In Vitro Regeneration of *Indica* Rice (*Oryza sativa* L.) Cultivar Sangyod and Its Transformation by *Agrobacterium tumefaciens*

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A Thesis Submitted in Fulfillment of the Requirements for the Degree of Master of Science in Plant Science

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Thesis Title	*In Vitro* Regeneration of *Indica* Rice (*Oryza sativa* L.) Cultivar Sangyod and Its Transformation by *Agrobacterium tumefaciens*
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ABSTRACT

Indica rice cultivar Sangyod is one of the most important commercial rice cultivars in Thailand. However, a review of the literature was unable to trace any published research considering the improvement by gene transformation of Sangyod using *Agrobacterium* tumefaciens. The present study investiagted to develop a tissue culture and plantlet regeneration systems, established *Agrobacterium*–mediated transformation systems and molecular analaysis to confirm introduction of foreign gene into putative rice transformants. Mature embryos from seed were used as explant for callus induction and plantlet regeneration system. The result of experiments showed that the highest frequency of callus induction (73.08 ± 2.65%) and mean callus fresh weight (67.5 ± 7.4 mg) were obtained on MS (Murashige and Skoog, 1962) medium supplemented with 2 mg/L 2, 4-dichlorophenoxyacetic acid (2,4 –D), 750 mg/L casein hydrolysate (CH) and 200 mg/L L-proline.

The combination of 0.5 mg/L α-naphthaleneacetic acid (NAA), 1 mg/L 6-benzyladenine (BA), 0.5 mg/L Kinetin (Kn) and containing solidified MS medium gave the maximum mean fresh weight of callus (938.9 ± 44 mg), the highest percentage of green spot formation (64.17 ± 7.08%), maximum shoot induction frequency (66.25 ± 6.80%) and mean number of shoots/callus (6.12 ± 0.36 shoots). Furthermore, the greatest mean number of shoots/explant (14.93 ± 0.97 shoots) and root formation (82.71 ± 3.03%) was observed in liquidified MS medium supplemented with 0.5 mg/L NAA and 1 mg/L BA.

The culture medium and plant growth regulators (PGRs) play a significant role in callus induction, fresh weight of callus, the callus growth index and the plantlet regeneration protocol of mature rice seeds. The highest callus induction (75.63 ± 5.28%) and fresh weight of callus (68.05 ± 20.04 mg) were achieved on agricultural

research development agency (ARDA) medium supplemented with 2 mg/L 2, 4-D, 750 mg/L CH and 200 mg/L L-proline after 4 weeks of culture. The optimum mean fresh weight of callus (1272.83 ± 48.63 mg) and the highest callus growth index (11.73-fold) were obtained on day 35 of sub-culture. The combination of 0.5 mg/L NAA, 0.5 mg/L 6-benzylaminopurine (BAP), 0.5 mg/L Thidiazuron (TDZ) and 1.0 mg/L Kn gave the maximum percentage of green spot formation (72.34 ± 8.75%), plantlet regeneration frequency (67.25 ± 6.14%) and the mean number of plantlets/ callus (6.63 ± 0.47 plantlets).

Finally, an efficient transformation system for the *indica* rice cultivar Sangyod was carried out by optimizing some key factors including inoculation time and optical density (OD) of *Agrobacterium* suspension, various concentrations of acetosyringone (AS) and cefotaxime. These results showed 6-week–old Sangyod calluses derived from mature seeds were inoculated at an OD_{600} at 0.6 for 30 minutes, Then the calluses were cultured on co–cultivation medium containing ARDA medium supplemented with 100 mg/L myo-inositol and 200 µM AS for 2 days. After co-cultivation, the explants were washed with liquid ARDA medium containing 300 mg/L cefotaxime for 20 minutes. Finally, the inoculated calluses were placed on selection medium (ARDA medium containing, 300 mg/L cefotaxime, 100 mg/L myo–inositol and 0.6 mM glyphosate). The highest transformation efficiency detected by measuring transient GUS expression (70.43%) and callus survival rate (43.5%) was achieved with the foregoing conditions, the stable transfer of the GUS gene were confirmed by PCR analysis.

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LIST OF ABBREVATIONS AND SYMBOLS

2, 4 - D	2. 4 - Diphenoxy Acetic Acid
BA	6-benzyladenine
NAA	α-naphthaleneacetic acid
CH	Casein hydrolysate
TDZ	Thidiazuron
BAP	6-benzylaminopurine
OD	Optical density
SE	Somatic embryo
DMRT	Duncan’s multiple range test
PGRs	Plant growth regulators
Kin	Kinetin
NN	Nitsch & Nitsch
MS	Murashige and Skoog
CIM	Callus induction medium
SCM	Sub-culture medium
ARDA	Agricultural research development agency
FW	Fresh weight of callus
CGI	Callus growth index
RM	Regeneration medium
GS	Green spot
^{o}C	Degree celsius
Fig	Figure
mg	Milligram

LIST OF ABBREVATIONS AND SYMBOLS (continued)

mg/L	Milligram per Liter
Wt	Weight
µg	Microgram
µL	Microliter
µM	Micromole
Tris	Tris (hydroxymethyl) aminomethane
DMRT	Duncan's multiple range test
DNA	Deoxyribonucleic acid
EPSPS	5-Enolpyruvylshikimate 3-phosphate sysnthase
EDTA	Ethylenediaminetetraacetic acid
GUS	β-glucuronidase
HCl	Hydrochloric acid
M1	DNA ladder 1000 bps
$NaCl_2$	Sodium chloride
PCR	Polymarese chain reaction
TE	Tris EDTA
TAE	Tris-acetic acid-disodium ethylenediaminetetraacetic acid
TBE	Tris- boric acid –disoum ethylenediaminetetraacetic acid
Tris	Tris (hydroxymethyl) aminomethane

LIST OF PAPERS AND PROCEEDINGS

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Dear Ho Thi Linh

On behalf of the Scientific Committee of the Forth Plant Science Symposium (4PSS), I am pleased to inform you that your abstract entitled *"The effect of culture media and plant growth regulators on callus induction and regeneration of indica rice (Oryza sativa L.) cultivar Sangyod"* has been accepted for **oral presentation**. Your abstract is currently under peer review by the Scientific Committee and if necessary we will contact you for further information or minor revision. The final version of the Abstracts will be printed and distribute during the conference.

The time and date of your presentation will be announced in the **Final Scientific Program** (http://natres.psu.ac.th/Department/PlantScience/symposium) by the first week of August 2017. You are invited to submit the full paper in the conference proceeding before 18 August 2017. You will need to Register Online and make payment by Credit Card or Bank Transfer. Once the organizing committee receives your payment, we will confirm your registration and payment. Please take note that it is the policy of the Organizing Committee that only papers submitted by registered and paid authors will be published in the conference proceeding.

We are looking forward to seeing you in Prince of Songkla University, THAILAND. Please feel free to contact me if you need further information regarding the preparation and presentation of your paper.

Yours sincerely,

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The 4th Plant Science Symposium

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เรื่อง *Effect of culture media and plant growth regulators on callus induction and regeneration of indica rice (Oryza sativa L. cv. Sangyod)*

ให้ไว้ ณ วันที่ ๑๘ สิงหาคม พ.ศ. ๒๕๖๐

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CHAPTER 1
GENERAL INTRODUCTION

General Introduction

Rice (*Oryza sativa* L.) belongs to the family Poaceae. It is a primary staple food for a half of the world's population. It is also the most important cereal crop in Asia regions where nearly 90% of global rice production and consumption (Evans, 1998). It accounts for 23% of world's total crop area, over 20% of global human daily calories intake (Song, 2003). Gobal rice demand is projected to rise 26% in the next 25 years and achieve nearly 555 million tons in 2035. Driven by both population growth and climate changes traditional plant breeding cannot meet the rising demand of rice production. Among the two most popular sub-species (ssp.) of rice, the indica group are largely grown in hot climates of the tropical, south and southeast Asia such as India, Thailand, Vietnam.

Sangyod rice is one of special rice varieties with dark-red color dehusk seed, soft and aromatic of cooked, grown in Pattalung province, Thailand for hundred years. Red rice had more minerals (iron), vitamins, bioactive compounds (anthocyanin, flavonoid, phenolic compounds) than white rice. Pigmented rice also contains the highest antioxidant activity (Nam *et al*., 2006) that helps reduction in the risk of some chronic diseases for people such as diabetes, cancer and cardiovascular syndrom. It has been reported that the chemical compositions of dehusked pigmented rice gains of three non- waxy varieties as Hom Kra Dang Ngah, Kamyan, Sangyod in Table 1 (Yodmanee *et al*., 2011). Currently, the demand for healthier rice products are being increasing globally. Providing specially rice varieties for market will rise economic profits to farmers and nutritional benefits to consumers.

Highlighting a need for the research and development of efficient mass propagation tools for Sangyod rice variety in the future is required. Crop improvement through tissue culture techiques are being widely applied for large scale plant multiplication including rice. Tissue culture techniques have become popular improvement tools used as alternative means of plant vegetative propagation and genetic engineering. Techniques for the transformation of rice are a suitable target for development in cultivars (Rao *et al*. 2009) and *Agrobacterium*-mediated gene transformation is the favored mode which has emerged as being the most effective technique because it is a relatively accurate mode of DNA transfer, leading to plant

regeneration with fewer transgene copies, higher transformation efficiency and lower costs, and is a simple procedure with decreased gene silencing (Hiei *et al*., 1994).

It is commonly used for many dicotyledonous plants and several monocots including rice (Mohanty *et al*., 1999). The success of *Agrobacterium*-mediated transformation has been reported in some *Indica* and *Japanica* rice cultivars such as IR 64, IR 72 (Kumar *et al*., 2005) and BRS Primavera (Bevitori *et al*., 2014). However, many *indica* rice cultivars often perform poorly in response to in vitro regeneration and produce low levels of transformation when compared to *Japanica* sub-species (Zhang *et al*., 1998; Khanna and Raina., 1998; Kumar *et al*., 2005). Several reports have focused on the assessment of the totipotency and transgenic capacity of *indica* genotypes (Lin and Zang, 2005).

Table 1 The chemical compositions of dehusked pigmented rice gains of three non-waxy varieties as Hom Kra Dang Ngah (HK), Kamyan (KN), Sangyod (SY)

Rice varieties	Protein (g/ 100db)	Lipid (g/ 100db)	Fiber (g/ 100db)	Ash (g/ 100db)	Iron content (g/ 100db)	Polyphenol (mgGAE/100gdb)	Anthocyanin (mg Cy-3 glc/100g db)
HK	6.96 ±0.10^{b}	1.47 ± 0.09^{ab}	0.28 ± 0.00^{b}	1.44± 0.10^{ab}	1.16 ± 0.03^{b}	80.44 ± 6.61^{b}	10.68 ±2.31^{a}
KN	6.63 ±0.11^{a}	2.17 ± 0.04^{c}	0.35 ± 0.05^{c}	1.64 ± 0.13^{ab}	1.26 ± 0.03^{cd}	58.89 ± 6.89^{a}	9.79 ± 1.54^{a}
SY	8.06 ±0.03^{d}	1.65 ± 0.56^{ab}	0.26 ± 0.01^{b}	2.15 ± 0.05^{c}	1.21 ± 0.04^{bc}	82.01± 7.90^{b}	15.14 ± 0.19^{b}

There are many factors which affect the efficiency of transformation of rice by *Agrobacterium* such as explant types, the strain and density of bacteria, the expression vector-plasmid, the inoculation period, the elimination of *Agrobacterium* infections after co-cultivation, selection agents and culture medium conditions (Cheng *et al*., 2004; Tie *et al*., 2012). The genotype of explants has been regarded as the main factor that influences transformation efficiency (Tie *et al*., 2012). Gene transformation is dependent on genotype. Therefore, the development of the *Agrobacterium* host range and increasing the transformation frequency is the primary objective in research in higher plants with the aim of decreasing costs and the resources required (Cheng *et al*., 2004). At present, the indica rice cultivar Sangyod is one of the most important commercial rice cultivars in Thailand. However, a review of the literature was unable

to trace any published research considering the improvement by gene transformation of Sangyod using *Agrobacterium tumefaciens*. Thus, to address this problem, this study were to establish an improvement callus induction and plant regeneration system for pigmented rice cv. Sangyod and to investigate the main factors affecting the efficiency of *Agrobacterium*–mediated transformation system for Sangyod rice.

Literature Review

1. Rice as an important crop

Rice commonlly is known as a crop of the species *Oryza sativa* L. (2n = 24) The genus *Oryza* L. is classified under the tribe Oryzeae, subfamily Oryzoideae, of the grass family Poaceae (Gramineae) (Lu, 1999). Rice is the most important staple food for more than a half of the world's population after wheat and maize. It provides 50-80 % of their daily calorie intake, 20% of dietary protein and 3% of dietary fat (Khush, 2005). Approximately 80% of the world rice production is based on *indica* rice varieties which are cultivated under subtropical and tropical regions.

Rice can use for food in many forms. Grains are heated in water to inducing cooked rice. Rice flour is often dissolved with water, boiled and used for other productions. The bran is an main source of oil for food and manufacture. Husks are applied for fertilizers and animal feed, and rice straw is utilized as an important animal feed and for making various wrapping materials and mats.

