant hormones are synthesized from root tip and moved to the leaves. Zeatin (Z) is the most abundant naturally occurring free cytokinin. Kinetin (Kn) and 6-benzyl-amino purine (BAP) are synthesized and widely used in laboratories. Cytokinin effects on chloroplast development, cell division, cell expansion in leaves, modification of apical dominance, promote lateral bud growth and shoot differentiation (Shah et al., 2019).

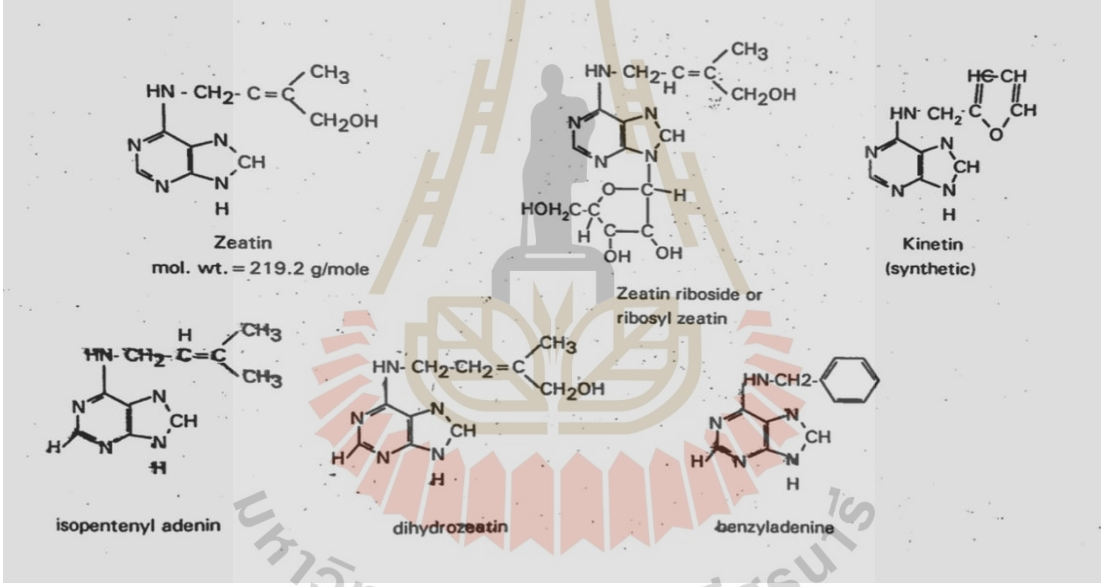

Figure 2.5 Structures of common natural and synthetic (kinetin) cytokines.

2.1.3 Jatropha regeneration

Plant tissue culture is a method that can regenerate a plant after passing through plant transformation. Many parts of Jatropha can be used as explants, such as apical bud, shoot, stem, and petiole. The cotyledonary leaf is the most used tissue for Jatropha transformation. Tissue culture method can rapidly produce sterile plantlets. Most,

plantlets are induced via tissue culture methods after transformation. Macronutrients, micronutrients, vitamins, hormones, and others components are needed for tissue culture medium. They are the key components for plant regeneration. Moreover, temperatures, lights, and other factors affect the *in vitro* plant growth.

Jatropha can be regenerated from several different types of explants. *Jatropha* callus can be induced via cotyledons on MS medium supplemented with 1 mg/L BAP and 0.10 mg/L IBA. After 4 weeks, Jatropha shoots can be induced on MS medium supplemented with 2 mg/L BAP, 0.05 mg/L IBA and 0.5 mg/L GA_3 (Subroto et al., 2014). Jatropha leaf explants can be used to induce to form calli. Leaf explants were cultured on MS medium containing 2.5 mg/L BA and 1.0 mg/L NAA for 6 weeks generated 97.5% green friable callus (El-Sayed et al., 2020).

Then, shoot can be regenerated. Jatropha shoots direct organogenesis can be induced from cotyledon on MS medium supplemented with 2 mg/L thidiazuron (TDZ) however, if petiole was used, only 0.5 mg/L TDZ was needed (Kumar et al., 2010). Axillary shoot buds can be induced from node on MS medium supplemented with 5 mg/L BA and 22 mg/L adenine sulfate (Datta et al., 2007). Moreover, Jatropha shoots direct organogenesis can be induced from cotyledon by soaking in 20 mg/L TDZ solution for 20 min. Then, cultured on hormone-free MS medium for 35 days.

Root induction is needed to produce plantlets. A 40% root induction rate was obtained after culturing shoot on MS medium supplemented with 0.5 mg/L IBA and 30 mg/L phloroglucinol for 6 weeks (Daud et al., 2013). Moreover, roots could be induced via direct shoot organogenesis. Shoot explants transferred to half-strength MS salts medium supplemented with 2 mg/L IBA can induce roots. Roots were produced within 20-25 days of culture. This medium could regenerate 4.25 roots per shoot (Tsegay et al.,

2017). In addition, axillary buds were used to generate shoot. After that, shoots were cultured and transferred to root induction medium. IBA and NAA were combined to induce roots. The result showed that 89.7% root induction was presented from Jatropha culturing on MS medium supplemented with 1.0 mg/L IBA and 1.0 mg/L NAA. But, the highest number of roots per shoot (6.5 roots) and root length (4.5 mm) were found on culturing of Jatropha shoot on MS medium supplemented with 1.5 mg/L IBA and 1.0 mg/L NAA (Mishra., 2018). This thesis investigated optimum disinfection protocol for Jatropha leaf explants and optimum media for Jatropha regeneration.

2.2 *Arabidopsis thaliana*

2.2.1 Morphology of *A. thaliana*

A. thaliana is a small plant that is a member of the mustard family (Figure 2.6). Europe is the origin of this small plant, which has widely spread to other continents. It's life cycle is around 6-8 weeks, depending on the condition of growth. It is a self-pollinated plant, since the stamens and pistils are in the same inflorescence. The flower consists of four white petals, surrounding with four sepals, six stamens, and two carpels (Meyerowitz., 1987). At the center of the flower is an ovary that consists of two carpels (Figure 2.6A). After culturing on medium or soil for 10 days, two cotyledons, hypocotyl and primary root will develop. The ovary develops into a fruit called a silique after fertilization. Each silique contains 30-60 seeds. The stem can be up to 20-70 cm in height.

There are many reasons that Arabidopsis is an attractive experimental model. Firstly, Arabidopsis contains a small genome ranging from 100-120 $x10^6$ bp, which is 10 times smaller than Tobacco and pea. Secondly, Arabidopsis has a short life cycle.

Therefore, a short time is needed to produce homozygous lines. Thirdly, Arabidopsis is a simple plant to do transformation by the floral dip method. Fourthly, each silique contains up to 60 seeds, in which many offspring are provided. Fifth, it is self-pollinated plant, so that it is easy to maintain homozygous lines. Lastly, it has been widely studied and there is a lot of knowledge about mutagenesis strategies (Wilson., 2000). In laboratory work, there are many varieties of Arabidopsis ecotypes. Columbia has been the most widely used in laboratories.

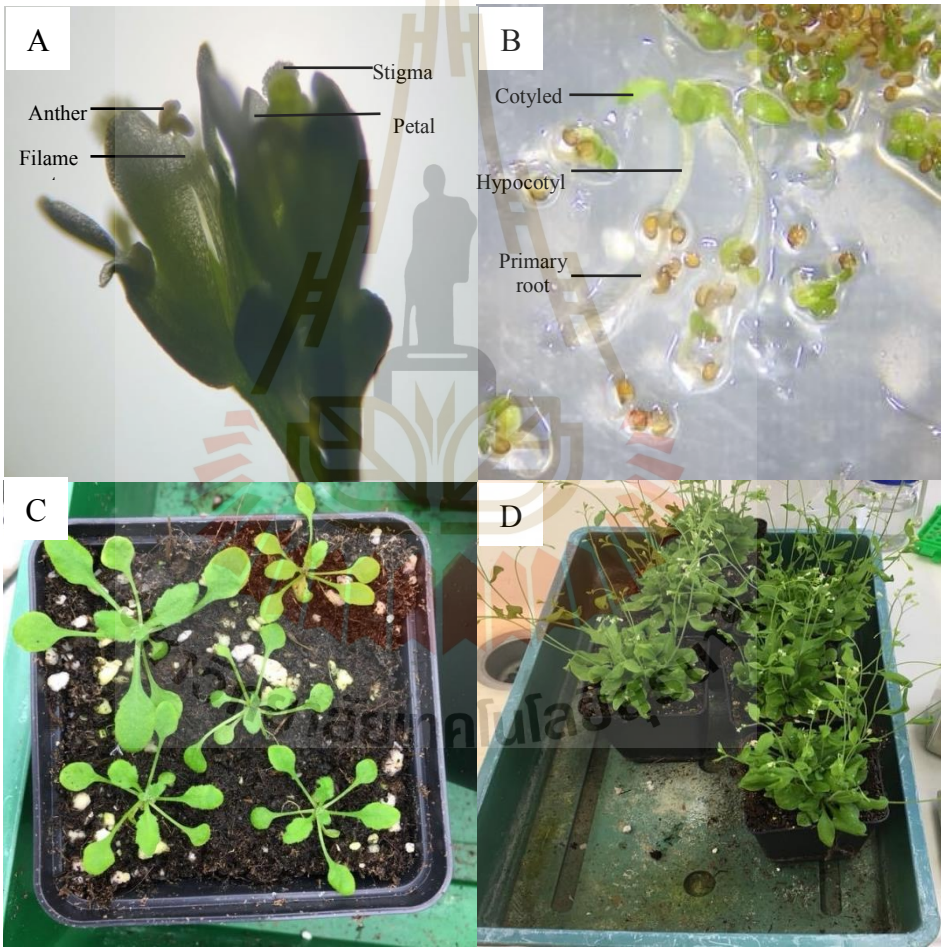

Figure 2.6 Arabidopsis morphology (A) *A. thaliana* flowers; (B) Arabidopsis seedlings; (C) 2-3 weeks old Arabidopsis plants; (D) Mature plants ready for transformation by floral dip method.

2.2.2 Floral dip

The floral dip method is one plant transformation method that is done via flower parts using a bacterial carrier. *Agrobacterium tumefacien* is a gram-negative soil bacterium that is found in nature. This bacterium has the ability to transfer Transfer-DNA (T-DNA) a single stand DNA to female gamete or ovule at only one allele of a given locus. The flower developmental stage indicates the performance of transformation rate. The optimum stage for transformation by the flower dip method is when the flower starts to open. Agrobacterium enters into the female after the gynoecium is open and before seeds are developed. The effect of Agrobacterium strains for flower dip transformation has been investigated, but the investigation mostly focuses on the stage of flower development (Nagle et al., 2018).

Transformation performance can be improved by preparation of the right concentration of the Agrobacterium solution and chemicals used in the experiment. Silwet L-77 is a nonionic organosilicon surfactant that enhances the attachment of bacteria to the plant. 2-(N-Morpholino) ethane sulfonic acid (MES) is a good buffer to maintain the bacteria activity. Transformed plants are incubated in the dark to enhance transformation rate after dipped into Agrobacterium solution. Arabidopsis T_1 transformants are a hemizygous product of germline transformation. These seeds are then screened on agar medium containing antibiotics. Moreover, T_1 Arabidopsis needs to be self-pollinated to produce homozygous T_2 lines.

2.2.3 Transgenic *A. thaliana*

Most studies of protein localization and gene function have been investigated in Arabidopsis. Studying protein localization in Arabidopsis using fluorescent protein tags, such as Green Fluorescent Protein (GFP), Yellow Fluorescent Protein (YFP) and

other fluorescent proteins, as well as β-glucuronidase (*GUS*) reporter gene to detect location of their function has been widely done. Nuclear and subcellular localization have been studied.

The nuclear proteome of Arabidopsis has been studied to understand the functional diversity of nuclei by identify the nuclear envelops. The plant expression vector contains two putative transmembrane domains at the N-terminal and short coiled-coil region tags with GFP had shown high GFP expressed in vegetative and reproductive tissue. But the another construction contains a signal peptide, lectin domain, a putative transmembrane domain, and kinase domain which was shown highly expressed in senescent leaves. This study demonstrated that signal peptide, lectin domain, a putative transmembrane domain, and kinase domain involved in a specific function of nuclear envelop during the senescence process (Goto et al., 2019).