Rice is an deviation among the cereal crops tropical C3 grass that explanded in semi-aquatic, low-radiation habitat. Rice can carry an odd portfolio of tolerants and susceptivity to abiotic and biotic stresses as compared to other plants. The indica cultivars are generally grown in tropical, subtropical and temperate regions and accounts for 80% of the rice cultivation areas in the world (Ayres and Park, 1994). The agriculture in combination with suitable agro-technologies has improved in many sections of the worldwide that lead to an enhancing in food grain production globally approximately 2350 million tons in 2007 (Godfray *et al.*, 2010).

1.1 Challenges for rice breeding in the future

The population of rice consumers is enhancing nearly 1.8% annually. demand of rice production in worldwide will increase to 850 million tons by 2025 (FAO, 2002). Food security is under challenges in almostly parts by facing claims for land, water, labour, energy and capital for industrialization and urbanization that causes many pressure to produce more food per unit of land (Godfray *et al.*, 2010). In addition, the global climate change seems to enhance affecting food insecurity, hunger and malnutrition for millions of people in developing countries (Nelson *et al.*, 2010). Futhermore, abiotic stresses have taken place such as salinity, drought as well as flooding correlated with climatic changeability, the biotic stresses involved pests, diseases and insect have also enhanced in recent years. Crop systems are highest risk particularly wheat and rice. Thus, we need to create rice cultivars with desire agronomically traits, high yield capacity and substantially for meeting these challenges of increasing rice production in future.

The major advantages of plant breeding techniques help to increase rice production through the application large-scale rice cultivars with high-yield and modern cultural practices. Using genetic engineering and conventional plant breeding techniques as hybrid rice technologies can enhance rice production rapidly.

It is projected that the demand of global rice production may be approximately 533 million tons of milled rice by 2030 (FAO, 2002). To meet this target, rice cultivars with high yield capacity, substantial resistance to diseases and insects, and tolerance to abiotic stresses are needed.

To overcome these problems, it requires integration approaches via using the new tools at molecular level. These consist of plant transgenic and the transfer of novel genes aim to resistance abiotic and biotic stresses as well as applying of molecular markers in breeding programs to achieve genetic improvement quickly.

1.2 Rice tissue culture

History of rice tissue culture dates back to mid-twentieth century. Chu *et al.* (1975) were among the first to show in vitro culture of immature embryos of rice. The in vitro embryo culture methods were mainly developed to rescue interspecific Fl hybrid embryos. Embryos rescue methodologies had a history of wide applications in introgression breeding and gene transfer from wild relatives to cultivated species in many crop plants. Hiei *et al.* (1994) reported that scutellar callus derived from mature rice seeds are more amenable for *Agrobacterium-mediated* transformation when compared to other explants. Thereafter, all the rice transformation protocols reported have used these callus explants for further improvements in rice transformation.

Since mature seed is the source for callus initiation, seed quality plays a pivotal role in rice tissue culture. Chu *et al.* (1975) showed that rice seeds that were surface sterilized using sodium hypochlorite showed greater seedling growth than those that were sterilized using mercury chloride. The superiority of 2,4-D (2.0 to 4 mg/l) and Kn (0.5 to 2 mg/L) inducing callus induction in *Indica* genotypes was recently reported (Rajesh *et al.*, 2008). Moreover, High percentage of callus induction at 97% was obtained when seeds of rice (*Oryza sativa* L.) cv. RD6 were cultured on modified N6 medium supplemented with 3% (w/v) sucrose, 22.5 µM 2,4-D and 0.8% agar under light condition (Pipatpanukul *et al.*, 2004. Another study reported sucrose (3%) to be the best carbon source for both callus induction and regeneration (Tariq *et al.*, 2008).

L-Proline is commonly included as organic supplement in tissue culture media. The use of 500 mg/L of L-glutamine and L-proline, joined to casein hydrolysate, has been successful for the induction of two lines of wheat callus (Bhausaheb *et al.*, 2015). Zaidi *et al.* (2006) demonstrated that the addition of the casamino acid promoting callus induction and regeneration of numerous genotypes of indica rice cv. MDU 5. Casamino acid is an essential amino acid which acts as a precursor of the IAA, an important auxin for somatic embryogenesis in cereals (Zaidi *et al.*, 2006). Plant regeneration in rice is affected by several factors such as genotype, development al stage of explant hormonal composition of the medium, carbohydrate

source, partial desiccation, and water stress. By increasing the agar concentration and supplementing the media with mannitol, the frequency of plant regeneration can be increased (Jain *et al.*, 1997).

Zhuo *et al.* (1996) observed that replacing 2,4-D with phenylacetic acid did not affect. The frequency of callus induction, however it significantly improved the shoot differentiation from callus in *Indica* rice. The pronounced recalcitrance can be minimized with changes in media composition for the culture of *indica* rice varieties which until recently, proved farles responsive than *japonica* rice (Rueb *et al*., 1994). ABA and sorbitol were reported to be effective in somatic embryo formation from rice callus (Eapen and George, 1990). Oinam (1993) reported that partial desiccation of rice callus leads to somatic embryo development and plant regeneration plantletsSome reports suggested the addition of 6-BA could enhance the quality of the initiated callus (Tian, 1994).

1.3 Genetic engineering techniques

Crop transformation technology becomes an adaptable platform for varieties improvement for researching gene function in crops. The development of an effective approach for genetic transformation is requisite to molecular biology application for the improvement of crop species. Genetic engineering is known as a direct approach of plant breeding that selective targets several traits to introduce into the plant. Genetic engineering in combination with conventional breeding programs allows good traits encoding by transgenes to introduce into commercial crops within an economic reasonable time frame.

The principal concept of plant genetic transformation was using genetic engineering methods to introduce foreign genes into crops. To achieve transgenic crops, some techniques have been seeked such as electroporation (He *et al*., 2001), *Agrobacterium* mediated transformation (Gelvin 2003; McCullen and Binns 2006), protoplasts mediated by polyethyleneglycol or calciumphosphate (Negrutiu *et al*., 1987; Datta *et al*., 1990), particle bombardment (Altpeter *et al*., 2005), liposome-mediated transformation (Caboche 1990), silicon carbide fibers (Frame *et al*., 1994)

and in planta *Agrobacterium*-mediated transformation through the vacuum infiltration of wholly plants (Bechtold *et al*., 1993).

2. The application of genetic Transformation

Many genetic engineering programs have been carried out aim to increasing potential environmental stress tolerance, pest and disease resistance in many plants species due to the successful rate of conventional breeding methods are not sufficient to overcome the requirements (Hansen and Wright, 1999). Thus, there is a need to improve rice cultivars that may tolerate high concentrations of salt, drought and water stress with optimum yield production. In this aspect, crops genetic transformation can be necessary tools in breeding programs. It allows access to an unlimited genes pool by the transferring of desirable genes. The development of crops transformation approachs during the past decades has improve crops by introducing of cloned genes. The two most important steps to be the master for transformation of crops are to transfer of foreign DNA into plant cells and regeneration of crops from transformed cells (Yookongkaew *et al.*, 2007).

The improvement of genetically engineering crops with enhancing tolerance abiotic and biotic stresses is the major challenges in plant biotechnology research. Rice transformation is a vital goal in cereals biotechnology, because the most it is also known as model of cereal genomics and one of the most important food crop in worldwide (Kohli *et al*., 2003). Genetic engineering has been utilize as a outstanding tool for rice improvement. Until now, the number of copies of genes inserted and chromosomal locations of the integrated genes are not control, the expression of the introduced genes varies among specific transformants. Therefore, a relatively large number of transgenic crops must be promoted aim to choice desirable transformants as well as to investigate the expression of introduced genes (Li *et al.*, 1993). The most commonly used method for transformation are biolistic approach and *Agrobacterium*-mediated transformation.

2.1 Purpose of genetic transformation

Major purpose of genetic transformation is to create plants containing useful phenotypes. Several reasons for genetic modifications are yield improvement, high resistant to disease and pest, herbicides tolerance, better nutritional value, increased shelf-life, better climatic survival by enhancing tolerance to drought, salinity conditions to allow the recovery of previous unfavorable land. Genetic transformation technology have expanded in the phase that most of all selected plant would be transformed, However, there were limitted less transformation efficiency and low transgene expression in some species in practical (Century *et al*., 2008)

The key factors of studying in crop transformation are to focus on high gene expression and stable of the transgenes on host plant rather than on the method for introducing foreign DNA aim to improve gene transferring methods in species. Replacement of ex-traneous DNA which is not important as the selection marker genes. Silencing gene transformation are via co-suppression mechanism and elimination the position impact thought to be response for various expression level of transgene in transformation crops are main areas for the development process in future (Hansen and Wright, 1999).

The transgenic crops released for commercial traits that expresse interest genes of conferring dominant phenotype with the selectable marker gene. Many characteristics of commercial genes importance, Although, they required expression of some transgene, genetic transformation technique can not sufficiently solve. The potential benefits of plastid transformations as mean of circumventing some of the various combine with nuclear gene expression have touched upon and will not continue to be investigated such as plastid transformations that become regular for varieties others than model systems. Chloroplast gene transformations have been recommended such as a available mechanism for polycistronic expression investigation. The capacity integration multigenes and assure their stable heritance and expression are important for the successful polygenic agronomically traits and manipulation of complex biosynthetic pathways in the future (Sidorov *et al*., 1999).

2.2 The herbicide glyphosate

Glyphosate (N-phosphonomethyl-glycine) is one of the active element nonselective systemic herbicide “roundup” to use manage a wide range of weeds Franz *et al*., (1997) reported that Henri Martin firstly synthesized glyphosate molecule in Swiss and was developed in 1972 by the Monsanto company. However, the firstly glyphosate was synthesized and tested as a herbicide by Franz in 1970. After that, it was early after dominated for using herbicide (Grossbard and Atkinson, 1985). Glyphosate was anionic at physiology pH levels. It is action as a salt with different cations such as. the isopropylamine salts or sodium. In 1974, the isopropylamine salt of glyphosate firstly introduced on market (Duke, 1988). After that, it applied in many crops as new method to control weeds because of large – scale the use of certain agriculture system as no till cropping, and expanded of glyphosate-resistant genetically modified crops. Glyphosate was obtainable some of features of the weed management system, inclusive of low level toxicity on non-target organisms, flexibility of utilization, non-selective herbicide, post-emergence control, crop safety, lack of soil mobility (Franz *et al.*, 1997).

2.3 Mode of the action of glyphosate

Glyphosate was unique the mode of action which the only molecule was more effective on inhibition the enzyme EPSPS (5-enolpyruvyl-shikimate- 3-phosphate synthase) of the shikimate pathway (Figure 1). Glyphosate was also a transition phase clone of phosphoenylpyruvate, one of the crucial EPSPS. The inhibitining of EPSPS reachs to decrease response inhibition of this pathway (Duke, 1988). Consequence, enormous of carbon abound to shikimate-3-phosphate that was converted to high level of shikimate. The high level of shikimate which fastly accrued to glyphosate-treated plant tissues was the indication that led. EPSPS was known as molecule target site of glyphosate that were discovered by Amrhein and co-workers (Steinrucken and Amrhein, 1980). Glyphosate would create the inhibiting of the shikimate pathway to kill plants was not entirely clear. It was assumed that incomplete of the aromatic amino acids production to keep needed protein synthesis was the basic effect, and this was constant in the slow improvement of symptoms.

Some researchers had indicated to support that views the promoted carbon flow to shikimate pathway by deregulation of pathway and inhibition EPSPS consequence in carbon shortages for other pathways (Siehl, 1997). The fast cessation of carbon infatuation glyphosate-treated sugarbeet was better defined for this mechanism than the decrease of aromatic amino acids production.

The EPSPS of most of all higher plant occured inhibition by glyphosate, making its a non-selective herbicide, action on extensive range of plant species. EPSPS inhibitor without analog or different chemical classes targets of this enzyme have been commercial. Couple with many others desirable traits of glyphosate provied ideal herbicide and a unique (Servaites *et al*., 1987). The commonlly effectiveness of glyphosate for weed control in combination with herbicide tolerant crops has improved environment as conservation horticulture for the management of soil erosion.

Two main components needed for the progress of commercial possible glyphosate-tolerant crops are a resistant enzyme and tolerable expression of enzyme in the transgenic plant, various challenge require understanding quantitative, structural, and developmental elements of gene expression (Caseley and Coupland, 1985). The goal enzyme for glyphosate inhibition in the aromatic amino acid biosynthetic pathway is EPSPS (5-Enol-pyruvylshikimate-3-phosphate synthase). Separation of this pathway builds a insufficiency in protein synthetic precursors, it also impacts much other plant cell which are obtained from intermediates and derivatives such as auxins, anthocyanins and flavonoids.

So that, plant must be created a resistant enzyme to sustain flux for growth and development. Numer of EPSPS enzymes from native, mutagenized microbes and plant sources were investigated to choice enzyme with high catalytic effective in the existence of glyphosate (Ruff *et al*., 1991). *Agrobacterium* sp. strain CP4 EPSPS (CP4 EPSPS) was established an special enzymes through this screening process. The second element to successful herbicide tolerance is definition of a resistant enzyme in all cell types which obtain a dose of herbicide and desire a function aromatic amino acid pathway (Hetherington *et al*., 1999).

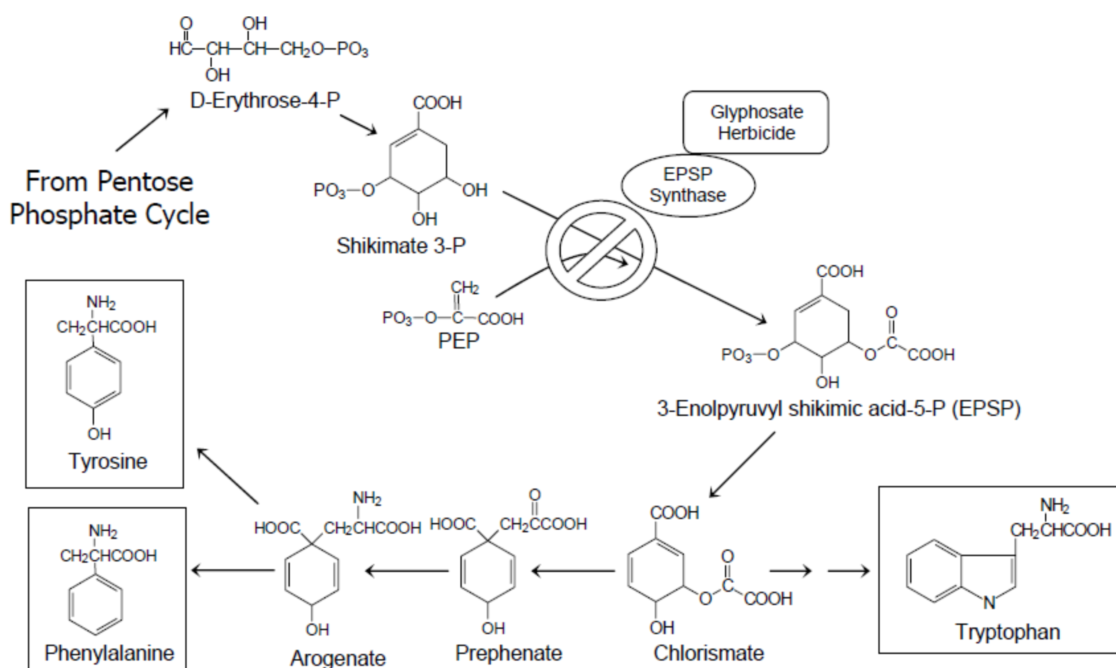

Figure 1 The primary mode of the action of glyphosate. Products of pathway and regulatory feedback inhibition.
Source: Devine *et al*. (1993)

2.4 The uptake and translocation of glyphosate

Glyphosate was taken up relatively fast on plant surfaces. The rate of leaf uptake differ considerable with species have at least some of the various in glyphosate between species. The dispersion was the most alike the mode of transport through plant cuticle. The physico-chemical of glyphosate allow it to translocated from leaves via the phloem or tissues which are metabolism sink for sucrose. Therefore, phytotoxic glyphosate levels reach meristem, young leaves and roots, storage organs and others active growth tissue. Good uptake and translocation to grown sites or limited degradation and a slow the mode of action are the basic reasons for effictively of glyphosate. Some species where it acts rapid as sugarbeet, glyphosate could limit its translocation (Duke and Powles, 2008).

3. *Agrobacterium* - mediated genetic transformation

Agrobacterium-mediated genetic transformation is one of the dominant technologies using for the creation of genetically modified transgenic plants. *Agrobacterium tumefaciens* (*A. tumefaciens*) is a Gram-negative soil pathogenic bacterium that causes crown galls or tumours on a large range of dicotyledonous plant species (Figure 2). Many studies aimed to improve the response of molecular engineering of *Agrobacterium* for the transport and generation of the bacteria DNA into the host cells have resulted in the formation of many recombinant *Agrobacterium* strains and technologies applied for the successful transformation of diverse plant species currently.

Agrobacterium-mediated transformation has some benefits such as higher transformation efficiency rather than protoplast transfer (Rao *et al.*, 2009), the competence to transfer large pieces of DNA, minimum rearrangement of transferred DNA, and characteristic insertion into the recipient genome of a individual segment of DNA at fewer copy number (Kumar *et al.*, 2005; Tyagi *et al.*, 2007) compared to particle bombardment technique (Shrawat *et al.*, 2006; Rao *et al.*, 2009). *Agrobacterium-mediated* transformation efficiency and delivery of T-DNA into plant cells is impacted by several parameters including explant sources (Owens and Cress, 1985; Hiei *et al.,* 1994). *Agrobacterium*-mediated transformation depends on the strain and integrates lower copy numbers of DNA into the plant genome in comparison particle bombardment. (Shou *et al.*, 2004).

Single copy transgenes could turn to be stable expressed rather than multiple gene copies (Iglesias *et al.*, 1997). The approval of *Agrobacterium*-mediated transformation in monocot and dicot plants are increasing due to may induce fewer transgenic actions. *Agrobacterium*-mediated gene transfermation was commonly established simpler integration patterns, lower arrangements both inserts and decreased problems within unstable or co- suppression over generations compared to other techniques using directly gene transfermation (Zambryski, 1988). The results from the production of one specific stranded copy of transferring T- DNA by the bacterial virulence systems. It transfers into the host cell according to integrated on host genome (Gelvin,

2003). The virulence (*vir*) genes of tumor with Ti plasmid are important genes mechanistical related to T-DNA transfermation. The *vir* genes admit border sequences containing of T-DNA disregarding other borders sequence (Zhu, 2000). The transferring of T-DNA depends upon *cis*-acting T-DNA region sequences and *trans*-acting of vir functions encoding by *Agrobacterium* chromosome and Ti plasmid (Binns and Thomashow, 1988). Thus, the vectors transformation maybe generate from wild kind *A.tumefaciens* plasmids by tumorigenic region from the T- DNA or removing entire oncogenes set. The interest genes may be organized into binary vector among the T-DNA regions and transferred into plant cells.

3.1 Protocol for Gene Transfer

The processes of gene transfer from *A. tumefaciens* into plant cells involves several steps (Figure 2 and 3):
(1) Bacterial colonization
(2) Induction of bacterial virulence system
(3) Generation of T-DNA transfer complex
(4) T-DNA transfer
(5) Integration of T-DNA into plant genome

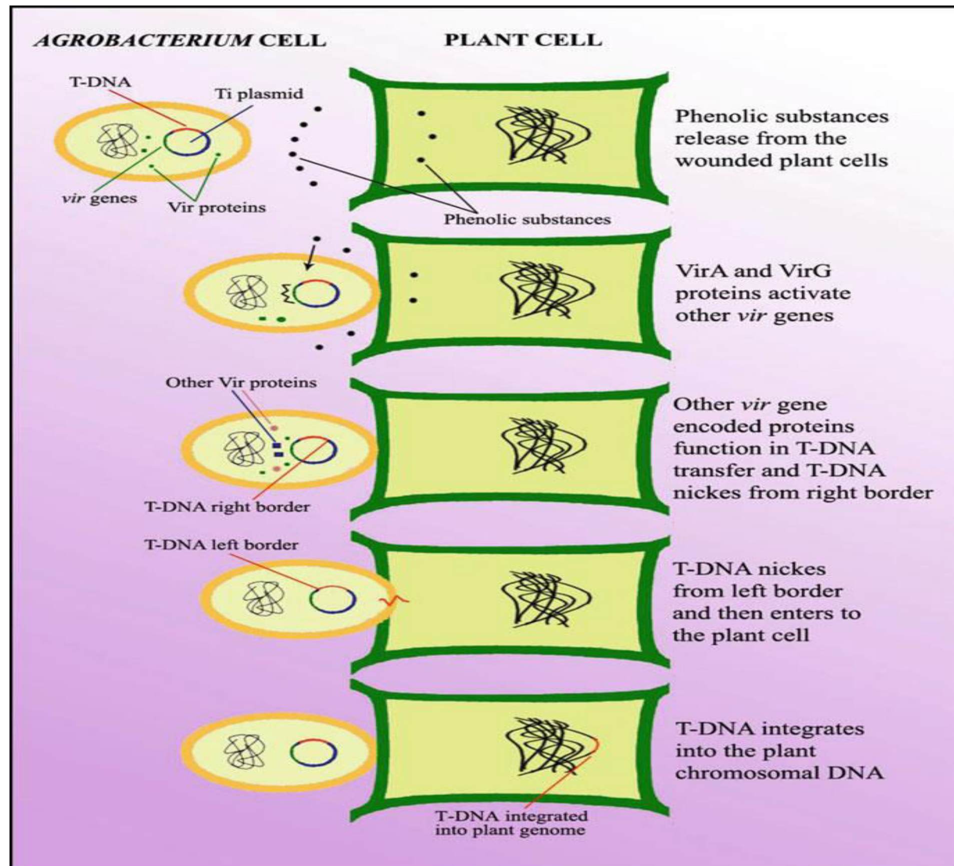

Figure 2 Natural gene transfer from *Agrobacterium* tumefaciens to plant cell. Source: Özcan *et al*. (2004)

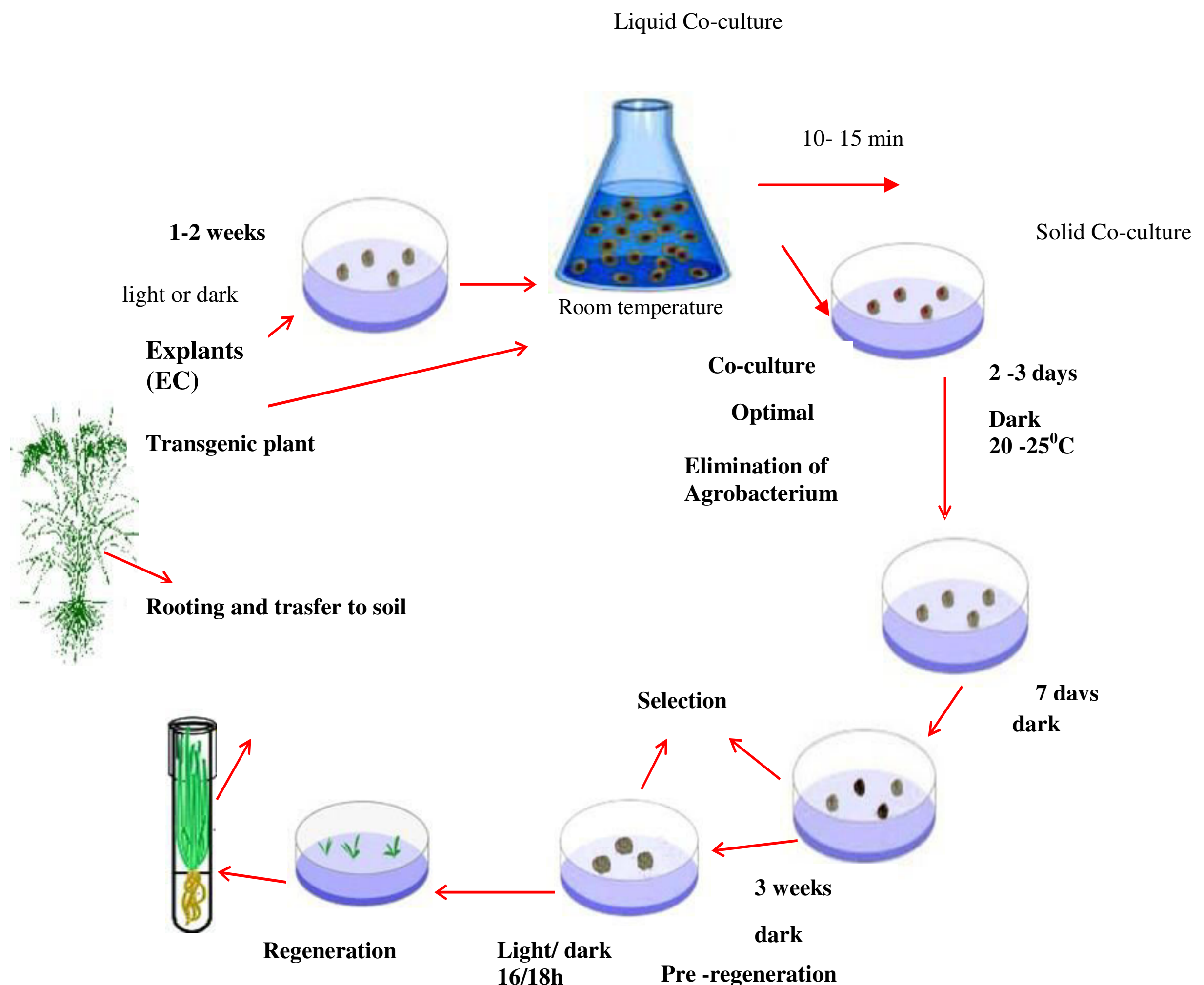

Figure 3 Scheme for *Agrobacterium*-mediated transformation of cereal plants

Sources: Karami (2008)

3.2 The origin and function of CP4 – EPSPS

The EPSPS family of enzymes are universal in microorganisms and plants. The EPSPS proteins catalyze the transfer of the enolpyruvyl group from phosphoenol pyruvate (PEP) to the 5-hydroxyl of shikimate-3-phosphate (S3P) through the yield inorganic phosphate and 5-enolpyruvylshikimate-3-phosphate (Alibhai and Stallings, 2001). This is only known as metabolic product and S3P is the production of penultimate from the shikimic acid pathway. It has been evaluated that aromatic

molecules were derived from shikimic acid pathway nearly 35% or higher of the dry weight of a plant (Franz *et al*., 1997).