A subcellular localization study of chlorophyllase (CLH) has been performed. Chlorophyllase (CLH) is an enzyme that catalyzes the degradation of chlorophyll to form chlorophyllide during the senescence of leaves. The localization of CLH1 and CLH2 isoform of CLH were studied to prove their function. A CLH1 tagged with YFP was detected under confocal microscopy and was located in the ER and tonoplast. (Hu et al., 2015). Then, localization of CLH2 was also studied using a YFP tag later. The localization of CLH2 was shown to be external to the chloroplast. Moreover, overexpression of CLH2 is expressed in the tonoplast and ER. Consequently, the study of CHL was concluded that both CHL1 and CHL2 isoforms were located in tonoplast and ER and were not involved with the degradation of Chlorophyllase (Hu et al., 2020).

Moreover, recombinant protein expression has been investigated in Arabidopsis as well. In the aspect of bacterial resistance, the function of the S -

adenosylmethionine decarboxylase (*SAMDC*) gene was investigated by its overexpression in *A. thaliana.* Transgenic Arabidopsis overexpressing *SAMDC* was found to respond to disease and jasmonic acid metabolism. Then, transgenic plants have increased tolerance to infection by *Pseudomonas syringae* (Marco et al., 2014).

In addition, the aspect of drought and stress tolerance has been of interest. Pectin methylesterase gene (*PtoPM35*) was cloned from *Populus tomentosa* and overexpressed in Arabidopsis. The overexpression result proved that pectin methylesterase could promote pectin demethylesterification in polar and enhance the response to osmotic stress. But stomata opening was inhibited (Yang et al., 2020).

Moreover, superoxide dismutase (*SOD*) gene was driven under ubiquitin promoter and overexpressed in Arabidopsis. Expression of *SOD* gene cloud against methyl viologen-induced oxidative stress. Additionally, the transgenic plants were treated with stress conditions to activate *CBF2*, *RD22*, *DREB1A*, *RD29A* and *RD29B* transpiction factor. Those transciption factors could reduce reactive oxygen species (ROS) accumulation. Then, transgenic plants led to enhance chilling tolerance. After chilling at 4 °C and ABA treated for 0-192 hr. The results showed *CBF2*, *RD22*, *DREB1A*, *RD29A* and *RD29B* transpiction factor were involved in chilling stress response. Moreover, transgenic plants were detected ROS activity which these chemically reactive chemical species could damage plant cell. ROS level was lower. Lastly, Arabidopsis transgenic plants were green and healthy under chilling stress conditions. However, this finding is important for growing plants in low temperature areas and contribute the adapting the plant to tolerance with stress condition (Lin et al., 2019).

Overexpression of high-value clinical products was also achieved in Arabidopsis. The human acidic fibroblast growth factor was produced and tested for stimulation of NIH/3T3 cell proliferation via the MTT assay. Recombinant protein from transgenic plants had a dose-dependent cell proliferation effect on the cell lines (Yang et al., 2015).

2.2.4 Promoter characteristic

Plant promoters are responsible for gene expressions. A promoter contains a DNA sequence for RNA polymerase II binding and the binding of transcription factors. There are two types of promoters used for recombinant protein expression. Firstly, constitutive promoters, such as *Act2,* CaMV 35S and Ubiquitin promoters. They are highly expressed in many cell types in the plant. Secondly, inducible promoters, for example, Rd29a and Rd29b. These inducible promoters need inducers to promote the expression. The inducer can be chemicals, plant hormones, such as Abscisic acids (ABA), or stress conditions. Most scientists preferred to use inducible promoters, since higher products can be obtained (Potenza et al., 2004).

In 2004, three constitutive promoters were compered in transgenes in alfalfa (*Medicago sativa*). The β-glucuronidase (*gusA*) reporter gene was driven by 35S promoter from cauliflower mosaic virus (CaMV), the cassava vein mosaic virus (CsVMV), and the sugarcane bacilliform badnavirus (ScBV). The results showed that GUS was modestly expressed in leaves, roots, and stem by 35S promoter. GUS activity was high in vascular tissue when expression was driven by the ScBV promoter. Interestingly, GUS expression from the CsVMV promoter was shown to be higher than that driven by the 35S (24-fold) and ScBV (38-fold) promoters (Samac et al., 2004). Moreover, two other promoters, the double 35S promoter and nopaline synthase

promoter (NOS) with and without tobacco etch virus TEV translational enhancer were compared in soybean. Quantitative real-time PCR results showed that without enhancer, the 35S promoter gave a higher expression level than that driven by the NOS promoter. But TEV could enhancer the NOS promoter higher than the 35S promoter (Testroet et al., 2017).

In addition, four constitutive promoters (35S, 35Somega, UBQ1 and BTH6) and one inducible promoter (Gmhsp17.3) were compared in Arabidopsis. The result found that 35S promoter containing omega elements could give the highest *GUS* expression. The 35Somega is therefore considered a very strong promoter for constitutive expression in Arabidopsis (Holtorf et al., 1995).

2.2.5 Plant cell suspension culture

Plant cell suspensions culture should be prepared from callus cultured on solid medium properly. The calli would be transferred to liquid medium. Culturing of plant cells in liquid medium is used to study optimum nutrition, growth and cell differentiation of callus tissue. However, culturing plant suspension cells are suitable for small organ or small pieces of the tissue rather than larger organs. Due to shoot proliferation can occur under the medium that lack aeration. Therefore, many tissue are not suitable to culture in liquid medium but other tissues need agitation of moving or shaking to prevent cell and cell aggregates settling to the bottom of the flask.

Recombinant protein for pharmaceutical products in plants can be produced via both whole-plants and plant cells. There are several advantages to produce recombinant proteins in plant cells, such as rapid growth, less space needed, and easier to manipulate. Antibodies, vaccines, cytokines, growth factors, and growth hormones are high-value products that have been produced in plants. Recombinant proteins

produced in plants also contains post translation modification that can cause less allergic reaction in humans when recombinant proteins are compared to a prokaryote (Xu et al., 2011).

Recombinant protein can be easily harvested from suspension culture due to plant cell wall can be represented as a net to entrap other non-specific proteins. That can be less work for purification step. Moreover, the protein of interest can be specifically secreted into suspension culture by inclusion of a signal peptide. As well as, GFP and mouse granulocyte-macro-phase colony-stimulating factor (mGM-CSF) were successfully produced in rice suspension by inclusion of the rice 33 KD signal peptide at the beginning of the coding sequence (Huang et al., 2015). In addition, recombinant protein secreted can be produced into solution without passing through an extraction process.

A recombinant human hormone erythropoietin (*EPO*) protein gene was introduced into Arabidopsis by vacuum infiltration. The purified EPO protein was observed on SDS-PAGE and western blot analysis (Pires et al., 2012). In addition, Arabidopsis suspension was used to produce heterologous proteins by using GFP as a reporter gene. Hypocotyls were transformed by *Rhizobium rhizogenes*. The GFP was secreted and produced up to 130 mg/L in suspension medium. Moreover, the human serpinal (α-1-antitrypsin) gene was produced via hairy roots through suspension medium by introducing a plant signal peptide. The protein product was detected by SDS-PAGE as a 50 kDa α-1-antitrypsin protein (Mai et al., 2016). Recently, Arabidopsis hairy root culture was used to produce human gastric lipase. Recombinant protein was highly expressed after treated with 2,4-D plant hormone. The stability of transgenic hairy root was studied by detection of lipase activity. The result showed high activity

even if the hairy roots were kept at room temperature for 2 months (Guerineau et al., 2020). Therefore, this thesis was investigated an efficiency of signal peptide from rice 33 KD and the optimum promoter for recombinant protein production system.

CHAPTER III

SIMPLE AND ECOLOGICALLY FRIENDLY METHODS FOR *Jatropha curcas* TISSUE CULTURE

3.1 Abstract

The use of toxic chemicals causes pollution and is not environmentally friendly. This research has been conducted to find the optimum non-hazardous disinfectant chemical with high efficiency to decontaminate Jatropha and to find the optimum hormones for Jatropha tissue culture regeneration. The optimum disinfectant of Jatropha leaf explants was 5% hydrogen peroxide (H_2O_2), which gave 47.0% green explants without contamination. The plant regeneration process was conducted with the combination of 6-benzyladenine (BA) and indole-3-butyric acid (IBA). Jatropha shoots were induced on Murashige and Skoog (MS) medium supplemented with 2 mg/L BA. This condition provided 43.7% regeneration with an average of 2 shoots per callus and the average shoot length was 17.8 mm. Calli with shoots were transferred to the root induction medium containing half MS medium supplemented with 0.5 mg/L IBA and 100 mg/L phloroglucinol (PG).

3.2 Introduction

Physic nut or *Jatropha curcas* L. is a drought-resistant plant that belongs to the Euphorbiaceae family. It is mostly considered to be an alternative plant source for

biodiesel production. Jatropha has been shown to have high fatty acid components when compared with other alternative biodiesel plants (Akbar et al, 2009). Jatropha's oils have similar characteristics to standard biodiesel, therefore, it is suitable to be used as an alternative fuel resource (Parawira, 2010). However, Jatropha can also be used for soap production and erosion control (Heller, 1996). A methanolic extract of Jatropha has been recovered and found to have the ability to inhibit cancer metastasis in mice (Balaji et al, 2009). To improve the quality and quantity of Jatropha, genetic modification is one of the high potential techniques.

To create a genetically modified plant, a tissue culture method is needed for plant regeneration. Plant tissue culture (PTC) consists of two main processes, sterilization, and plant regeneration. Sterilization is an essential part of the process that will improve plant regeneration, as well as achieving greater success in genetic modification (Silva et al, 2015). Many disinfectants have been used to eliminate contaminations. Most plant samples are normally collected from an *ex vitro* environment with high contaminations. $HgCl_2$ has been one of the many choices for removing bacteria and fungi contamination (Shukla, 2013: Zhao et al, 2016: Yadav et al,2017: Nguyen et al, 2018). However, it is quite toxic to both the environment and lab personnel.

Plantlets are regenerated and developed from calli using hormones. Auxins and cytokinins are plant hormones that are regularly used in PTC. Auxin is an effective hormone that is responsible for the necessary processes of cell division and cell elongation. Generally, auxin is co-activated with cytokinin to produce calli. Cytokinin is accountable for protein synthesis, as well as the activation of enzyme. Most cytokinins are synthesized from the roots and transported via the xylem to other parts of the plant, and very little is synthesized from the shoot (George et al, 2008). TDZ is a cytokinin like

compound used for callus and indirectly for shoot induction. However, using a high concentration of TDZ could inhibit shoot induction (Cao and Hammerschlag, 2000). Therefore, a combination of BA and IBA is more appropriate due to the toxicity of TDZ (Paranjape, 2014).

In this work, the effect of various disinfectants was observed to achieve optimum disinfection protocol for Jatropha explants. Tissue culture media with appropriate hormones to regenerate Jatropha plantlet was also determined.

3.3 Materials and methods

3.3.1 Optimization of leaf disinfectants

Jatropha samples were collected from Suranaree University of Technology, organic farm. Leaves were harvested, washed thoroughly with tap water for 15 min, and then cleaned with dishwashing detergent. The sterilization process was done using two main disinfectants; hydrogen peroxide (H_2O_2) and sodium hypochlorite (NaClO) in a range of 2.5-10% for 5 min. Then, the leaves were rinsed three times with sterile distilled water. The optimization of leaf disinfectant experiments were repeated three times with five replicates each.

3.3.2 Media optimization for callus induction

Sterilized leaves were cut into pieces approximately 0.5 x 0.5 cm^2 and put on Murashige and Skoog (MS) medium supplemented with combinations of BA and IBA at 0.5-2.0 mg/L and 0.7% (w/v) American bacteriological agar (Pronadisa; Laboratorios Conda, Madrid, Spain). The percentage of callus formation was recorded after 4 weeks of culture and the calli were transferred to new plates with similar media. The experiments were repeated five times with five replications each.

3.3.3 Media optimization for shoot and root induction

Shoot induction was performed with the same medium as callus induction medium. The percentage of shoot regeneration, shoot number per callus, and shoot length (mm) were recorded after 4 weeks of culture on shoot induction medium. The experiment was repeated five times with five replications. After 4 weeks, the shoots were transferred to half MS medium supplemented with 100 mg/L PG and 0.5 mg/L IBA. After 1 week, the rooted shoots were transferred to half MS medium supplemented with 25.0 mg/L AC.

3.3.4 Statistical analysis

The experiment was set up in a completely randomized design (CRD) and the statistical analysis of data and one-way ANOVA followed by Duncan's multiple range tests were performed with the P value of 0.05.