The CP4-EPSPS gene was isolated from *Agrobacterium* sp. strain CP4, a common soil-borne bacterium. It has contained sequenced and encodes a EPSPS protein including of a single polypeptide of 455 amino acids. The CP4-EPSPS protein performed in genetically engineered glyphosate tolerance in plants was functional equal to endogenous plant EPSPS enzymes, thus, the CP4 -EPSPS shows decreased affection for glyphosate (Franz *et al*., 1997).

For plants which were not glyphosate tolerance, glyphosate binded to the endogenous plants EPSPS enzyme and blocked the biosynthesis of 5-enolpyruvyl-shikimate-3-phosphate, therefore starving plants of necessary secondary metabolites and amino acids (Steinrücken and Amrhein, 1980). The inhibition of EPSPS enzyme activity has shown via the induction of a binary complex of EPSPS and 5-hydroxyl of shikimate -3 – phosphate (S3P) glyphosate. The effective of glyphosate binding blocks the binding of PEP and inhibits EPSPS catalysis of S3P and PEP in CP4-EPSPS gene. Whereas, the affection for PEP is more higher than the impact of glyphosate. This various in the glyphosate binding affection is the primary for glyphosate tolerance in CP4 EPSPS-transformed plants. The function of CP4 -EPSPS gene will continue in the presence of glyphosate, producing the aromatic amino acids and other metabolites that are essential for normal plant growth and development (Figure 4).

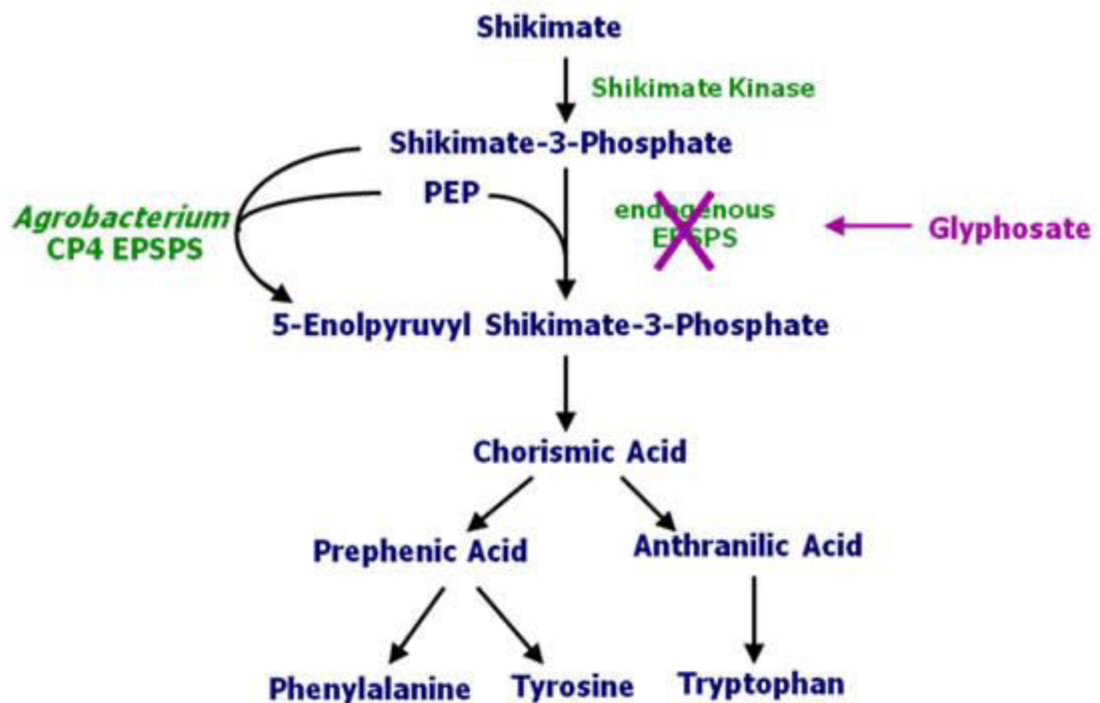

Figure 4 Schematic the action of glyphosate mode and mechanism of CP4-EPSPS gene mediated tolerance
Source: Kishore *et al*. (1988)

3.3 Factors affecting *Agrobacterium* –mediated transformation of plants

At present, *Agrobacterium*-mediated transformation has been applied to transfer many plant species e.g economic crops, fruit, vegetables, ornamental, medicinal plants and forest trees. Many factors can influence on the *Agrobacterium*-mediated transformation such as type of bacterial strains and cell density, plant growth regulators, antibiotics, genotype, explant wounding, light and temperature (Li *et al*., 1997). The efficient genetic transformation protocol is to determine the suitable combination of many factors together. These factors could help the improvement of new methods to extend the host range of *Agrobacterium* and control the transformation process during transformation of crops. Many factors affecting *Agrobacterium*-mediated transformation of crops have been examined. Before the tackle of stable genetic transformation of new species, it was useful to optimum the factors affecting transformation efficiencies as well as maybe reduce materials and costs in further.

Explant

There are various of explants that may use as material sources for *Agrobacterium*-mediated transformation e.g immature embryos, mature embryos, leaf blades, shoot apices, roots, stem segments and callus suspension. However, the type of explants is a key factor and could be suitable for regeneration process that recovers cells in almost the transformation of plants systems. In maize, the mesocotyls derived intercalary meristem region were initiated for transgenetic (Ritchie *et al.,*1993). In *Pisum sativum,* only the differentiating cells near vascular system of epicotyl and cotyledon regions effected on *Agrobacterium* transformation (de Kathen and Jacobsen, 1995). The transient GUS expression was performed at scutellum side of barley embryos (McCormac *et al.,* 1998). The type of explants were strongly influence on efficiency transformation frequency of Sorghum (Zhao *et al*., 2011). Immature embryos from greenhouse grown plants showed lower transformation rate than Immature embryos from field grown plant (Kondo *et al*., 2000). Some researchers reported that many plants species the excellent explant for *Agrobacterium*-mediated transformation was embryogenic callus derived from mature seeds (Suzuki and Nakano, 2002; Cheng *et al*., 2004).

Particularlly, in barley and cereals, either immature embryos or callus derived scutellum have been the favored explants because of their good morphogenetic capacity (Cheng *et al*., 2004; Shrawat *et al*., 2006). The dessication of explants in *Agrobacterium* infection period is main factor to promote transformation of plant species. In rice, the effective transformation rate of air-drying callus derived cells suspension increased 10 –times compared to control treatment (Urushibara *et al*., 2001). Similarly, the air-drying of cells suspension prior to agrobacterium infection had possitive on transformation efficiency of sugarcane (Arencibia *et al*., 1998). The desiccation of pre -cultured of embryogenesis callus of wheat and maize improved T-DNA delivery and recover plant cells after co-culture, therefore, they helped to enhance stable transformation rate (Cheng *et al*., 2003). In several recalcitrant dicot species as cells suspension of soybean was also promoted responsive T-DNA delivery (Cheng *et al*., 1997).

The desiccation suppressed the bacteria growth during co-culture period even though the molecular mechanism of desiccation remained imprecise. The transformation efficiency was impacted by pre-culture of several explants such as wheat, canola, pepper, cotton, barley, *Arabidopsis* (Cheng *et al*., 1997; Shrawat *et al*., 2006). The active of cells division were initiated consequent to wounding and promoted attaching *Agrobacterium* on the freshly synthesized of cell wall at wound sites and produced v*ir*- induction compounds via the metabolic of active cells were offered as essential factors. The different response of plant tissues to bacterium was applied to the potential of this agrobacterium that attachs to cells or variations in T-DNA transfer engine (Lippincott *et al*., 1977). Generally, it has been revealed that only plants with the responsive wound will expand populations of woundbordering cells which were efficient transformation (Potrykus, 1991). However, cells necrosis were commonly observed in the cultures of plant cells with exposuring to bacterium.

The modified transformation parameters may optimize the prospect of thoroughly transferring in some recalcitrant cell types. Besides, cells necrosis according bacterium infection still remain significantly inhibition (Gelvin, 2003). The cells browning or death with exposuring *Agrobacterium* appears in many dicot and monocot plants such as grape, wheat, tomato and lettuce (Wroblewski *et al*., 2005). *Agrobacterium* transformation prompts the expression of genes in host cells as the component of plant protection machinery (Veena *et al*., 2003). wheat embryos and root cells rapidly induced hydrogen peroxide (H_2O_2) after *Agrobacterium* infection, exhibitted modified cells wall structure and the consequence of cells necrosis and death (Parrott *et al*., 2002). A interaction between the decreasing of cells death and the promoted transformation rate has been indicated in sugarcane, rice and maize (Enríquez-Obregón *et al*., 1997; Zhao *et al*., 2000).

In Poaceae, *Agrobacterium* produced necrosis which may be prevented by using inhibition agents as silver nitrate (Hansen and Durham, 2000). The anti-necrotic of tissues may supply sufficient environment for the correlation of plant cells and *Agrobacterium* by inhibiting necrosis and result in increased transformation efficiency (Enríquez- Obregón *et al*., 1997). It has been reported that the infection of maize callus with *Agrobacterium* sustains rapidly, both sensitive and responsive type

of cells death were suppressed by two baculovirus genes as iap and p 35 (Hansen 2000). However, the browing and necrosis of cells after *Agrobacterium* infection are still main difficult in the genetic transformation of plants and molecular of cell death through apoptosis processes may minimize bacteria inducing cell death.

Explant wounding

The physical explant wounding is generally known as the greatly affects transformation efficiency in *Agrobacterium*-mediated transformation systems (Rashid *et al*., 1996). Bacteria were used to cut surfaces of plants many times after wounding. If plants were infected within 3 days of wounding, tumor production was relative efficiency. They resulted in low rate of the plants growning tumors infection 4 days after wounding. The tumorigenesis was vanished after 5 days. However, tomato continued affecting to tumorigenesis to 2 weeks after wounding (Braun, 1954). Tomato still remained nearly 25% of the susceptivity of plants inoculated after 6 days of wounding (Davis *et al.,* 1991).

Despite of the suberisating of the cell walls, which may appear a physical transformation boundary, occurred 4 days after wounding, the suberized cells still maintained high transformation susceptivity. The approach and kind of wounding ranges can make during the explants preparation to particle gun mediated micro-wounding (Zuker *et al*., 1999). Tissues may be wounded applying *Agrobacterium*-filled syringe that permit some levels of transmission of *Agrobacterium* (Chee *et al*., 1989). Another methods were using sonication to wound and adapt the tissue system to increase *Agrobacterium* infection. This method related to control the plant tissue of short ultrasound periods in the appearance of *Agrobacterium*.

The strength of these techniques are that the sonication consequences of thousands of microwounds on and under the surface of plant tissue are caused by cavitation. This wounding patterns admit bacteria to move deep and complete throughout the plant tissue to increase the possibility of infection plant cells. Furthermore, the ultrasound acts micro wounding on the sub- surface and surface layer of plant tissue to s phenolic compound that increases transformation efficiency in soybeans (Santarem *et al*., 1998). Sonication was used to enhancing

Agrobacterium-mediated transformation of some plant species (Pathak and Hamzah, 2008). The wound site also caused entry point for *Agrobacterium* and resulted in the decrease essential phenolic elements for active *vir* gene (Joubert *et al*., 2002). The supplementary of phenolic elements had also been reported in soybean, barley, rice and cotton and barley to increasing of transformation efficiency (Kumlehn *et al*., 2006). However, acetosyringone did not promote effective transformation in poplars and tea (Mondal *et al*., 2001). In monocots, the supplementary of acetosyringone during the interaction of plant and bacteria supported the gene transfer that phenolic compounds did not synthesized (Cheng *et al*., 1997; Hiei *et al*., 1997; Wu *et al*., 2003).

It has been reported that *Vir* gene producing signals were delivery from only wound sites of plants as anti –microbial. Phenolics are related in healing and lignification of the wound (Dixon and Paiva, 1995). Two phenolic compounds, acetosyringone and hydroxyacetosyringone were transferred in higher quantity of tobacco leaves than un-wounded leaves (Stachel *et al*., 1986). Plant tissues wounding can be vital to understanding physical obstacle that could block T-DNA transferring. The waxy cuticle that coats plant epidermis may block production physical connects *Agrobacterium* and host cells which were required for T-DNA transferring (Zhu *et al*., 2003). T-DNA may be transferred to leaves mesophyll cells of un -wounded plants (Escudero and Hohn, 1997).