3.4 Results

3.4.1 Leaves sterilization

Leaves disinfection was performed using sodium hypochlorite and hydrogen peroxide. These 2 disinfectants showed different adverse effects on leaf explants. Leaf explants treated with sodium hypochlorite gave the highest rate of green explants without contamination (51.2%) (Figure 3.1). However, all leaf explants were damaged. After 4 weeks of tissue culture process, the leaf explants become dried and wilted. Finally, they were brown and died (Figure 3.2A). Sodium hypochlorite had stronger effect on the contaminations and the leaves explants than hydrogen peroxide. Using low concentration of hydrogen peroxide of 2.5% could not control the contamination of microorganisms. But, with hydrogen peroxide of higher than 5%, leaf explants were all

dead, similar to the use of sodium hypochlorite. Therefore, 5% hydrogen peroxide was the optimum concentration to use to eliminate contaminants and survival of the leaf explants. It provided up to 47.0% of green explants without contamination (Figure 3.2B). This condition was further used in the next experiment.

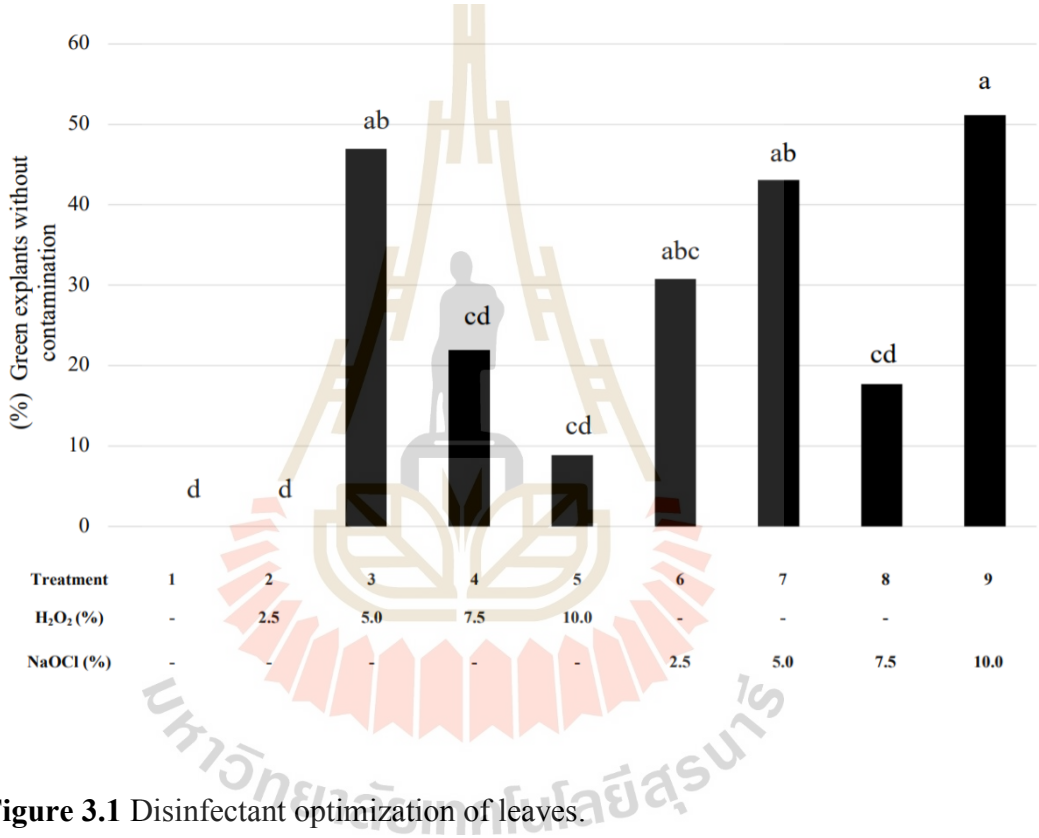

Figure 3.1 Disinfectant optimization of leaves.

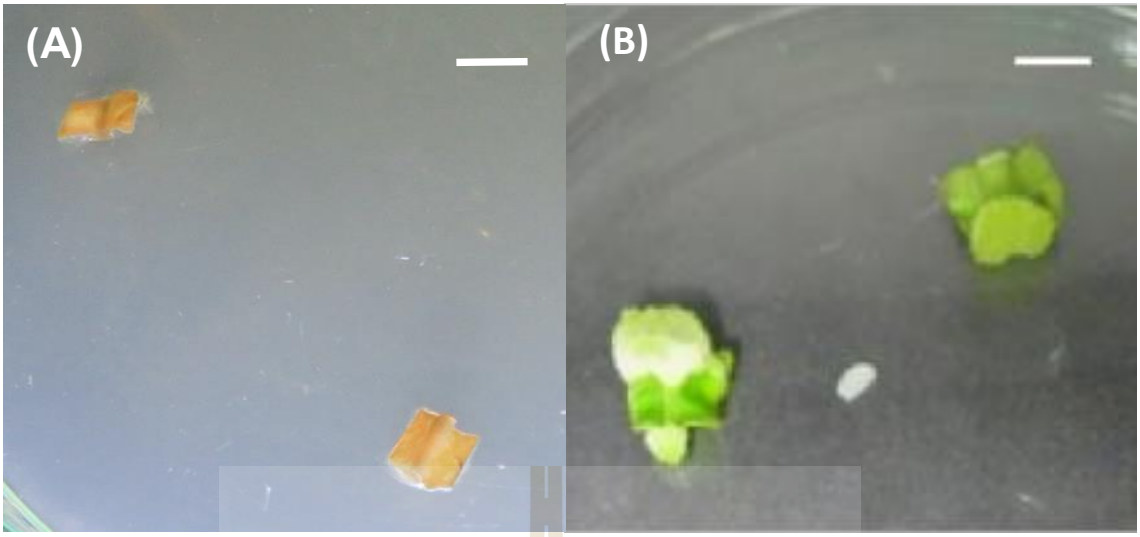

Figure 3.2 Disinfection of leaf explants. (A) leaf explants cleaned with 10% sodium hypochlorite (treatment 9); (B) leaf explants cleaned with 5% hydrogen peroxide (treatment 3), Bar = 0.5 centimeter.

3.4.2 Callus formation

Jatropha callus was induced with the combination of 0.5-2.0 mg/L BA and 0.5-2.0 mg/L IBA (Figure 3.3). No callus was induced with the use of BA alone (Figures 3.4B-E). However, with the combination of BA and IBA, 90.5% of the leaf explants expanded and green compact calli were formed on the edges after culturing for 4-5 days (Figure 3.3A) (Figures 3.4F-I).

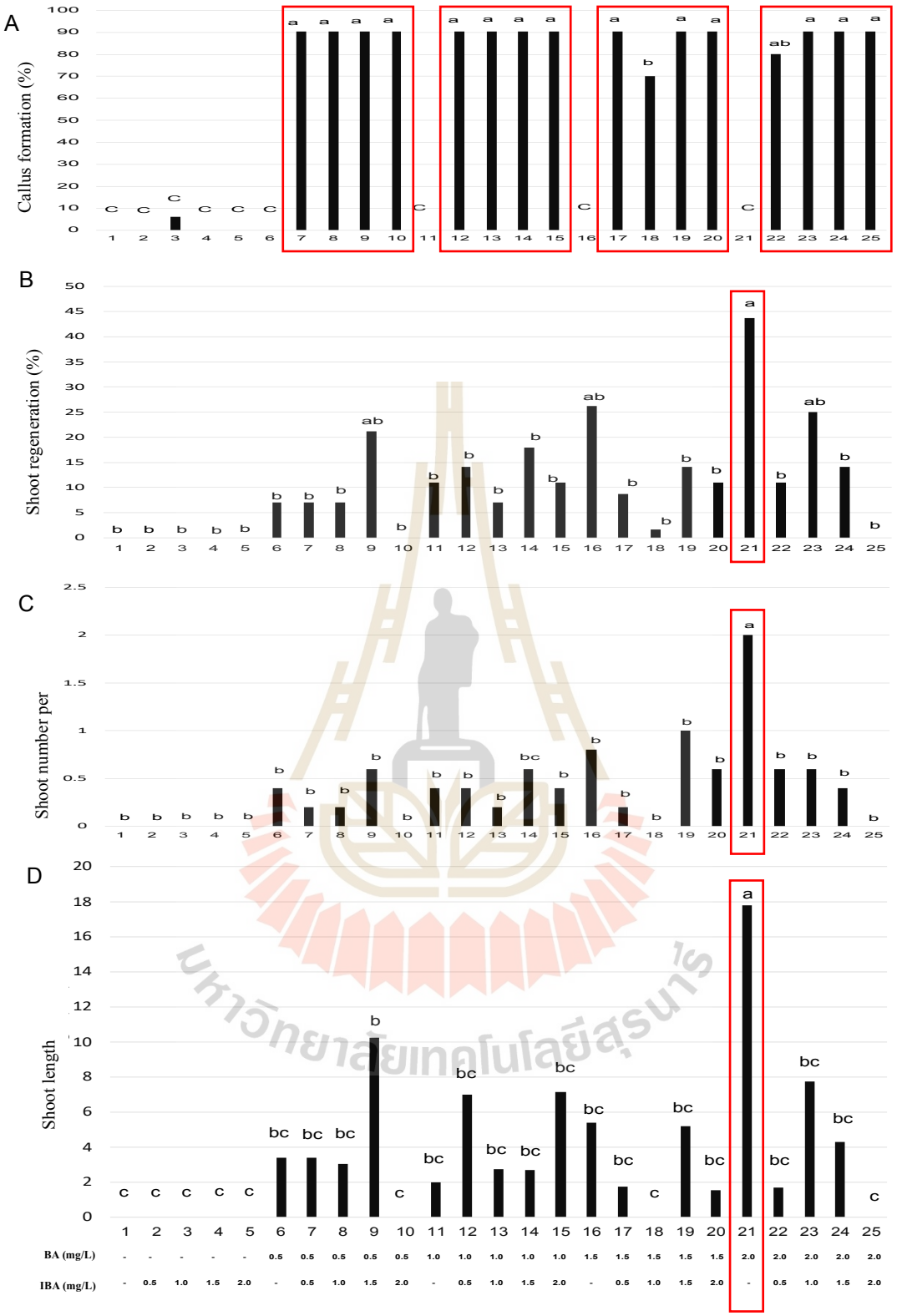

Figure 3.3 Effect of the combination of BA and IBA. (A) % callus formation; (B) % shoot regeneration; (C) shoot number per callus; (D) shoot length (mm). The data was recorded after 4 weeks of culture.

3.4.3 Shoot and root induction

After 4-6 weeks, Jatropha calli were induced with the combination of BA and IBA to produce shoots. Supplementation with only BA at 2.0 mg/L gave rise to 43.3% shoot regeneration with 2 shoots per callus. The longest shoot length was 22.1 mm with an average of about 17.8 mm per shoot (Figures 3.3B-D) (Figure 3.5A).

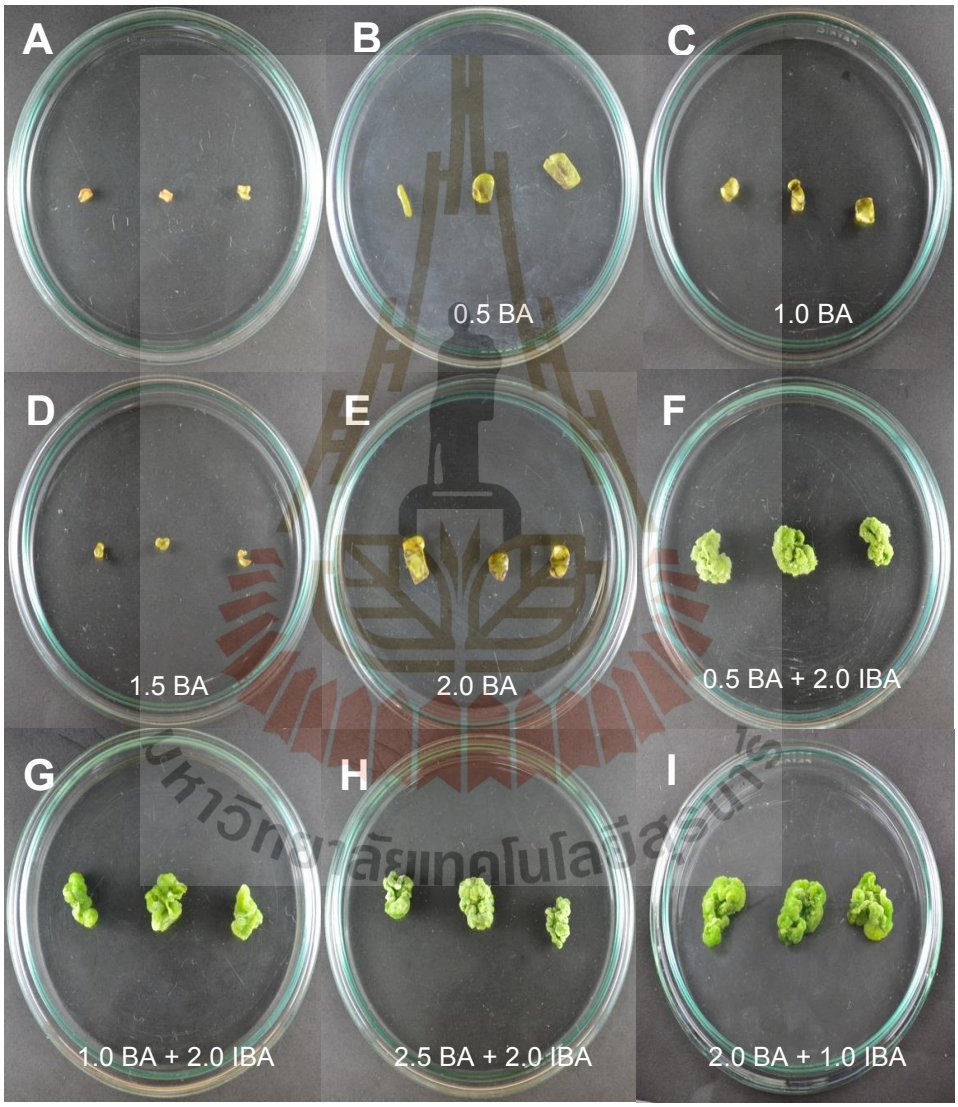

Figure 3.4 Effect of the combination of BA and IBA on callus induction. (A) induced calli on MS medium without plant hormone; (B-E) induced calli on MS medium supplemented with only BA; (F-I) induced calli on MS supplemented with combinations of BA and IBA. (The values are in mg/L).

For root induction, the shoots were transferred to half MS medium supplemented with 0.5 mg/L IBA and 100 mg/L PG for 7 days (Figure 3.5B). During root induction, long white roots were observed from the callus connected with the shoots. After 7 days the rooted shoot were transferred to root elongation medium containing half MS medium supplemented with 25.0mg/L AC to elongate the root (Figures 3.5C-D). In the root elongation medium, production of branch roots and root hairs were observed.

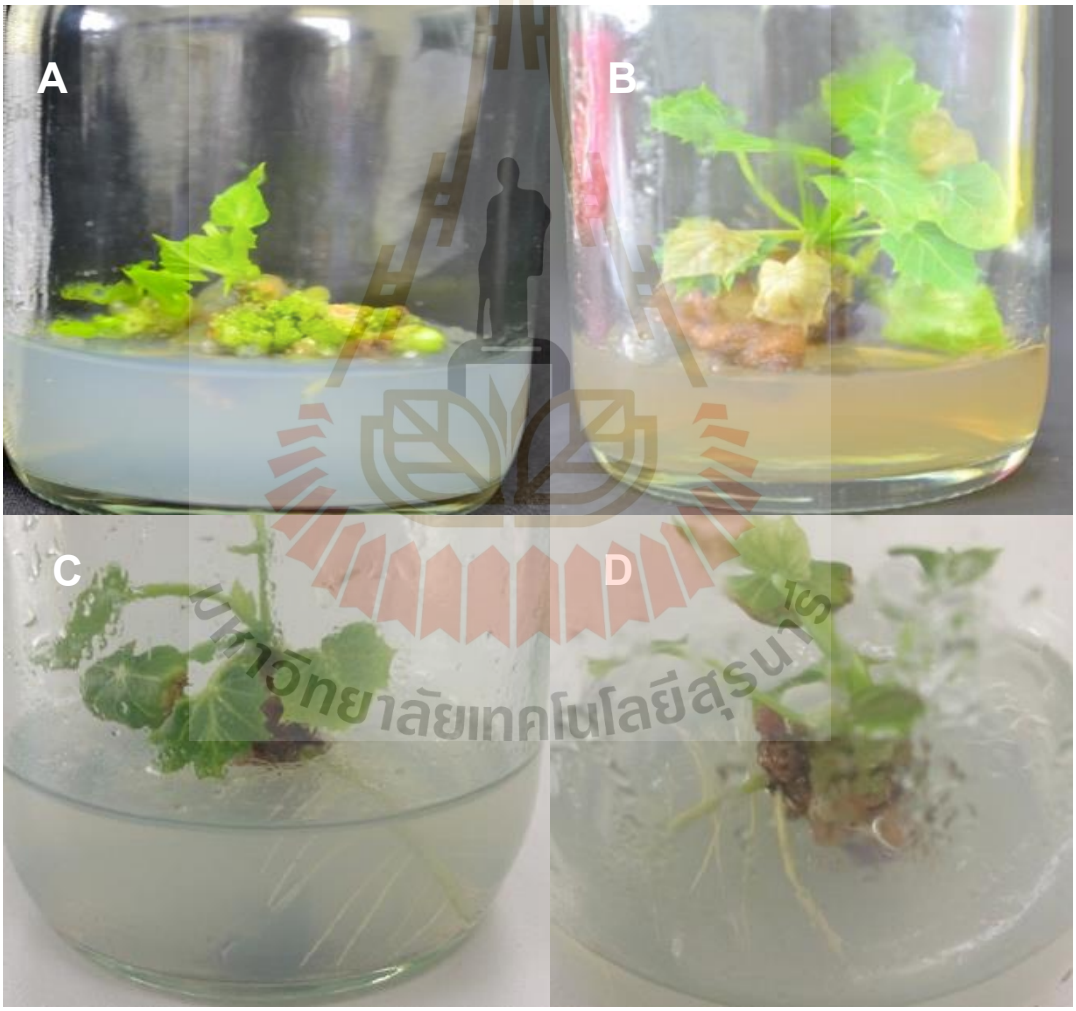

Figure 3.5 Regeneration of shoot and rooted shoots. (A) regenerated shoot on medium supplemented with 2 mg/L BA; (B) root formation activation on MS medium supplemented with 0.5 mg/L IBA and 100 mg/L PG; (C-D) root elongation on half MS medium supplemented with 25 mg/L AC.

3.5 Discussion

Sterilization of leaves for plant tissue culture with non-hazard sanitizer can be done. Hydrogen peroxide of 0.5% was the optimum concentration for *ex vitro* Jatropha leaf explants sterilization. Jatropha calli were produced from leaves on MS medium with the combination of BA and IBA. Indirect shoots were developed on MS medium supplemented with only BA. Then, root was induced on half MS medium supplemented with IBA and PG. The roots were able to elongate and branch on half MS medium supplemented with AC.

Sterilization is the key of success in plant tissue culture. Therefore, chemicals and cleaning procedures should be considered. Simple and ecologically friendly methods were studied to find the process for Jatropha tissue culture via leaf explants. The result indicated that sodium hypochlorite was not suitable to sterilize Jatropha leaves. However, 5% of sodium hypochlorite was successfully used to sterilize *Coccinia abyssinica* leaves (Guma et al., 2015). The performance of sodium hypochlorite was tested on grapevine axillary buds. The contamination rate was only 19.8% when sterilized with 1.3% sodium hypochlorite for 1 hour (Lazo-Javalera et al, 2016). Thus, sodium hypochlorite might be suitable to use with grapevine axillary buds but not Jatropha leaf explants.

While, hydrogen peroxide has rarely been used for leaf sterilization. In this work, hydrogen peroxide was used and the efficiency for leaves explant sterilization was tested. The result showed that hydrogen peroxide was better than sodium hypochlorite for Jatropha leaf sterilization.

Calli can be formed with the combination of the plant hormone auxin and cytokinin. Compact calli are the best phenotype to induce shoots. The work of Mazumdar et. al in

2010 has shown that after 3 weeks, Jatropha cotyledonary tissue can form 70.8% callus after culturing on MS medium supplemented with 1.5 mg/L BAP and 0.05 mg/L IBA. Our results showed that the combination of BA and IBA gave 90.5% callus formation from leaf explants. However, other types of auxin and cytokinin have also been used to induce Jatropha callus. A combination of NAA and kinetin could induce callus from epicotyl. MS medium supplemented with 1 mg/L NAA and 0.1 mg/L Kinetin gave rise to high callus formation rate (Li et al, 2012). These experiments showed that the combination of auxin and cytokinin in MS medium can induce Jatropha calli formation.

Shoots induced from Jatropha callus on MS medium supplemented with 1.5 mg/L 6-Benzylaminopurine (BAP), 0.6 mg/L kinetin, 0.3 mg/L Indole-3-acetic acid (IAA), and 0.1 mg/L TDZ, after 8 weeks was up to 8.25 cm (Cecilia et al., 2016). The work of Subroto, 2014 showed 45.8% shoot induction on MS medium supplemented with 2 mg/L BAP, 0.05 mg/L IBA, and 0.5 mg/L GA_3. However, IAA is a heat sensitive hormone and TDZ is somewhat a toxic hormone. These two hormones are not simple to prepare and use. Another alternative way of direct shoot organogenesis induction from Jatropha juvenile cotyledon was achieved with MS medium supplemented with 0.5 mg/L BAP, 0.2 mg/L IBA, and 1.0 mg/L TDZ. Shoots were induced up to 3 shoots per explant within 6 weeks (Tsegay et al, 2017). However, TDZ is a herbicide that is toxic to fish and aquatic organisms and it is not easily degraded. It also causes irritation to eyes, skin and mucous membranes in human (Paranjape, 2014). In our work only BA and IBA were used to induce shoot from compact Jatropha callus. Both single hormone or the combination of the two hormones can give rise to Jatropha shoot within 4 weeks.

Similarly, 52.0% Jatropha root could be induced within 2-3 weeks with MS basal medium supplemented with 0.2 mg/L IBA with 5.6 roots per shoot, and 8.7 cm of root

length (Datta et al, 2007). IBA, which is an auxin, plays a critical role in root initiation. Moreover, the balancing of auxin and cytokinin is an important for root induction. The highest efficiency of root induction was by using half wood plant medium (WPM) supplemented with 0.5 mg/L IBA and 30.0 mg/L PG. Root formation was up to 83.0%, which could generate root up to 3.1 roots per shoot within 6 weeks (Daud et al, 2013). In our study, roots were also regenerated from modification of the above root induction medium. A few plantlets were obtained.

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CHAPTER IV

GREEN FLUORESCENT PROTEIN EXPRESSION IN SUSPENSION CELL CULTURE

4.1 Abstract

The promoter is an important part to control recombinant protein expression. However, to localize the produced recombinant protein outside the cell depends on the signal sequence (SS) or signal peptide. The Cauliflower Mosaic Virus (CaMV) 35S constitutive promoter and the Rd29a inducible promoter were used to drive *Oryza sativa* 33 KD protein signal peptide (SS) fused with Green Fluoresce reporter Protein (GFP) in *Arabidopsis thaliana*. In suspension culture the GFP fluorescent intensity in the media was found to be higher when driven by the Rd29a promoter than the CaMV 35S promoter. After plasmolysis of transgenic seedling, the GFP was found to be between the plasma membrane and the cell wall. The Rd29a promoter could be used to drive the SS fused with GFP or other high value proteins in plant. In suspension culture, the high value recombinant protein, such as a growth factors, antibodies or vaccines can be easily harvested and purified.

4.2 Introduction

Recombinant proteins used for pharmaceutical products can be produced in plants via both whole-plants and plant cells. There are several advantages to produced recombinant proteins by plant cells such as rapid growth, used less space and effort.

Antibodies, vaccines, cytokines, and growth factors are high-value products that have been generated in the plants. Post-translation modification of the recombinant products can be easily obtained in plant with less allergy to humans (Xu et al., 2011).

Promoter is responsible for controlling the gene expression, two types of influential promoters have been mostly used to achieve high recombinant protein; constitutive and inducible promoters. The CaMV 35S promoter is one of the most highly used promoter to drive constitutive expression of recombinant gene in whole plants. The CaMV 35S promoter has been challenged by other constitutive or inducible promoters (Holtorf et al., 1995: Van der Fits and Memelink., 1997: Samac et al., 2004). Inducible promoter such as Rd29 promoter needs to be induced by chemicals or stress conditions. Higher production of target proteins can be seen when the promoter is activated effectively (Potenza et al., 2004). Interestingly, Rd29a promoter driven GFP expression in plant suspension culture has not been published.