Although the transferring appeared only when *Agrobacterium* were pre - induced with phenolic compound. Cells division of wound curative can play a role in tumourigenesis (Binns and Thomashow, 1988). DNA replication may promote T-DNA integration, the division of cells can be hypersensitive to the mitogenic influences of T- DNA encoding phytohormone. Moreover, the utilization of exogenous auxin triggers plant cells transformation in prior to infection (Chateau *et al*., 2000). *Agrobacterium*-produced tumours were limited to cambium that was meristematic cell and active to cells division. The wound of explant can cause basically plant cells to differentiate and emerge meristematic tissue like a cambium (Ghorbel *et al*., 2000).

Plant species and genotype

The capacity of bacterium strains to transfer plant cells that is described by their plasmid and genomes chromosomal encoding all the mechanism of attchment and DNA-transferring. However, Plants can create various inducer molecule which differ their producing capacity and cellular.

The variability causes differences the *vir* gene expression levels in various hosts, through impacting hypersensitivity to infecting by bacterium. A low concentration of *vir* gene expression can produce plant recalcitrant via the character of the incompetence of the *Agrobacterium* to synthesize and transfer sufficient T-DNA necessary for infection successfully. Many plant species vary greatly in their sensitive to infection through *Agrobacterium* (Cheng *et al*., 2004). Despite of a species, various cultivars may perform greatly different the levels of sensitivity to tumorigenesis by particularly *Agrobacterium* strains. These various have been indicated in maize, legumes, tomato, Arabidopsis and other species (Ritchie *et al*., 1993; Nam *et al*., 1997). *Agrobacterium*- mediated transformation of higher plants was established for dicotyledonous species. In monocotyledonous species, The frequency of Agrobacterium-mediated transformation efficiency has been greatly promoted in maize, rice, wheat, sorghum (Hiei *et al*., 1997; Toki *et al*., 1997; Zhao *et al*., 2000; Carlos *et al*., 2004).

The variation of capacity bacterium to infection genotype has been a main disadvangtage of transformation in elite varieties of monocotyledonous, particularlly in spreading the host range to commercial cultivation. At present, the cereal crops transformed, rice occurs least genotypes dependent, such as more than 40 genotypes of *japonica*, *indica* and *javonica* have been transferred. However, other cereal crops have been successfully only few genotypes by using the *Agrobacterium*-mediated transformation such as maize cv. A188, sugarcane cv. Ja 60 -5. Even through the transgenic crops have recovered from elite varieties of sorghum or maize (Zhao *et al*., 2000; Gordon-Kamm *et al*., 2002). The generally transformation frequency is lower compared to model cultivars. The studies revealed that the response of genotype

dependent and *Agrobacterium*-mediated transformation of cereals was main disadvantage some elite varieties of economical cereal crops.

The relative various in the resistant of agronomically crucial plant species to *Agrobacterium*-mediated- transformation can be the appearance of inhibitors rather than to inadequate active of the *Agrobacterium vir* machanism through host cells exudates. The natural appearance of inhibitors conducted against signal perception through the *VirA* to *VirG* that was two basic regulatory system can play an vital role in host defense (Zhang *et al*., 2000). MDIBOA (2-hydroxy-4,7- dimethoxybenzoxazin-3-one) was one of the natural inhibitor of *vir* gene production and the indoleacetic acid was also performed to inhibit *vir* gene production (Liu and Nester, 2006). However, crops can identify bacterium and transferred transgenetic like foreign alien which apply their defense structure to attack the infection progress and foreign genes expression.

Antibiotic

For genetic transformation, plant tissues were infected by the co-cultivation with *Agrobacterium* habouring a interest gene in antibiotic free media for 2–3 days. After co- cultivation, the *Agrobacterium* requires to suppress and not to inhibit with the growth and development of transformed plant cells. The successful *Agrobacterium* mediated- transformation depends on the plant regeneration efficiency and subsequent elimination of *Agrobacterium* from transformation plant cells.

The elimination of *Agrobacterium* is commonly obtained through the addition of antibiotics in the culture media because the maintained appearance of *Agrobacterium* may be a issue for establish transformation or inhibit with the growth and development of the transformed plant cells or lead to the cultures death (Matzk *et al*., 1996). Carbenicillin and cefatoxime are the most generally applied as antibiotics for elimination of *Agrobacterium*.. It has been reported that the infection of bacterium enhanced the leaves explant resistant to selective agent in barley (Shrawat *et al*. 2007). Crops have various resistance mechanism to abiotic stresses such as cell wall components (lignin and cellulose), tannins, phenolics and other

defense mechanisms (Muthukrishan *et al*., 2001). There is problematic to select the right concentrations of selective agents to facilitate organogenesis and avoid breakout. Antibiotics can prohibit cell walls synthesis (Holford and Newbury, 1992). Contamination decreases regeneration frequencies and produces plants death (Estopa *et al*., 2001). These influences would cause to reduce transformation efficient. Thus, it is important to have the suitable rate of antibiotics to obtain antibiotic protection to selection and sufficient rate of organogenesis. Many studies indicated that the toxicity of antibiotics influenced to somatic embryogenesis, callus growth and shoot regeneration (Tang *et al*., 2000).

The using of several antibiotics (carbenicillin and ticarcillin) limit for elimination *Agrobacterium.* They may be inactivated through producing f3-lactamases by *Agrobacterium* whereas, others antibiotics as cefotaxime that have high resistance to f3-lactamases and prohibit regeneration systems (Shackelford and Chlan, 1996; Ling *et al*., 1998). At present, some reports revealed that antibiotic timentin is the combination of ticarcillin and a f3-lactamase inhibitor clavulanic acid that may effectively elimination *Agrobacterium* without inhibition plant regeneration (Cheng *et al*., 1998; Ling *et al*., 1998; Tang *et al*., 2000).

Plant growth regulators (PGRs)

PGRs are necessary in transgenetic plants. The select of PGR is one of the most critical factors impacting on transformation efficiency such as the frequency of transient expression and stable integration. The competency of transformation can not be occurred or low in recalcitrant explants; however, it can be increased by PGR treatments (Sangwan *et al*., 1992; Villemont *et al*., 1997). The explants become responsive to *Agrobacterium* when they were pre-cultured on media containing PGRs (Chateau *et al*., 2000). In many monocots, the appearance of 2,4-D in co-cultivation medium promote transformation (Wu *et al*., 2003). A significant high frequency of callus generation by applying picloram performed transient GUS expression in Typha latifolia (Nandakumar *et al*., 2004). In barley, using dicamba in the callus formation and preservation media promoted transient GUS activity and genetic transformation (Trifonova *et al*., 2001). The pre-culture of explants in the medium containing benzyl

adenine that was observed to increase the transient GUS activity in kenaf (Herath *et al.*, 2005). PGRs stimulate cells division and differentiation in some tissues. The stimulant of cells division through PGRs propose *Agrobacterium* mediated-transformation efficiency can appear at a particularly phase of the plant cells cycle (Chateau *et al.*, 2000).

The plant cells cycle of Petunia mesophyll cells have been integrated to the specific stage of inhibitors in cells cycle for transformation (Villemont *et al.*, 1997). If the absence of cells cycle without PGRs treatment could not occur transformation or the expression of T- DNA-encoded GUSA. Plant cells deal with mimosine lead to block at late G1 stage in cells cycle. The greatest transformation competency was shown at high ratio of S and G2 stage to M stage in these cells. In case of the dis-appearance of cells division, T-DNA would be undertaken, translated to the nucleus, and expressed in cells carrying DNA synthesis, therefore the transient of *Agrobacterium*-mediated transformation required S stage in DNA synthesis. Cells division were needed for stabilization of transferring and T-DNA integration.

***Agrobacterium* strain**

Many factors affecting the effective of *Agrobacterium*-mediated transformation, the *Agrobacterium* strain is one of the most important infection. A vast number of *A. tumefaciens* strains had been isolated as Ach5 and Bo542 and indentified the host range (Hood *et al.*, 1987). It was reported that the range and infection capacity were vulnerable upon Ti- plasmid (Hood *et al.*, 1993). *Agrobacterium* strain A281 was a super-virulent strain improved from Bo542 carrying pTiBo542 (Watson *et al.*, 1975). pTiBo542 was established by renewal the nature T-region with a kanamycin resistant gene (Hood *et al.*, 1986). EHA101 and EHA105, harboring pTiBo542, are determined to be vigorous in the transformation of plant (Hood *et al.*, 1993). These studies have focused to improve the efficient of T- DNA delivery to monocotyledons cells by using of ‘super- virulent’ strains or ‘super-binary’ vectors (Zhao *et al.*, 2000; Adelina *et al.*, 2001). The various in the responsive of *Agrobacterium* infecting genotype had been a main obstacle in the transformation of elite varieties of cereals e.g. an efficient of T-DNA transformation in wheat between two *A. rhizogenes* strains (LBA9402 and Ar2626) and two *A.*

tumefaciens strains (LBA4404 and EHA101) (McCormac *et al*.,1998). Cheng *et al.* (2004) revealed that only EHA101 transfers T-DNA into wheat successfully. Almost of cereal crops, the genetic transformation has been obtained by applying *Agrobacterium* strains LBA4404, EHA101 and their derivative (EHA105 from EHA101, AGL0 and AGL1 from EHA101). EHA105 is superior to LBA4404 in the transgenetic of maize and more suitable for other cereals (Rashid *et al*., 1996; Huang and Wei, 2006). The hyper -virulence of EHA105, AGL1 derives from pTiBo542 plasmid (Hood *et al*., 1993) where the *virG* and *virA* genes enhances *vir* genes induction for T-DNA transferring (Gelvin, 2000). The improved efficiency of plant transformation with EHA105, AGL1 was possibly relevanted to the increasing of vir genes induction

Chabaud *et al*. (2003) were indicated that *Agrobacterium* transformation of the model legume *Medicago truncatula* cv. ‘Jemalong’ for strains LBA 4404, C58pGV2260 and AGL1. The maximum transformation efficiency was observed in the hyper-virulent strain AGL1. For other species, e.g alfalfa with the hypervirulent strain A281 (Chabaud *et al*., 1988) and rubber tree with AGL1 (Montoro *et al*., 2000). The successful of genetic transformation of rice has been resulted by using a standard binary vector in a super-virulent strain in combination with a regular strain in a super-binary vector (Hiei *et al*. 1994). Similarlly, several studies have been indicated in maize, wheat and sugarcane (Arencibia *et al*., 1998; Zhao *et al*., 2001).

The combination of a standard binary vector in a super-virulent strain and a super-binary vector in a regular strain has resulted in the successful transformation of rice (Hiei *et al*., 1994). As a result of the success in rice, identical or similar combinations were used for the genetic transformation of maize (Zhao *et al*., 2001), barley (Wu *et al*., 1998), sorghum (Zhao *et al*., 2000), wheat (Khanna and Daggard, 2002) and sugarcane (Arencibia *et al*., 1998). With some crops, such as maize and sorghum, an efficient transformation system was established only with super-binary vectors in LBA4404, whereas a standard binary vector in a superviru- lent strain showed low transformation frequency even with improved co-culture conditions in maize (Frame *et al.,* 2002). Ishida *et al*. (1996) reported that high transformated efficiency of maize A188 with a super-binary vector in *Agrobacterium*. The recovery of sugarcane transgenic was the co-cultivation of callus with *Agrobacterium*

habouring super-binary vectors including both EHA105 and A281 (Zhangsun *et al.*, 2007). The essential active *vir*G mutant gene of a binary vector promotes T-DNA delivery in monocot and dicot species (Ke *et al.*, 2001). The application of a combination of the super-binary or binary vectors containing active *virG* gene can improve the transformation efficiency in recalcitrant plants in the further.

Agrobacterium cell density

After the explants tissue are suffused, enhancing of the innoculum of *Agrobacterium* density (OD_{600}) will not increase production further. When the density of *Agrobacterium* overgrowth can harm explants due to infecting of *Agrobacterium* may cause expanded enzymatic browning and cell death (Olhoft *et al.*, 2003). Besides, , the optimal *Agrobacterium* density can depend on other factors as genotypes, explants, cells viability, the strain of *Agrobacterium*.

The enhancing OD600 of *Agrobacterium* AGL1 from 1.0 - 1.5 leads to significant increases in the number of explants inducing spots in wheat (Amoah *et al.*, 2001). In tea, the optimal *A. tumefaciens* LBA 4404 and EHA105 was at 0.6 (Mondal *et al.*, 2001). For the transformation and regeneration in rice, OD600 of pJB90GI plasmid was in the range of 0.4 -0.6 and 0.3 – 0.4 for pTOK233 (Kumria *et al.*, 2001). High percentage of contamination and reducing plant regeneration were showed at bacterium overgrowth in cotton (Jin *et al.*, 2005). Thus, the regarding multifactors impacting transformation, the optimum of bacterium inoculum density is important for specific transformation systems.