Signal peptides are small peptide of about 20-30 amino acids. Their application has been used to display recombinant proteins. Three signal peptide, aAmy3, CIN1, and 33 KD protein driven by Ubiquitin (Ubi) constitutive promoter have been compared to display GFP in rice suspension culture. GFP was found in the medium. The use of 33 KD signal peptide showed more GFP in the media than the use of aAmy3 and CIN1 signal peptide. The 33 KD protein signal peptide fused with granulocyte-macrophage colony-stimulating factor also showed the highest secretion into the medium. Secretion into the medium is suitable for manufacture high-value recombinant protein in plant suspension culture system (Huang et al., 2015). It helps decrease the process of extraction and purification as well as lower chemical contamination.

In this work, CaMV 35S promoter was compared with Rd29a promoter to secrete GFP into suspension culture by using the 33 KD protein signal peptide (SS) from *Oryza sativa*. The efficiency of SS was observed under confocal microscope. Comparison of promoters was done by measure the GFP fluorescent in the media.

4.2 Methods

4.2.1 Plasmid construction

pMDC83 plant expression vector was used as a backbone vector. The T-DNA region of the vectors are shown in Figure 4.1. The SS gene was amplified by PCR using cDNA prepared from *Oryza sativa japonica* and cloned to *Pac*I and *Apa*I restriction site of pMDC83 vector using Forward primer: 5' AATTTAATTAAATGGCGGCATTAA GCCAGCTG 3' and Revere primer: 5' AATGGGCCCCCGATGGGATACGTCGTC GCCGC 3' to make pMDC83-SS (Figure 4.1b). The *Rd29a* inducible promoter gene was amplified by PCR using Forward primer: 5' CCAAGCTTCATGTTAAAT GGCTATGC 3' and Reveres primer: 5' TGAGATCTAGTAAAACAGAGGAGG GTCTC 3'. The cDNA prepared from *A. thaliana* was used as template and then the PCR product was cloned and into the *Hind*III and *Xba*I sites of pMDC83-SS to replace the 35S promoter to make Rd29a:SS:GFP (Figure 4.1C).

4.2.2 Flora dip method

Arabidopsis thaliana Columbia (Col-0) wild-type (WT) seeds were cleaned with 15% commercial bleach (DanKlorix, Hamburg, Germany) for 20 min, washed three times with sterile distilled water and sown on half-Murashige and Skoog (MS) (Duchefa Biochemie, Haarlem, The Netherlands), containing 0.5% (w/v) sucrose and 0.8% (w/v) phytagel (Sigma, St. Louis, USA). After 10 days at 22°C, the seedlings were

transferred and grown in mixed peat moss and perlite (1:1) with 16/8 h (day/night) photoperiod for 5-6 weeks until inflorescences appeared.

Agrobacterium tumefaciens strain GV3101 carrying the binary vectors were grown in the dark at 28°C on LB plate supplemented with 100 µg/mL rifampicin and 50 µg/mL kanamycin. Then, a single colony was transferred to 5 mL liquid medium. 3 mL culture were transferred into 500 mL medium and grown for another 2 days. After centrifugation, the pellet was resuspended in 100 mL of transformation buffer containing half-MS, 0.5 g/L MES (Duchefa Biochemie, Haarlem, The Netherlands), 0.03% (v/v) silwet L-77 and 0.5% (w/v) sucrose (Zhang et al., 2006). Inflorescences of *A. thaliana* were dipped into the Agrobacterium solution, then transformed plants were warped in plastic bags overnight to increase humidity. T_0 *A. thaliana* seeds were selected on half-MS containing 0.5% (w/v) sucrose, 0.8% (w/v) phytagel, 50 µg/mL hygromycin and 100 µg/mL cefotaxime and transfer to peat moss and pelite.

4.2.3 PCR Check Transgenic Plants

The T_0 seedlings were then potted in peat moss mixed with perlite and the T_1 seeds were collected. The T_1 and T_2 seeds were selected on half-MS agar containing 50 µg/mL hygromycin. Total genomic DNA from T_2 leaves and Col-0 WT (control) were extracted using 50 mM EDTA pH 8.0, 50 mM Tris-HCI pH8.0 and 0.2% w/v SDS (Tsugama et al., 2011). PCR analysis was conducted to detect the presence of the GFP fragment in the T_2 leaves using Forward primer: 5’ ATGAGTAAAGGAGAAGAA CTTTTCACTGGAGTTG 3’ and Revere primer: 5’ TTTGTATAGTTCATCCAT GCCATGTGTAATCCC 3’. PCR was performed in a 15 µL reaction volume. Temperature cycling was 95°C for 3 min and 35 cycles of 95°C for 30 sec, 54°C for 30

sec, 72°C for 40 sec and final extension at 72°C for 5 min. PCR samples were subjected to electrophoresis in 1% (w/v) agarose gel and visualized.

4.2.4 Plant tissue culture and cell suspension

T_3 Transgenic *A. thaliana* seeds were cleaned and germinated as mention above. After 21-28 days, the cotyledons were collected and cultured on Gamborg B5 medium (Gamborg et al., 1968) containing 2% (w/v) sucrose, 0.5 mg/L of dichlorophenoxyacetic acid (2,4-D) and 0.05 mg/L kinetin. After 4 weeks, calli were transferred and cultured in Gamborg B5 liquid medium.

4.2.5 Bradford protein assay

GFP expressed in *Escherichia coli* was purified and quantified for the total protein by Bradford method. The bradford solution contained 50 mg of Coomassie brilliant blue G-250 in 50 mL 85% phosphoric acid. The solution was adjusted to 500 mL and filtered thought Whatman #1. The protein concentration of purified GFP expressed from *E. coli* was measured using a Labsystems VarioscanTM LUX (Thermo Fisher Scientific) at 595 nm.

4.2.6 Fluorescent measurements

The fluorescent intensity of different amount of GFP expressed from *E. coli* were measured using a Labsystems VarioscanTM LUX (Thermo Fisher Scientific) and a standard curve was generated. The proteins were excited at 488 nm and fluorescence emission recorded at 520 nm. The GFP fluorescent from the suspension culture media were analyzed and concentration was calculated from purified GFP expression in *E. coli* standard curved.

One-month-old WT, 35S:GFP, 35S:SS:GFP, and Rd29a:SS:GFP suspension culture were sub-cultured. On day 0, 3, and 6, one milliter of suspension culture were

collected and spinned down at 14,000 rpm for 10 min and the fluorescent intensity was measured as mentioned above. The experiments were repeated five times with three replications.

300 mg of fresh callus of WT and Rd29a:SS:GFP were cultured in 10 mL liquid medium. The suspension cultures were collected at day 0, 3, 6, 9, 12, and 18, then the fluorescent intensity was measured as mentioned above. The experiments were repeated three times with three replications.

4.2.7 SDS-PAGE

Three milliters of day 6 the suspension culture was collected and spinned down at 4,000 rpm for 10 min. Media was precipitated with 4 volume of acetone and was incubated at -20 °C. After 1 hr, they were centrifuged at 14,000 rpm for 10 min and washed with 500 µL of acetone. The pellets were dried and resuspension in 100 µL of loading dye consists of 250 mM Tris-HCI pH 6.8, 10% (w/v) SDS, 30% (v/v) glycerol, 500 mM DTT, 0.05% (w/v) bromophenol blue and 10% (w/v) 2-mercaptotanol. Fifteen microliters were loaded on 15% SDS-PAGE. The gel was run at constant voltage of 100 volts for 90 min. The gel was stained with Coomassie brilliant blue solution for 10 min, then was destained in destaining solution.

4.2.8 Confocal Microscopy

Five-day-old T_3 seedlings grown on half-MS were observed using laser scanning confocal microscopy (Nikon A1Rsi). Plasmolysis was induced with 0.8 M mannitol for 30 min, and then incubated in 0.8 M mannitol containing 20 µg/mL propidium iodide for cell wall straining for 15 min. GFP fluorescence was detected in the 500–550 nm range using 488 nm laser excitation.

4.2.9 Statistical analysis

All experiment was set up in a completely randomized design (CRD) and the statistical analysis of data and one-way ANOVA followed by Duncan's multiple range tests were performed with the P-value of 0.05.

4.3 Results

4.3.1 CaMV 35S Constitutive promoter and Rd29a inducible promoter in Arabidopsis cell

A. thaliana transformations were produced by floral dip method Agrobacterium carrying 3 different vectors (Figure 4.1) were used in this work. T_2 *A. thaliana* were checked by PCR (data not shown) using GFP primers. All transgenics plants showed positive results. T_3 generation plants were used in further experiments.

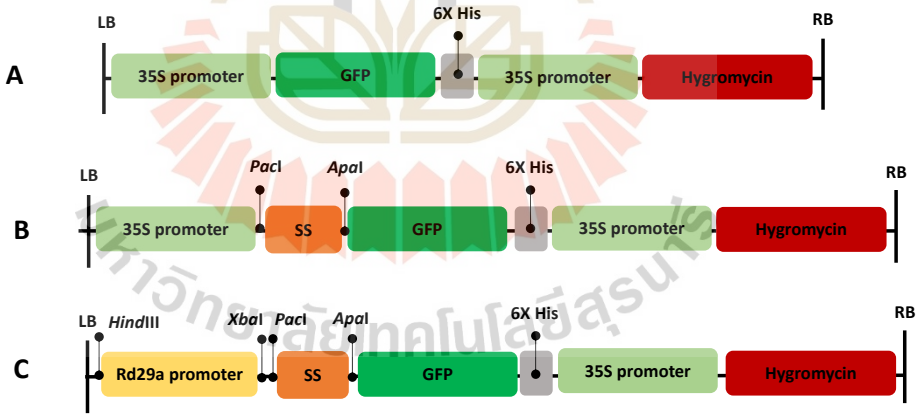

Figure 4.1 Schematic diagram of the T-DNA region of the vector used. (A) 35S:GFP; (B) 35S:SS:GFP; (C) Rd29a:SS:GFP, LB; left border, RB; right border, 35S; 35S CaMV constitutive promoter, Rd29a; Rd29a inducible promoter SS: 33 KD Rice signal peptide, GFP; Green Fluorescent Protein, 6X His; 6 histidine sequence tag, Hygromycin; Hygromycin resistance gene driven by CaMV 35S promoter.

4.3.2 Subcellular localization of GFP

The localization of GFP led by SS was investigated in WT and T_3 seedling. The result showed that GFP was not detected in WT seedling (Figures 4.2A-C). After plasmolysis, GFP was detected in the cytoplasm of the transgenic seedling of 35S:GFP (Figures 4.2D-F). While transgenic seedling of 35S:SS:GFP and Rd29a:SS:GFP showed GFP at the extracellular matrix (Figures 4.2G-L) and the cytoplasm. Interestingly in Rd29a:SS:GFP transgenic seedling GFP was also detected inside the plasma membrane.

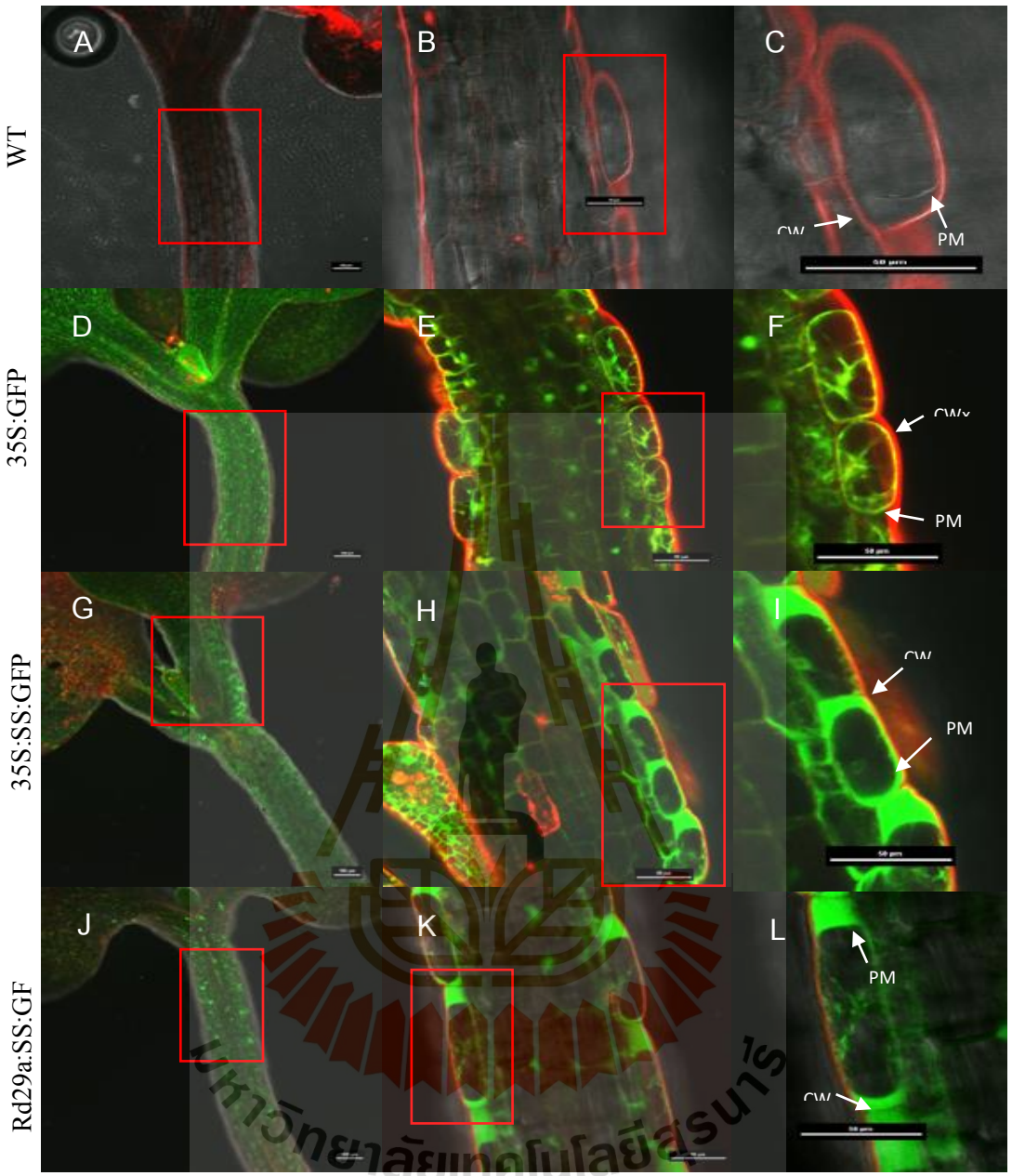

Figure 4.2 Confocal microscope analysis of GFP emission after treated with mannitol. (A-C) Seedling of WT non-detected; (D-F) Seedling of 35S:GFP; (G-I) Seedling of 35S:SS:GFP; (J-L) Seedling of Rd29a:SS:GFP. The image detected under both green fluorescence and red fluorescence channel (propidium iodide staining) and overlaid (yellow color). CW; cell wall, PM; plasma membrane. Scale bar (A, D, G, J) = 100 μm, Scale bar (the second and third column) = 50 μm.

4.3.3 Fluorescence expression in suspension culture

GFP of the suspension cultures of T_3 calli were measured in relative fluorescent unit (RFU). Very low fluorescence was detected in suspension of WT calli. At day 3, the RFU was low in all samples. However at day 6, RFU of media from Rd29a:SS:GFP (2.5) was higher than the media of 35S:SS:GFP (1.4) which was not significant difference from the media of 35S:GFP (0.8) (Figure 4.3).

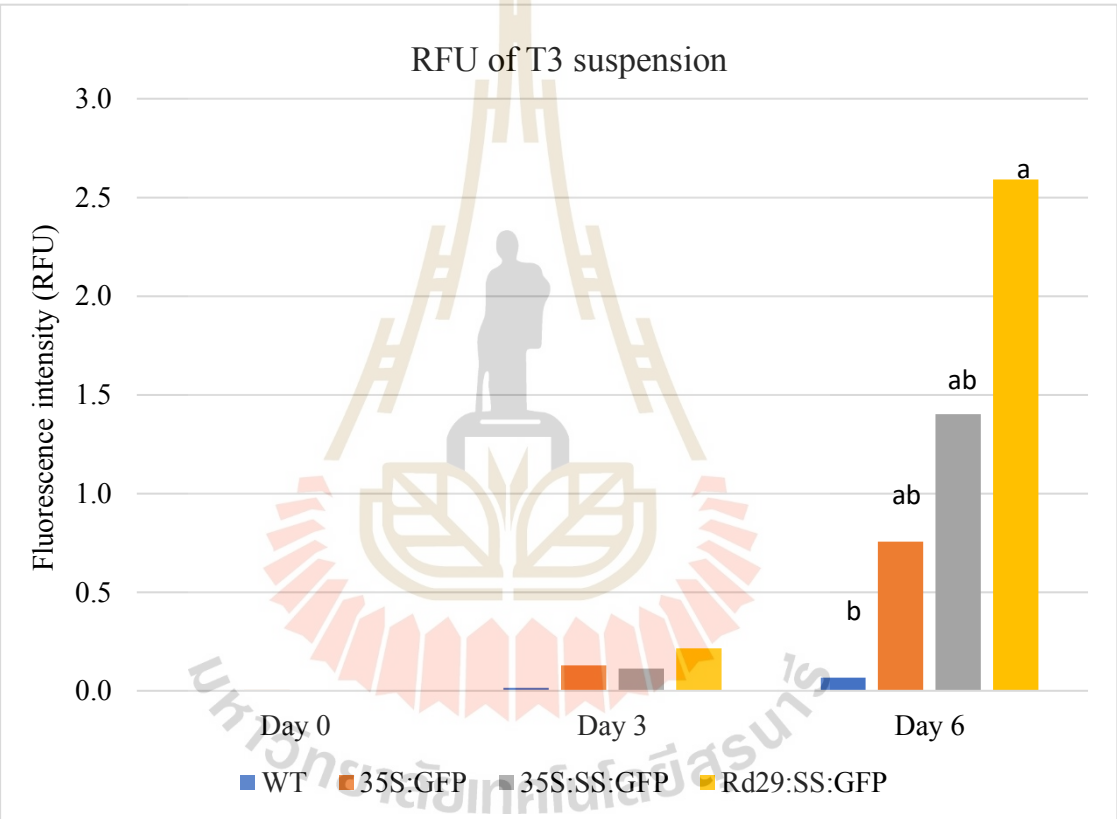

Figure 4.3 Comparison of GFP expression in T_3 in suspension culture. Suspension from 4 weeks old calli culture harvested at day 0, 3 and 6.

4.3.4 Comparison of WT and Rd29a promoter

The expression of GFP in the media of freshly prepared T_3 calli containing Rd29a:SS:GFP was compared to wildtype suspension. No fluorescence was detected

from WT media. However, high level of RFU was detected from day 3 onward up to 25 RFU and leveled of at the day 18 (Figure 4.4).

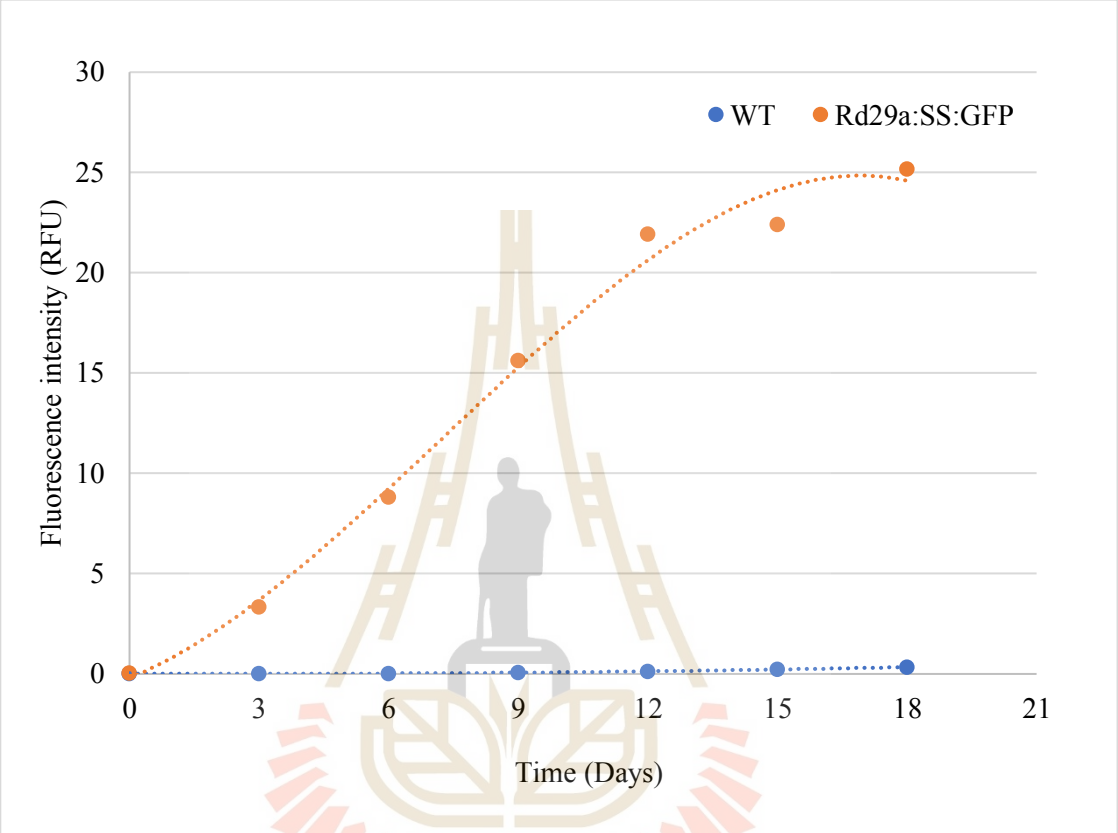

Figure 4.4 RFU of suspension culture of T_3 Rd29a:SS:GFP and WT. Fresh calli of Rd29a:SS:GFP cultured for 18 days and compared with WT via RFU.

4.3.5 Quantification of recombinant GFP expression

Suspension culture medium was precipitated with acetone and analyzed by SDS-PAGE (Figure 4.5). The 27 kDa GFP from *E. coli* was used as positive control (GFP) to compare the size. GFP band was detected from suspension culture of 35S:SS:GFP and Rd29a:SS:GFP at approximately 27 kDa but not detected in WT.

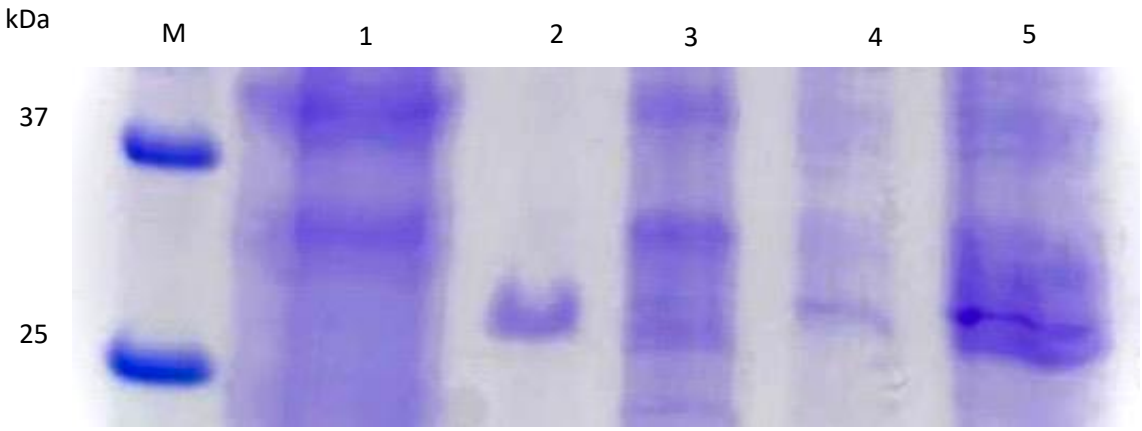

Figure 4.5 Identification of GFP in suspension medium by SDS-PAGE. M: molecular weight marker; Lane 1: WT; Lane 2: GFP purified expressed in *E. coli* Lane 3: 35S:GFP; Lane 4: 35S:SS:GFP. ; Lane 5: Rd29a:SS:GFP.