Light

Clercq *et al.* (2002) reported that in bean, the *Agrobacterium*-mediated transformation was inhibited at 24 hours continuous darkness compared to 16 hours per day under light. Light is combined with some physiological factors, such as plant phytohormone concentrations, cells proliferation, cells cycle phase (Villemont *et al.*,

1997; Zambre *et al*., 2003). The positive influences of light were discovered by physiological factors that an efficient of T-DNA transformation depends on widely inducing *vir-* genes. Light can helpt to increase a amount of the phenolic *vir* gene inducing from the orchid *Dendrobium* (Nan *et al*., 1997). However, It has been reported that the transient expression of a CaMV p35S-*uid*A gene transferred to embryos of *Picea* via particle bombardment method that was not impacted by light conditions. Thus, the optimum influences of light can be apply for *Agrobacterium*-mediated T- DNA transfer specifically (Gelvin,2003).

The protocols of *Agrobacterium-* mediated transformation for plants were used under darkness co-culture conditions without specify affecting transformation frequency (Compton, 1999). The promising morphogenic of calluses or explants can improve through the incubation in dark, particularly the endogenous of light sensitive hormones or the inhibiting of phenolic compounds accumulation (Arezki *et al*., 2001). Shoot regeneration from the cotyledonary of nodal explants may be promote under dark pre-treatment. High transient GUS expression of callus appeared under darkness culturing rather than light condition in *Typha latifolia* species (Nandakumar *et al*., 2004). Dark pre-treatment caused fewer cells wall and vascular tissue (Hartmann, 1997) thus, the infiltration of *Agrobacterium* could be increased. It has been reported that the influence of illumination on *Agrobacterium* transformation efficiency was obtained from photo-period.

Light greatly improved genetic transformation from bacterium to plant cells, even the transgenic rate of co-cultivation under 16 hours light/ 8 hours dark was lower than consecutive light system (Zambre *et al*., 2003). The high correlation between *Arabidopsis* and *uid*A gene expression in combination with using positive photo-period during co-culture, it was sharply prevented by dark and increased more consecutive light than under a 16 hours light /8 hours dark photoperiod (Zambre *et al*., 2003). The different results from many studies about light affecting transformation could be interact between light systems and other factors.

Temperature

Temperature is also known as a element influencing the ability of bacterium which transfer T- DNA to plant cells. Some studies on the efficient of *Agrobacterium tumefaciens* tumorigenesis were reported that the negative affecting of tumor development were high temperatures (Braun, 1958). The elimination of tumor development was the conformation change of *vir* A at 32°C (Jin *et al*., 1993). Kudirka *et al*. (1986) indicated that co-cultivation temperature beween soybean explants and *Agrobacterium* was shown as main element that the co-cultivation transformation was eliminated at 30°C for 48 h, whereas the successful transformation was at 25°C. the maximum thermosensitivity was during the T-DNA transferring from bacterium to the host cells followed 25°C co- cultivation. In *Phaseolus acutifolius, Nicotiana tabacum* the temperature impacts on gene transformation to plant cells by using callus and leaf disc respectively.

After co-cultivation, the GUS gene was applied to to discover transient expression, the optimum temperature was at 22°C in both these species. Whereas, the reducing of transient GUS activity was observed at 25°C (Dillen *et al*., 1997). However, in case of the higher survival rate of shoot aper of *Hibiscus cannabinus* on selection medium was obtained at 25 and 28°C as compared to temperatures of 16 and 19°C (Srivatanakul *et al*., 2001). Jin *et al*. (2005) reported that low temperature has significantly enhanced *Agrobacterium*- mediated transformation efficiency in cotton. The affecting of temperature on stable T-DNA integration and transient GUS activity during co-cultivation period, the optimum number of stable transformed plant cells was showed at 25°C (Sales *et al*., 2001). Therefore, the optimum temperature for stably *Agrobacterium*- mediated transformation efficiency could be tested with each specificly *Agrobacterium* strain and explants.

Vectors and markers in gene transfer

Most of all the transgenic crops of transformation systems require distinguish processes to introduce cloning DNA into plant cells in order to isolate cells which integrated the DNA into the suitable plant genome and recovery fully the development of plant from the transformed cells. Selectable marker genes are crucial

the development of crops transformation technology because they allow scientists to determine cells that are revealing the cloned DNA and choice for the transformation. As the selectable marker genes are expected action in range of cell types, they are generally established like chimeric genes applying regulatory sequences that ensure fundamental expression throughout plants. The selectable marker genes are often co-transformed with genes of interest. Once the transgenic crops have been bred, generated and characterized through conventional genetic (Amoah *et al*., 2001)

Binary vectors are standard tools for delivery of a wide range of genes into the cells of higher plants. These vector have certain important characters like multiple cloning sites, bacterial origin of replication, unique restriction sites and selectable markers gene cassettes enabling the recognition of untransformed cells (Dafny-Yelin and Tzfira, 2007). The most used genes in rice are dominant selectable markers that confer resistant to antibiotics or herbicides i.e. *ble* (glycopeptides binding protein), *dhfr* (dihydrofolate reductase), *hpt* (hygromycin phosphotransferase), *nptII* (neomycin phosphotransferase). In addition to selectable marker genes, plant researchers also used reporter genes known as screenable marker, a visual marker or a scorable marker, such as gus A (ß-glucuronidase, GUS), *luc* (firefly luciferase), *gfp* (green fluorescent protein).

Reporter genes are used to identify and evaluate the expression of genes in the transgenic plants. The GUS (*uidA*) gene originating from *E. coliis* commonly used for genetic transformation of plants (Godwin *et al.*, 1992). Through histological staining, one can examine the expression of GUS gene in transformed tissue. Binary vector is the usage of constitutive promoters providing transgene expression in majority of the plant tissues. Studies have been done on rice transformation by using CaMV35s constitutive promoters.

Transgenic Analysis

After selection of plants carrying the selectable marker and reporter genes, molecular analysis is completed to validate the presence of the genes in transformants. The polymerase chain reaction (PCR) is commonly used for genetic transformation. Still, when analyzing the T0 lines, one could obtain fake results caused by the

Agrobacterium infection of untransformed plants (Nagy *et al*., 1989). To avoid confusion, further PCR test can be carried out with the unique gene specific primers to see if the later are present in tested plants.

Perspectives and advances in *Agrobacterium* – mediated transformation

Regarding the most important role of crops in the human diet, food security in the future can not be obtained without main increasing in crop production. Thus, crops genetic transformation has become an meaningful technique for the improvement of varieties species with desirable agronomically traits in recent years. Even though, there are enormous the plants genetic transformation, an important challenge facing crops transformation is the improvement of approachs to producing a high frequency of crops typically containing stably and precisely transgenetic expression. The using of *Agrobacterium* has advanced for genetically transformation crop (Gelvin, 2000). Today, many horticulturally and agronomically major crops are usually transformed using this method.

There also have some challenges, particularly economically major crops, elite cultivar recalcitrant to transformation. Tissue browning and death according *Agrobacterium* infection are still a main drawback in *Agrobacterium*-mediated transformation of cereals. Whereas, the transformation efficiency systems have been well- established in several crops and some important factors affecting *Agrobacterium*-mediated transformation of crops have reported and optimum improvement the transformation parameter e.g. co-culture condition, optimize inoculation, reaching the range of transform genotype and using of ready available explant (Lowe and Krul, 1991).

At present, the establishment and molecular of the plant genes included for successful *Agrobacterium*-mediated transformation are new chances for better understanding of the respontive crops to *Agrobacterium* infection. Many information can help to improve methods to increasing the transformation rate of economically important plant species (Cheng *et al*., 2004).

Objectives

The objectives of this study were to:

1. Establish the protocol for tissue culture and efficient plant regeneration system from mature seeds of indica rice cultivar Sangyod
2. Investigate an *Agrobacterium*–mediated transformation system for Sangyod rice to be applied in transformations involving various essential commercial genes in the future

CHAPTER 2

Improved Callus Induction and Plantlet Regeneration Systems in *Indica* Rice (*Oryza sativa* L.) Cultivar Sangyod

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Introduction

Rice (*Oryza sativa* L.) belongs to the family Poaceae. It is a primary staple food for a half of the world's population. It is also the most important cereal crop in Asia regions where nearly 90% of global rice production and consumption (Evans, 1998). It accounts for 23% of world's total crop area, over 20% of global human daily calories intake (Song, 2003). Gobal rice demand is projected to rise 26% in the next 25 years and achieve nearly 555 million tons in 2035. Driven by both population growth and climate changes traditional plant breeding cannot meet the rising demand of rice production. Among the two most popular sub-species (ssp.) of rice, the *indica* group are largely grown in hot climates of the tropical, south and southeast Asia such as India, Thailand, Vietnam.

Sangyod rice is one of special rice varieties with dark-red color dehusk seed, soft and aromatic of cooked, grown in Pattalung province, Thailand for hundred years. Red rice had more minerals (iron), vitamins, bioactive compounds (anthocyanin, flavonoid, phenolic compounds) than white rice. Pigmented rice also contains the highest antioxidant activity (Nam, 2006) that helps reduction in the risk of some chronic diseases for people such as diabetes, cancer and cardiovascular syndrom.

Currently, the demand for healthier rice products are being increasing globally. Providing specially rice varieties for market will rise economic profits to farmers and nutritional benefits to consumers. Highlighting a need for the research and development of efficient mass propagation tools for Sangyod rice variety in the future is required. Crop improvement through tissue culture techiques are being widely applied for large scale plant multiplication including rice. However, *indica* varieties are generally difficult to culture and requires a longer period as compared to *japonica* varieties. Most of *indica* subspecies are recalcitrant to *in vitro* response due to poor

callus formation and regeneration capacity (Chen *et al.*, 1991; Ramesh *et al.*, 2009). Successful of callus induction and regeneration *in vitro* commonly depend on many factors such as genotype, types of explants, culture media, plant growth regulator (PGRs), carbon sources and culture conditions (Ge *et al.,* 2006; Saharan *et al.*, 2004). Genotype and culture media are the two key factors that determine the fate of *in vitro* raised culture (Lin and Zhang, 2005). In rice, plant regeneration has been obtained from different type of explants such as immature seeds (Feng *et al.*, 2011), mature seeds (Khanna and Raina, 1998), anther (Hiei and Komari, 2008), leaf (Islan *et al.*, 2014) and root (Xa and Lang, 2011).

Node or shoot tip has less potential for callogenesis than mature seeds (Karthikeyan *et al.*, 2011). In rice, mature embryos are generally applied for callus formation and efficient plant regeneration system *in vitro* cultures (Yatazawa *et al.*, 1967) as the best explant source for genetic transformation in comparison with other explants. MS medium (Murashige and Skoog., 1962) is the most commonly used for the propagation of many plant species (Rashid *et al.*, 2000). The plant hormones and the nitrogen source have profound impact on the response of the initial explant (Verma *et al.,* 2011). PGRs play a crucial role in deciding of the improvement pathway of plant cells in culture medium. Amino acids also offered positive impacts on rice callus growth (Hussain *et al.*, 2012) particularly proline and casein hydrolysate (CH) was reported by several researchers (Chowdhury *et al.*, 1993; Shahsavari *et al.*, 2010).

Establishment of a highly efficient plant regeneration for *indica* varieties system are prerequisite for the application of genetic transformation technology of rice for high yield and quality improvement. Hence optimization of plant regeneration protocol for desired genotype is essential. To our best knowledge, protocol for high frequency plant regeneration is still lacking in the other pigmented rice cultivars in Thailand using embryogenic callus cultures. Moreover, establish an improvement callus induction and plant regeneration system for pigmented rice cv. Sangyod have not been reported earlier.

The objectives of this study were to define the optimum concentrations of 2,4 –D, L-proline and CH for callus induction from culturing mature seeds of *indica* rice cultivar Sangyod and establish high plantlet regeneration protocol for applying gene transformation.

Materials and methods

Plant material and sterilization

Mature seeds of indica rice cultivar Sangyod (Figure 5a) were used as explant source. They were obtained from Department Plant Science, Faculty Natural Resources, Prince of Songkla University, Thailand. The seeds were dehusked, washed with running tap water for 20 minutes, then surface sterilized with 70% (v/v) ethanol for 2 minutes, followed by immersion in 20% (v/v) Clorox (commercial bleach) containing 2-3 drops of a wetting agent "Tween-20" on orbital shaker at 100 rpm for 10 minutes. Finally, the seeds were rinsed five times with sterile distilled water in laminar air flow hood before blottion dry on autoclaved tissue paper to remove excess water. Sterile seeds were then cultured on callus induction medium.

Callus induction

Three experiments were carried out to optimize concentration of 2,4-D, casein hydrolysate (CH) and L-proline in order to find out the best medum for callus induction of Sangyod rice.

Experiment 1: Disinfested seeds were cultured on callus induction medium (CIM) which was MS basal medium supplemented with different concentrations (0 – 4 mg/L) of 2,4–D in combination with 1000 mg/ L CH and, 100 mg/L L-proline.

Experiment 2: Disinfested explants were inoculated on CIM fortified with 2,4–D at the best concentration obtained from experiment 1, 100 mg/L L-proline and various concentrations (100, 250, 500, 1000 mg/L) of CH.

Experiment 3: Finally, the influence of different concentrations of L-proline on callus induction was investigated. Disinfested seeds were cultured on CIM with the

best concentration of 2,4-D from experiment 1, CH from experiment 2 and different concentrations (0, 50, 100, 200, 300 mg/ L) of L-proline.