4.4 Discussion

The protein secretion pathway started from the recognition of the signal recognition particle (SRP) to the signal peptide. Then, the ribosome-protein complex is translocated to an endoplasmic reticulum (ER) translocation pore or the membrane of the ER. Then the signal peptide sequence is cleaved off by signal peptidase. Then, nascent protein is transported to the Golgi apparatus from *cis* to *trans* direction with some modifications such as glycosylation and proteolytic processing. Then, the vesicles are formed and released to the extracellular space (Chung and Zeng., 2017). The GFP with SS expressed from both CaMV 35S and Rd29a promoters were found in the cytoplasm, while GFP without the SS was located in the intracellular space, as confirmed by both confocal microscopy and fluorescence of the suspension culture medium. Similarly, GFP fused with the signal peptide from tobacco extensin was found between the plasma membrane and cell wall (Zhang et al., 2019). However, Pr1b secretory signal peptide

from tobacco targeted the tag protein to only the ER (Joensuu et al., 2010; Häkkinen et al., 2018).

The CaMV 35S promoter has been used since the early days of transgenic plants. It has been shown to constitutively express recombinant products in almost all parts of the plant (Sanders et al., 1987; Pauli et al., 2004). However, Rd29a inducible promoter has recently been shown to highly express under stress conditions on solid media (Sarker et al., 2016), but to our knowledge no work in suspension culture has been performed. GFP expressed from a synthetic promoter of drought response element (DRE) from the cor 15a promoter of *A. thaliana* upstream of the core sequence of CaMV 35S promoter fused with TMV omega gave very high GFP expression (Gerasymenko and Sheludko., 2017) after cold induction. In 2014, Huet et al., has shown that GFP fused with an *A. thaliana* signal peptide produced from a double CaMV 35S promoter in turnip hairy root system was as high as 120 mg/L suspension culture. Improvement of gene expression can be made by using a strong promoter, and adding enhancers or activators. In our work, we have expressed GFP under control of the Rd29a inducible promoter. Normally, an inducible promoter will need chemical induction, for example mannitol or plant hormones such as abscisic acid (ABA), or stress condition to activate high level of expression. Shaking or agitation of suspension cell culture can also induce the stress-induced promoter because it is a stress condition (Sun and Linden., 1999). Hence, the Rd29a:SS:GFP in suspension culture was activated with a higher level of expression without any chemical induction.

Normally, initial stationary phase has been recommended to be the best period to harvest manufactured recombinant products to have high cell density, to avoid depletion of nutrient and protease production. At stationary phase, the production of toxic by-

products can be found (Galáz-Ávalos et al., 2012). Our result showed that GFP reach stationary phase of production at day12 to day15.

Different plant suspension starter culture systems provided very different amounts of recombinant protein product. Starting the suspension culture from fresh compact calli was shown to produce much higher productivity than pipetting cells from old suspension (sub-cultured). Fresh calli from different transgenic leaves provided similar cell density. In contrast, transferring suspension cells by pipetting/pouring gave rise to different amounts of cells and clump of calli into the new suspension medium (Mustafa et al., 2011). The activation of the Rd29a promoter has been shown with NaCl, mannitol, ABA, ABA signaling, cold, and drought stress conditions (Hua et al., 2006; Li et al., 2013), however, in this study, we have shown that shaking of the suspension cell Rd29a can also induce expression of GFP at high level when fresh calli were used.

4.5 Conclusion

The signal peptide of *O. sativa* can be used to secrete recombinant protein from *Arabidopsis* cell into suspension culture, which is more convenient and more efficient to harvest recombinant protein from a green cell factory. The Rd29a inducible promoter has been successfully used to control the recombinant GFP expression. This is the first publication of recombinant protein derivn by the Rd29a promoter in suspension culture. Next we will produce high value human growth factors in these cell factories, since it will be easily purify from the culture medium.

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CHAPTER V

CONCLUSION

Sterilization of leaves for plant tissue culture with non-hazard sanitizers can be done. Hydrogen peroxide of 0.5% was the optimum concentration for *ex vitro* Jatropha leaf explants sterilization. Jatropha calli were produced from leaves on MS medium with the combination of BA and IBA. Indirect shoots were developed on MS medium supplemented with only BA. Then, root was induced on half MS medium supplemented with IBA and PG for activation. The roots were able to elongate and branch on half MS medium supplemented with AC. This study will be suitable for Jatropha plantlet to be free from viruses and contaminations and also can be a part of Jatropha transgenic plant production.

The signal peptide from rice can be used for the secretion of protein products from Arabidopsis suspension cell. The GFP recombinant protein can be detected in the suspension medium. Moreover, The Rd29a inducible promoter was shown to control the recombinant GFP expression and gave higher expression of GFP than the double 35S promoter. This study will be used as a model to produce high value product for example; human growth factors which would be more convenient to harvest and purify when compare to other methods.

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APPENDIX

Optimization of leaf disinfectant

$$\text{Green explant without contamination (\%)} = \frac{\text{Number of green explant}}{\text{Total number of explant treated}} \text{ x100}$$

Media optimization for callus induction

$$\text{Callus formation (\%)} = \frac{\text{Number of callus}}{\text{Total number of explant cultured}} \text{ x100}$$

Media optimization for shoot induction

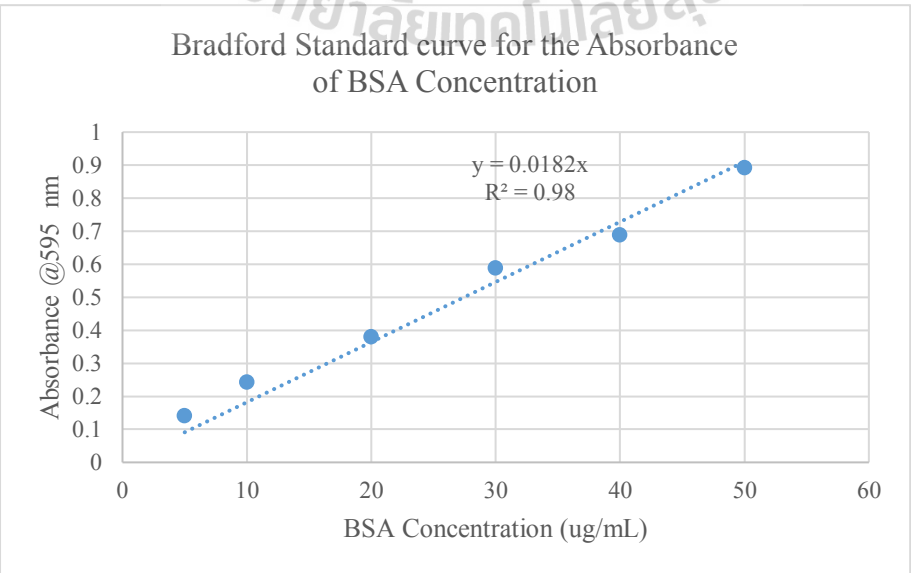
$$\text{Shoot regeneration (\%)} = \frac{\text{Number of shoots}}{\text{Total number of callus cultured}} \text{ x100}$$

$$\text{Shoot length} = \frac{\text{Length of all shoots}}{\text{Total number of shoots}}$$

$$\text{Shoot number} = \frac{\text{Number of shoots}}{\text{Total number of callus with shoots}}$$

Standard Curve BSA Preparation

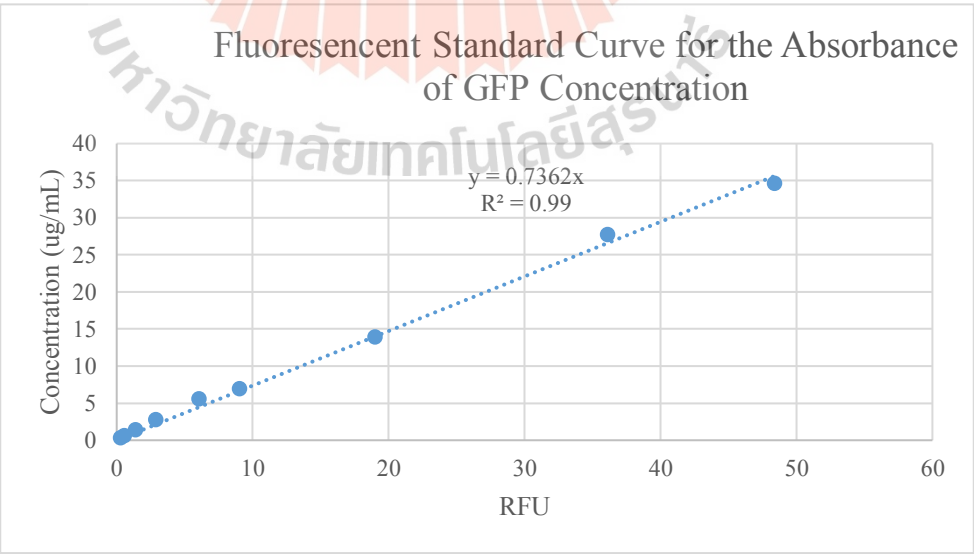
BSA(µg/ml)	Time 1		Time 2		AVE
	Rep1	Rep2	Rep1	Rep2	
5	0.124	0.184	0.095	0.161	0.141
10	0.295	0.184	0.269	0.223	0.243
20	0.431	0.314	0.341	0.432	0.380
30	0.584	0.541	0.718	0.513	0.589
40	0.706	0.692	0.681	0.678	0.689
50	0.767	0.916	0.938	0.949	0.893



Standard Curve for the Absorbance of GFP

Sample	Total protein			x20	Total protein ug/mL
	R1	R2	AVE		
STD GFP dilutute 1:20	0.280	0.224	0.252	5.040	276.923

RFU	ug/mL
0.280	0.277
0.580	0.554
1.404	1.385
2.875	2.769
6.064	5.538
9.032	6.923
18.998	13.846
36.118	27.692
48.388	34.615



Raw data: Fluorescence expression in suspension culture

BLANK	Fluorescence intensity			
	Rep1	Rep2	Rep3	AVE
Media	0.01827	0.01981	0.01733	0.01847

Day 0

Sample	Fluorescence intensity			AVE	AVE-BLANK	All AVE
	R1	R2	R3			
Col-o R1	0.02101	0.01938	0.01632	0.01890	0.00043	0.00073
Col-o R2	0.02016	0.01938	0.01727	0.01894	0.00048	
Col-o R3	0.01831	0.01681	0.01959	0.01824	-0.00023	
Col-o R4	0.02142	0.01937	0.02143	0.02074	0.00227	
35S_GFP R1	0.03130	0.02088	0.02624	0.02614	0.00767	0.00345
35S_GFP R2	0.01950	0.02293	0.02027	0.02090	0.00243	
35S_GFP R3	0.02081	0.01787	0.02916	0.02261	0.00414	
35S_GFP R4	0.01696	0.01860	0.01853	0.01803	-0.00044	
35S_33KD_GFP R1	0.01998	0.02675	0.01762	0.02145	0.00298	0.00095
35S_33KD_GFP R2	0.02317	0.01777	0.01721	0.01938	0.00091	
35S_33KD_GFP R3	0.01904	0.02164	0.01705	0.01924	0.00077	
35S_33KD_GFP R4	0.01815	0.01854	0.01610	0.01760	-0.00087	
Rd29_33KD_GFP R1	0.01484	0.01750	0.01671	0.01635	-0.00212	0.000000
Rd29_33KD_GFP R2	0.01370	0.01787	0.01302	0.01486	-0.00361	
Rd29_33KD_GFP R3	0.01716	0.01996	0.01721	0.01811	-0.00036	
Rd29_33KD_GFP R4	0.01502	0.01847	0.01713	0.01687	-0.00160	

Day 3

BLANK	Fluorescence intensity			
	Rep1	Rep2	Rep3	AVE
Media	0.02396	0.02647	0.01502	0.02182

Sample	Fluorescence intensity			AVE	AVE-BLANK	All AVE
	R1	R2	R3			
Col-o R1	0.03380	0.03459	0.03569	0.03469	0.01288	0.01442
Col-o R2	0.03668	0.03385	0.03386	0.03480	0.01298	
Col-o R3	0.03856	0.02949	0.02939	0.03248	0.01066	
Col-o R4	0.04284	0.04162	0.04445	0.04297	0.02115	
35S_GFP R1	0.09133	0.07657	0.08730	0.08507	0.06325	0.12807
35S_GFP R2	0.23000	0.25310	0.19930	0.22747	0.20565	
35S_GFP R3	0.15930	0.15200	0.15270	0.15467	0.13285	
35S_GFP R4	0.11730	0.15380	0.12590	0.13233	0.11052	
35S_33KD_GFP R1	0.11940	0.10040	0.10910	0.10963	0.08782	0.11258
35S_33KD_GFP R2	0.14140	0.11380	0.11640	0.12387	0.10205	
35S_33KD_GFP R3	0.11810	0.11560	0.13360	0.12243	0.10062	
35S_33KD_GFP R4	0.18800	0.16070	0.19620	0.18163	0.15982	
Rd29_33KD_GFP R1	0.22040	0.21050	0.17560	0.20217	0.18035	0.21606
Rd29_33KD_GFP R2	0.14850	0.14350	0.13710	0.14303	0.12122	
Rd29_33KD_GFP R3	0.31110	0.26940	0.19320	0.25790	0.23608	
Rd29_33KD_GFP R4	0.32060	0.44130	0.28330	0.34840	0.32658	