CIM from each experiment was added with 3% (w/v) sucrose and solidified with 0.75% (w/v) agar, the pH of the culture medium were adjusted to 5.7 before autoclaving at 121 ^{0}C, 1.07 kg/ cm^{2} for 20 min, culture bottles were sealed by Parafilm. All cultures were maintained at 26 ± 2 ^{0}C in the culture room under 14 hours photoperiod with irradiance of 25 µmol/m^{2}/s provied by cool white fluorescent tubes. After four weeks of culture frequency of callus induction, morphology of callus and mean fresh weight of callus were recorded and statistically compared.

Callus proliferation and shoot formation

The calluses (0.1 gram fresh weight) obtained from the most suitable medium of previous experiments were transferred to regeneration medium (RM) which was MS supplemented with various concentrations of PGRs (NAA, BA and Kn) as shown in Table 5. The medium was supplemented with 3% (w/v) sucrose and adjusted to pH 5.7 prior to addition with 0.75% (w/v) agar and autoclaving at 121 ^{0}C, 1.07 kg/cm^{2} for 20 min. The cultures were maintained at the same conditions as mentioned in previous experiments. The calluses were sub-cultured to fresh medium with the same composition every 2 weeks for 10 weeks. At the end of culture period mean fresh weight of callus, the percentage of green spots (GS) formation, the percentage of shoots formation and mean number of shoots per callus were determined and statistically compared.

Multiple shoot formation and root induction

Shoots tip at approximately 5 mm in length were transferred to liquidified RM with different concentrations (0.5, 1.0 mg/L) of BA or Kn alone or in combination with 0.5 mg/L NAA (Table 6). All PGRs containing RM were supplemented with 30 g/ L sucrose, adjusted to pH 5.7 prior to addition with 0.75% (w/v) agar and autoclaving at 121^{0}C, 1.07 kg/ cm^{2} for 20 min. All cultures were incubated on rotary shaker at 100 rpm under 14 hour photoperiod in the culture room in order to optimize plantlet regeneration. Mean number of shoots per cultured shoot

tip and the percentage of roots induction were recorded and statistically compared after 4 weeks of culture.

Statistical analysis

All the tissue culture experiments of callus induction, shoot proliferation, and root induction were arranged in completely randomized design (CRD) with 8 replicates per treatment (4 bottles/ replication). Data were tested by using one–way analysis of variance (ANOVA) and the significant differences among means were separated by Duncan's multiple range test (DMRT) ($p \leq 0.01$) using program R statistical package version 2.14.

Results and discussions

Effect of different concentrations of 2,4–D on callus induction

For callus induction, CIM supplemented with various concentrations of 2,4-D (0 –4 mg/ L) in combination with 1000 mg/ L CH and 100 mg/ L L-proline gave different frequency of callus formation and callus morphology. Mature seeds can be selected as explant source for callus induction and regeneration effectively due to they are accessible all the time of year. The seeds swelled and clearly observed from the scutellum region after 10 days of culture under 14 hours photoperiod. Based on results (Table 2), the percentage of callus induction was from 17.29 to 64.38% across various concentrations of 2, 4-D after 1 month of culture. No callus initiation was obtained on the CIM without 2, 4-D (control). Our result revealed that 2,4–D in the presence of CH and L-proline stimulated callus induction of Sangyod rice similar to those reported previously (Wetherell *et al.,* 1976; Raval and Chatto, 1993). The concentration of 2, 4-D higher than 2.0 mg/ L caused the decrease in callus induction frequency.

Different characteristics of callus were found in different concentrations of 2,4-D. CIM containing 2.0 mg/ L 2,4-D gave the best response in callus formation (64.38%) and desired morphology of embryogenic callus as white or yellowish color with globular structure (Table 2, Fig. 5b and 5c). Our results are in agreement with Shahsavari *et al*. (2010) who reported that the highest percentage of callus induction

was obtained on MS medium supplemented with 2.0 mg/ L 2,4-D in upland rice cultivars Selasi, Kusan, Siam and Lamsan, respectively. Several researchers showed that MS medium supplemented with 2.0 mg/ L 2,4-D was better for aromatic rice KDML105, Basmati 370 (Ageel and Elmeer, 2011). Appearance of non - embryogenic callus consisting of brown color, necrosis and rhizogenesis was observed on high 2,4-D containing CIM. Besides, the decrease in frequency of callus formation was obtained on high concentration of 2, 4-D (3 - 4.0 mg/ L) similar to those reports from Libin *et al.* (2012) and Din *et al.* (2016).

The choice and distinction of embyogenic callus is essential to obtain efficiency of plantlet regeneration (Jaseela *et al.,* 2009; Summart *et al.,* 2008). 2,4-D was known as a strong synthetic auxin and popular applied as growth regulator in plant tissue culture. It plays critical role for successful in callus initiation and sustainment in rice (Karthikeyan *et al*., 2011). 2,4-D alone was often used for callus induction. However, several researchers reported that supplementation of 2,4-D together with CH or L-proline enhanced the response of callus formation and proliferation rather than 2,4 – D alone (Yinxia and Te-Chato, 2012; Rattana *et al.*, 2012).

The combination of 300 mg/ L L-proline and 400 mg/ L CH in MS medium supplemented with 2.5 mg/ L 2, 4-D and 1.0 mg/ L Kn gave the highest callus induction frequency of rice cv. BRRI dhan32 (Khaleda and Al-Forkan, 2006). Each genotype of rice requires different hormonal composition. Therefore, CIM should be modified to suit for each variety of rice. The function of 2,4–D was reported to enhanced amount of callus through increasing cell division rates. Embryogenic callus initiation could be promoted through sufficient concentration of 2,4-D (Che Radziah *et al.*, 2012). Our result also revealed that the combination of 2,4-D with amino acids (CH and L-proline) could be useful for promoting a high percentage of callus induction in Sangyod rice.

Table 2 Effect of concentrations of 2,4-D on callus induction from mature seed of Sangyod rice on CIM supplemented with 1000 mg/ L CH and 100 mg/ L L-proline after 4 weeks of culture

2,4 –D (mg/ L)	Percentage of callus formation (%)	Morphology of callus
0.0	0.00 ± 0.00^{e}	-
1.0	30.62 ± 2.92^{c}	White, friable
1.5	45.79 ± 3.21^{b}	Yellow, friable
2.0	64.38 ± 2.87^{a}	White- Yellowish, nodular
3.0	17.29 ± 2.20^{d}	Yellowish-brown, compact, little rhizogenic
4.0	18.91 ± 2.97^{d}	Brown, compact, little necrosis
F -test	**	-
C.V. (%)	8.24	-

** Significant difference at $p \leq 0.01$ level

Values are means of 8 replicates ± SD. Means followed by different letters within column are significantly different by DMRT.

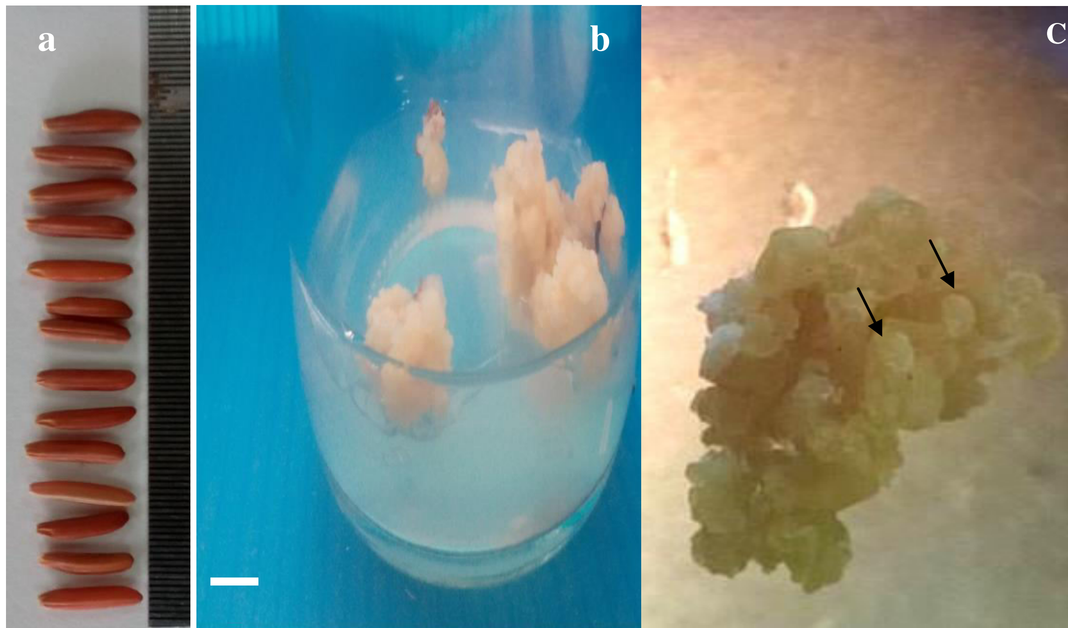

Figure 5 Morphological characteristic of callus of Sangyod rice on MS medium supplemented with 2 mg/ L 2,4 –D, 750 mg/L CH and 200 mg/ L L-proline after 4 weeks of culture

(a): Dehusked mature seeds of Sangyod rice

(b): Callus induction (bar = 0.5 cm)

(c): Characteristic of embryogenic callus with glubolar shape observed under microscope (X40)

Effect of different concentrations of CH on callus formation

The results in Table 3 revealed that increasing concentrations of CH promoted callus formation percentage from 25.37% (100 mg/ L) up to 68.56% (750 mg/ L) on MS medium supplemented with 2 mg/ L 2,4–D and 100 mg/ L L-proline. Callus induction frequency was significantly different ($p \leq 0.01$) among various concentrations of casein hydrolysate after 4 weeks of culture. The highest percentage of callus formation (68.56 ± 4.35 %) was obtained on MS medium supplemented with 750 mg/ L CH. However, the response of callus induction was slightly decreased to 61.67% when the concentration of CH was increased to 1000 mg/ L.

Our results was different to those previous reports in rice cv. Selasi which found that the combination of 300 or 600 mg/ L CH and 500 mg/ L L-proline increased callus induction percentage (Rashid *et al.*, 2003).Growth of rice calluses in terms of size and quality were obtained in the presence of CH whereas the number of embryogenic calluses did not increase. The result was_similar to the study reported by Raval and Chatto (1993) and Khaleda and Al-Forkan (2006). They reported that high callus induction percentage (87%) was obtained from rice cv. HA-8 on MS medium with 2 mg/ L 2,4–D and 0.6 % (w/v) CH. Moreover, NN medium (Nitsch and Nitsch, 1969) containing 2 mg/ L 2,4–D and 300 mg/ L CH increased callus induction in rice cv. KDML 105 (Liu *et al.*, 2001; Siddique *et al.*, 2014).

Although our results were in contrary to previous report of Che Radziah *et al.* (2012) different concentrations of CH (300 -1000 mg/ L) did not show significant impact on callus formation percentage of rice cv. MR 219 (72.5 % to 97.5 %). It has been earlier studied that addition of amino acids such as L-proline, CH can stimulate callus formation and plantlet regeneration frequencies (Afolabi *et al*., 2008).

CH is organic nitrogen source (Mohdin *et al.*, 2016). It also provides several vitamins, micronutrient, calcium and particular a mixture of 18 amino acids and have been reported to improve callus growth in the culture medium (Afolabi *et al.*, 2008). Base on this result, 750 mg/ L CH was selected due to the most suitable for induction of rice callus cv. Sangyod and this concentration was used for next experiment (Table 3).

Table 3 Effect of concentrations of CH on callus formation from mature seed of Sangyod rice on CIM supplemented with 2 mg/ L 2,4 -D and 100 mg/ L L-proline after 4 weeks of culture

CH (mg/ L)	Frequency of callus formation (%)
100	25.37 ± 2.76[c]
250	36.81 ± 3.63[bc]
500	46.67 ± 1.69[b]
750	68.56 ± 4.35[a]
1000	61.67 ± 4.29[a]
F-test	**
C.V. (%)	7.53

** Significant difference at $p \leq 0.01$ level
Values are means of 8 replicates ± SD. Means followed by different letters within column are significantly different by DMRT.

Effect of different concentrations of L-proline on callus formation

Based on data showed in Table 4, the percentage of callus initiation (34.38-73.08 %) and callus fresh weight (31.3-67.5 mg) were obtained on MS medium supplemented with various concentrations of proline, 2 mg/ L 2,4 – D and 750 mg/ L CH after 4 weeks of culture. Our result indicated that various concentrations of proline gave a significant effect on callus induction percentage and callus fresh weight. This finding is in approval with those earlier results reported by Chowdhury *et al.* (1993), Che Radziah *et al.* (2012) and Bhausaheb *et al.* (2015).

The absence of L-proline in culture medium (control treatment) showed the lowest frequency of callus formation and callus fresh weight at 34.38 % and 31.3 mg, respectively.