Day 6

BLANK	Fluorescence intensity			
	Rep1	Rep2	Rep3	AVE
Media	0.01444	0.01632	0.01149	0.01408

Sample	Fluorescence intensity			AVE	AVE-BLANK	All AVE
	R1	R2	R3			
Col-o R1	0.10350	0.11870	0.12060	0.11427	0.10018	0.06531
Col-o R2	0.07274	0.07361	0.06758	0.07131	0.05723	
Col-o R3	Contaminated					
Col-o R4	0.04966	0.0553	0.05284	0.05260	0.03852	
35S_GFP R1	0.11630	0.12010	0.13350	0.12330	0.10922	0.75663
35S_GFP R2	1.50600	1.71200	1.75200	1.65667	1.64258	
35S_GFP R3	0.29680	0.30330	0.28450	0.29487	0.28078	
35S_GFP R4	1.00800	1.06400	0.95210	1.00803	0.99395	
35S_33KD_GFP R1	1.73900	1.74300	1.75300	1.74500	1.73092	1.40039
35S_33KD_GFP R2	1.16500	1.27700	1.20100	1.21433	1.20025	
35S_33KD_GFP R3	1.59300	1.78300	1.53900	1.63833	1.62425	
35S_33KD_GFP R4	1.16400	1.12100	0.89570	1.06023	1.04615	
Rd29_33KD_GFP R1	0.17790	0.1889	0.14050	0.16910	0.15502	2.59188
Rd29_33KD_GFP R2	5.36000	5.60700	4.82100	5.26267	5.24858	
Rd29_33KD_GFP R3	5.09100	4.41000	5.05400	4.85167	4.83758	
Rd29_33KD_GFP R4	0.14720	0.13540	0.13860	0.14040	0.12632	

Raw data: comparison of WT and Rd29a promoter

Day 0

BLANK	Fluorescence intensity			
	Rep1	Rep2	Rep3	AVE
Media	0.02299	0.02846	0.02598	0.02581

Sample	Fluorescence intensity			AVE	AVE
	R1	R2	R3		
Col-o R1	0.01817	0.02343	0.02647	0.02269	0.02440
Col-o R2	0.01774	0.02029	0.02116	0.01973	
Col-o R3	0.02195	0.02810	0.02548	0.02518	
Col-o R4	0.02434	0.02495	0.02495	0.02475	
Col-o R5	0.02658	0.03459	0.02764	0.02960	
Rd29_33KD_GFP R1	0.02553	0.02537	0.02695	0.02595	0.02710
Rd29_33KD_GFP R2	0.02585	0.03522	0.03249	0.03119	
Rd29_33KD_GFP R3	0.02236	0.02799	0.02849	0.02628	
Rd29_33KD_GFP R4	0.02165	0.02470	0.02421	0.02352	
Rd29_33KD_GFP R5	0.02571	0.03028	0.02975	0.02858	

Day 3

BLANK	Fluorescence intensity			
	Rep1	Rep2	Rep3	AVE
Media	0.02719	0.01967	0.02218	0.02301

Sample	Fluorescence intensity			AVE	AVE-BLANK	All AVE
	R1	R2	R3			
Col-o R1	0.03765	0.04051	0.04536	0.04117	0.01816	0.01701
Col-o R2	0.04004	0.04041	0.04369	0.04138	0.01837	
Col-o R3	0.03527	0.03783	0.03419	0.03576	0.01275	
Col-o R4	0.03642	0.03786	0.04146	0.03858	0.01557	
Col-o R5	0.04149	0.04727	0.04093	0.04323	0.02022	
Rd29_33KD_GFP R1	3.81900	4.53100	3.97200	4.10733	4.08432	3.34183
Rd29_33KD_GFP R2	3.13300	3.12200	3.53800	3.26433	3.24132	
Rd29_33KD_GFP R3	1.67000	1.76900	2.24700	1.89533	1.87232	
Rd29_33KD_GFP R4	3.35260	2.45100	2.78700	2.86353	2.84052	
Rd29_33KD_GFP R5	4.52900	5.02700	4.52500	4.69367	4.67065	

Day 6

BLANK	Fluorescence intensity			
	Rep1	Rep2	Rep3	AVE
Media	0.03253	0.03006	0.03868	0.03376

Sample	Fluorescence intensity			AVE	AVE-BLANK	All AVE
	R1	R2	R3			
Col-o R1	0.06190	0.06431	0.05088	0.05903	0.02527	0.01865
Col-o R2	0.05552	0.04921	0.05125	0.05200	0.01824	
Col-o R3	0.04897	0.03535	0.03648	0.04026	0.00651	
Col-o R4	0.04377	0.04636	0.05122	0.04712	0.01336	
Col-o R5	0.06099	0.05480	0.07513	0.06364	0.02988	
Rd29_33KD_GFP R1	8.17000	8.71100	8.33100	8.40400	8.37024	8.81811
Rd29_33KD_GFP R2	8.55000	8.44700	9.10000	8.69900	8.66524	
Rd29_33KD_GFP R3	5.49100	5.72800	5.19300	5.47067	5.43691	
Rd29_33KD_GFP R4	8.05200	7.88500	7.03000	7.65567	7.62191	
Rd29_33KD_GFP R5	14.66000	14.36000	13.07000	14.03000	13.99624	

Day 9

BLANK	Fluorescence intensity			
	Rep1	Rep2	Rep3	AVE
Media	0.02350	0.02999	0.03332	0.02894

Sample	Fluorescence intensity			AVE	AVE-BLANK	All AVE
	R1	R2	R3			
Col-o R1	0.07638	0.08746	0.09659	0.08681	0.05787	0.07442
Col-o R2	0.06832	0.06482	0.12990	0.08768	0.05874	
Col-o R3	0.14790	0.16270	0.14650	0.15237	0.12343	
Col-o R4	0.08142	0.09075	0.14310	0.10509	0.07615	
Col-o R5	0.06530	0.08829	0.10090	0.08483	0.05589	
Rd29_33KD_GFP R1	13.71000	15.56000	16.11000	15.12667	15.09773	15.62760
Rd29_33KD_GFP R2	15.21000	18.83000	17.24000	17.09333	17.06440	
Rd29_33KD_GFP R3	7.24200	7.89400	8.68200	7.93933	7.91040	
Rd29_33KD_GFP R4	25.62000	27.05000	28.52000	27.06333	27.03440	
Rd29_33KD_GFP R5	10.22000	10.95000	12.01000	11.06000	11.03106	

Day 12

BLANK	Fluorescence intensity			
	Rep1	Rep2	Rep3	AVE
Media	0.02232	0.02607	0.02249	0.02362

Sample	Fluorescence intensity			AVE	AVE-BLANK	All AVE
	R1	R2	R3			
Col-o R1	0.08347	0.14590	0.34700	0.19212	0.16847	0.11046
Col-o R2	0.09186	0.11530	0.07202	0.09306	0.06940	
Col-o R3	contaminated					
Col-o R4	0.08470	0.11240	0.22240	0.13983	0.11621	
Col-o R5	0.09534	0.12150	0.11720	0.11135	0.08772	

Sample	Fluorescence intensity			AVE	AVE BLANK	All AVE
	R1	R2	R3			
Rd29_33KD_GFP R1	18.60000	20.17000	20.84000	19.87000	19.84637	21.93554
Rd29_33KD_GFP R2	22.34000	19.36000	23.55000	21.75000	21.72637	
Rd29_33KD_GFP R3	11.25000	12.22000	12.82000	12.09667	12.07304	
Rd29_33KD_GFP R4	15.00000	16.96000	17.50000	16.48667	16.46304	
Rd29_33KD_GFP R5	32.90000	34.87000	34.59000	34.12000	34.09637	

Day 15

BLANK	Fluorescence intensity			
	Rep1	Rep2	Rep3	AVE
Media	0.01475	0.03645	0.02498	0.02539

Sample	Fluorescence intensity			AVE	AVE-BLANK	All AVE
	R1	R2	R3			
Col-o R1	0.29440	0.23620	0.23520	0.25527	0.22987	0.23396
Col-o R2	0.28330	0.30700	0.25900	0.28310	0.25771	
Col-o R3	Contaminated					
Col-o R4	0.25440	0.20880	0.19890	0.22070	0.19531	
Col-o R5	0.36510	0.22310	0.24680	0.27833	0.25294	
Rd29_33KD_GFP R1	22.0000	23.03000	15.37000	20.13333	20.10794	22.39794
Rd29_33KD_GFP R2	20.17000	25.11000	24.32000	23.2000	23.17461	
Rd29_33KD_GFP R3	13.60000	16.05000	14.73000	14.79333	14.76794	

Sample	Fluorescence intensity			AVE	AVE BLANK	All AVE
	R1	R2	R3			
Rd29_33KD_GFP R4	Contaminated					
Rd29_33KD_GFP R5	30.09000	32.51000	32.10000	31.56667	31.54127	

Day 18

BLANK	Fluorescence intensity			
	Rep1	Rep2	Rep3	AVE
Media	0.02958	0.03487	0.03077	0.03174

Sample	Fluorescence intensity			AVE	AVE-BLANK	All AVE
	R1	R2	R3			
Col-o R1	0.39240	0.50730	0.46880	0.45617	0.42442	0.34064
Col-o R2	0.29230	0.39240	0.37330	0.35267	0.32092	
Col-o R3	Contaminated					
Col-o R4	0.40260	0.34980	0.35770	0.37003	0.33829	
Col-o R5	0.33130	0.37050	0.23020	0.31067	0.27893	
Rd29_33KD_GFP R1	22.91000	30.50000	24.56000	25.99000	25.95826	21.80159
Rd29_33KD_GFP R2	23.23000	22.07000	21.72000	22.34000	22.30826	
Rd29_33KD_GFP R3	17.25000	17.88000	16.38000	17.17000	17.13826	
Rd29_33KD_GFP R4	Contaminated					
Rd29_33KD_GFP R5	Contaminated					

Presentations

- Boonayanan, N. and Ketudat-Cairns, M. (2017) Tissue culture of *Jatropha curcas*. The 5th SUT International Agricultural Colloquium " SUT Agriculture Technology 4.0". Synchrotron auditorium hall, Suranaree University of Technology, 15-16 August, 2017 (Oral Presentation).
- Boonyanan, P., Imsoonthornruksa, S., Seifert, G. and Ketudat-Cairns, M. (2017) Recombinant Human Growth Factors Production in Plant. 6th SUT International Colloquium "Enzyme Biotechnology for food Feed and Fuel" . Suranaree University of Technology, 19-21 December, 2017 (Poster Presentation).
- Boonayanan, N. and Ketudat-Cairns, M. (2018) Jatropha tissue culture. Thai Student Academic Conference 2018 (TSAC) "Bridging Academic Research and Practical implication: knowledge creation and transferring", Belgium, 16-20 July, 2018 (Poster Presentation).
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- Boonyanan, P., Imsoonthornruksa, S., Seifert, G. and Ketudat-Cairns, M. (2019) Comparison of CaMV 35S Constitutive Promoter and Rd29a Inducible Promoter for Recombinant Protein Production. The 31th Annual Meeting of Thai Society for Biotechnology and International Cenference (TSB 2019)" Bioinnovation for sustainability". Patong Beach Phuket, Thailand, 10-12.

BIOGRAPHY

Miss Pasama Boonyanan was born on November 5, 1991 in Bangkok, Thailand. She graduated with a Bachelor degree in Crop Production Technology from Suranaree University of Technology in 2014. Then, she worked with Assoc. Prof. Dr. Mariena Ketudat-Cairns prior to her further study. She received the Royal Golden Jubilee Ph.D. (RGJ-PHD) Program (Grant no. PHD/0106/2557) to support her study in the Ph.D. program of Biotechnology, Institute of Agricultural Technology, Suranaree University of Technology. Her research consisted of tissue culture of *Jatropha curcas* and the production of GFP in cell suspension.