The supplement of L-proline in the culture medium at 200 mg/ L was an optimum callus induction percentage (73.08 ± 2.65) and callus fresh weight (67.5 ± 7.4 mg) followed by 100 mg/ L (61.67 ± 3.52 and 66.1 ± 11.6 mg, respectively). The increment in concentration of L-proline up to 300 mg/ L decreased callus induction percentage (40.21 ± 2.77) and callus fresh weight (39.0 ± 3.7 mg) significantly. Similar results were also reported in callus growth of some rice varieties such as Udayagiri, Pratikhya and Khandagiri (Subhadra *et al*., 2013). The positive effects of L-proline on the response of callus induction and regeneration have been demonstrated in rice cv. Hom Kra Dang Ngah (Yinxia and Te-Chato, 2012). However, the highest percentage callus formation (100) of cv. MR 219 was observed on MS medium supplemented with 3.0 mg/ L 2,4–D and 1000 mg/ L L-proline (Libin *et al*., 2012). In addition, NN medium supplemented with 1.5 mg/ L 2,4 –D, 300 mg/ L CH and 1000 mg/ L L-proline was reported to be the most suitable for increasing of callus formation in Supanburi 1 (Rattana *et al*., 2012) whereas the combination of 500 mg/ L CH and 500 mg/ L L-proline was suggested to have positive influence on callus formation in rice cv. HKR-46 and HKR-126 (Feng *et al*., 2011).

L-proline is a type of amino acids and also known as organic nitrogen source supplied growth and development of plant cells. The addition of L-proline in the medium acts as stress condition due to the reduction of water potential, thus,enhancing the development of embryogenic callus through the accumulation of nutritional items in cells (Subhadra *et al*., 2013). Moreover, L-proline was recommended to act as an osmoticum, source of $NADP^+$ and a nitrogen storage pool, essential for rapid embryo growth (Matsuta and Hirabayashi, 1989). Raval and Chatto (1993) reported that L-proline assisted increasing embryogenic callus induction and callus growth as similar to our observation as shown in Figure.6.

The highest embryogenic callus induction percentage was also observed on MS medium supplemented with 3.0 mg/ L 2,4-D, 2.0 mg/ L, Kn and 200 mg/ L L-proline in cv. Pratikhya and Swarna (Moghaddan *et al*., 2000). L-proline could provide a readily available nitrogen source to promote callus growth (Bhausaheb *et al*., 2015). In the present study, we recommend that 200 mg/ L L-proline containing MS medium together with 2 mg/ L 2,4–D and 750 mg/ L CH was the most effective for callus induction frequency and growth in rice cv. Sangyod (Table 4).

Table 4 Effect of L-proline on callus formation from mature seed of Sangyod rice on MS medium supplemented with 2 mg/L 2,4-D and 750 mg/ L CH after 4 weeks of culture

L-Proline (mg/ L)	Percentage of callus formation (%)	Mean callus fresh weight (mg)
0	34.38 ± 2.31^{b}	31.3 ± 5.4^{b}
50	46.04 ± 4.82^{b}	49.5 ± 8.1^{ab}
100	61.67 ± 3.52^{a}	66.1 ±11.6^{a}
200	73.08 ± 2.65^{a}	67.5 ± 7.4^{a}
300	40.21 ± 2.77^{b}	39.0 ± 3.7^{ab}
F-test	**	**
C.V. (%)	6.68	14.32

** Significant difference at $p \leq 0.01$ level

Values are means of 8 replicates ± SD. Means followed by different letters within column are significantly different by DMRT.

Effect of various concentrations of cytokinin and auxin on regeneration efficiency

The ratio of cytokinin (BA with Kn) to auxin (NAA) is essential for *in vitro* regeneration efficiency of rice cv. Sangyod. The combinations of NAA with BA and Kn are often used for plantlet regeneration in several rice varieties from embryonic callus (Ghanti *et al*., 2009). Both types of cytokinin and auxin affected cell cycles. Therefore, the ratio of cytokinin to auxin is key factor controlling many growth processes including organ regeneration from varied tissues (Rueb *et al.,* 1994). Several kinds of interaction may be antagonistic, additive or synergistic (Joyia and Khan, 2013). Calluses transferred to those PGRs containing regeneration medium showed different response of development (Table 5). Green spots were observed from embryogenic callus after 8 weeks of cultured (Figure 6).

Shoot induction percentage and mean number of shoots/callus varied based on the combination of PGRs which performed 2 weeks later. Green spots were produced from callus by the process of photosynthesis of the callus when they were emerged to the light (Inoue and Maeda, 1982). In Poaceae, the appearance of green spots has been recognized as predictors of shoot induction capacity (Coenen and Lomax, 1997). Our data showed that different concentrations of PGRs gave significant influence on callus growth and shoot regeneration (Table 5). The combination of 1 mg/ L BA, 0.5 mg/L Kn and 0. 5 mg/L NAA gave the maximum mean fresh weight of callus (938.9 ± 44 mg), the highest percentage of green spot formation (64.17 ± 7.08), optimum shoot induction frequency (66.25 ± 6.80 %) and maximum mean number of shoots/callus (6.12 ± 0.36 shoots) which was statistically different ($p < 0.01$) with the other concentrations of PGRs.

Decrease in callus fresh weight and shoot regeneration frequency in Sangyod rice were observed on high ratio of cytokinin (BA + Kn) to auxin (NAA) above 3:1 in Table 5 Conversely, in cv. Hom Kra Dang Ngah with high ratio of those PGRs (6:1) had the highest percentage green spot formation (75.5) and plantlets regeneraion (33.3) on MS medium supplemented with 0.5 mg/L NAA, 1.0 mg/L BA and 2.0 mg/L Kn (Yinxia and Te-chato, 2013).

Some authors reported that addition of 0.5 mg/ L NAA, 3 mg/ L BA and 0.5 mg/L Kn gave the most suitable for plantlet regeneration frequency at 80% and mean number of shoots/explant at 3.1 shoots/ callus in rice cv. Topa (Liu *et al.,* 2001). Our results showed that the combination of NAA and BA or Kn alone also promoted callus growth (fresh weight) and shoot regeneration in rice cv. Sangyod. Similarly result was reported in rice cv. Super Basmati on MS medium supplemented with 1 mg/L NAA and 3 mg/L Kn which provided number of shoots/callus at 9.66 ± 2.0 shoots (Bhausaheb *et al.*, 2015). The response of callus growth and shoots regeneration in rice cv. Sangyod *in vitro* was affected by various interactions and concentrations of PGRs (NAA, BA and Kn). Low ratio of cytokinin to auxin was suitable for this cultivar. The different responses of variety might be due to recalcitrance and genotype-dependence in *indica* rice. Therefore, it is essential to modify combination of PGRs base on different genotypes to inceasing regeneration efficiency.

Table 5 Effect of PGRs (NAA, BA, Kn) containing MS medium with 750 mg/L CH and 200 mg/L L-proline on regeneration efficiency from mature seed of Sangyod rice after 6 weeks of culture

Concentrations of PGRs (mg/ L)			Mean fresh weight of callus (mg)	Percentage of green spot formation (%)	Shoot induction frequency (%)	Mean number of shoots/ callus
NAA	BA	Kn				
0.5	2.0	0.0	526.0 ± 20.5^{bc}	28.33 ± 5.74^{ab}	42.50 ± 7.73^{ab}	3.13 ± 0.28^{b}
0.5	0.0	2.0	608.7 ± 18.2^{bc}	37.50 ± 13.21^{ab}	35.00 ± 9.45^{b}	2.68 ± 0.65^{b}
0.5	1.0	0.5	938.9 ± 44.0^{a}	64.17 ± 7.08^{a}	66.25 ± 6.80^{a}	6.12 ± 0.36^{a}
0.5	1.0	1.5	613.7 ± 16.2^{bc}	22.50 ± 9.59^{b}	36.25 ± 7.78^{b}	2.55 ± 0.50^{b}
0.5	1.0	2.0	653.0 ± 14.9^{b}	16.67 ± 3.56^{b}	21.25 ± 4.79^{b}	3.05 ± 0.48^{b}
1.0	1.5	1.0	571.7 ± 29.5^{bc}	40.00 ± 6.55^{ab}	38.75 ± 2.95^{b}	4.03 ± 0.42^{b}
1.0	1.5	2.0	563.3 ± 35.1^{bc}	47.50 ± 13.83^{ab}	32.50 ± 5.26^{b}	3.18 ± 0.14^{b}
F-test			**	**	**	**
C.V. (%)			3.98	25.14	17.61	12.75

** Significant difference at $p \leq 0.01$ level

Values are means of 8 replicates ± SD. Means followed by different letters within column are significantly different by DMRT.

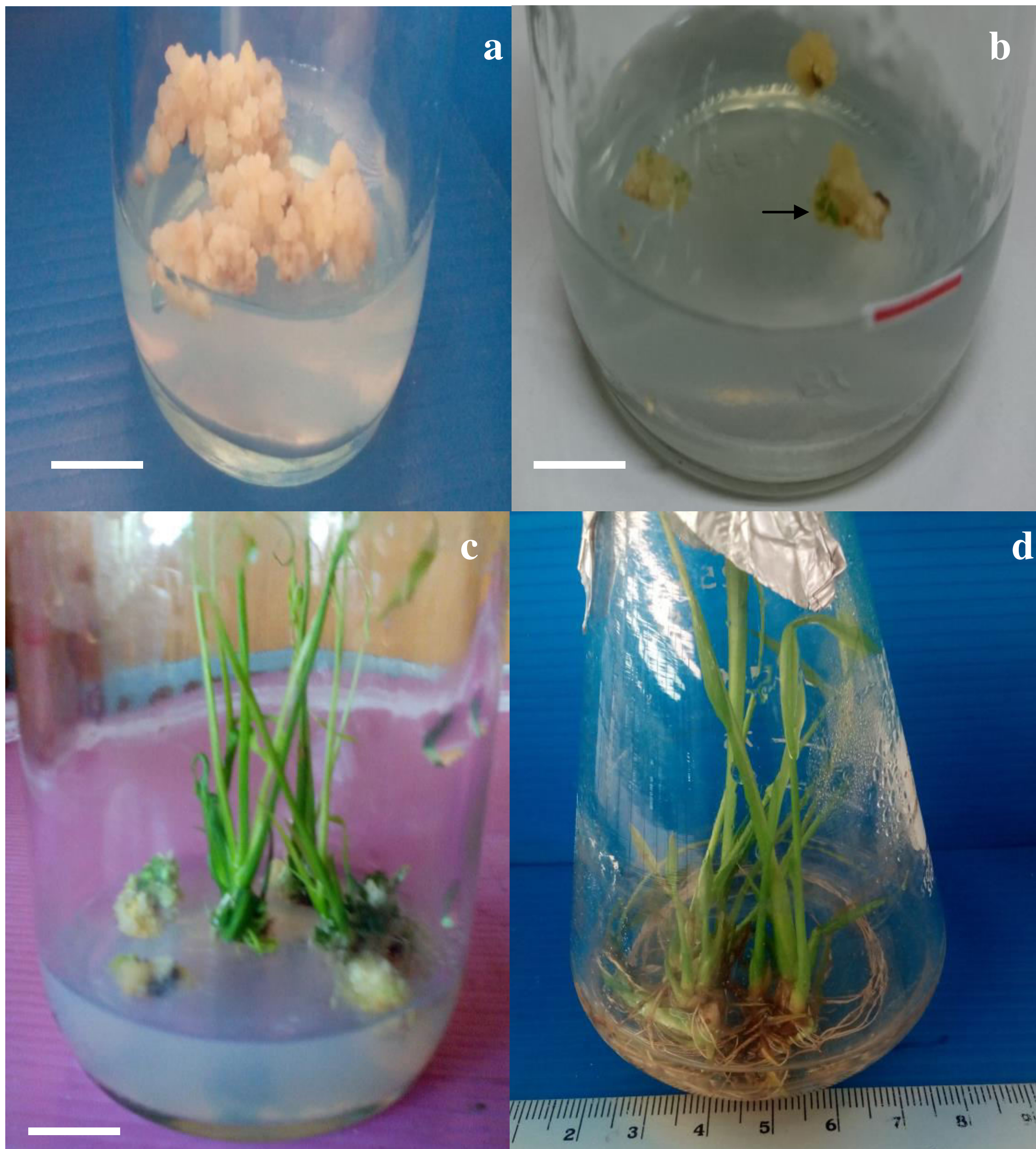

Figure 6 Morphological of callus and plantlet regeneration of Sangyod rice.

(a): Callus proliferation on solidified MS medium supplemented with 0.5 mg/L NAA, 1 mg/L BA and 0.5 mg/L KN (bar =1 cm)

(b): Geen spot formation

(c): Shoot formation

(d): Shoot multiplication and root formation in liquidified MS medium supplemented with 0.5 mg/L NAA and 1 mg/L BA.

Effect of different concentrations of PGRs (NAA, BA and Kn) on multiple shoot and root formation

In rice, plantlet regeneration efficiency affected by many factors such as genotype, physiological status of the explants, PGRs and culture environments (Menseses *et al.*, 2005). Shoot tip is considered as good explant and obtained strongly the dividing of meristematic cells that might easily maintain *in vitro* regeneration (Jubair *et al.*, 2008). A high cytokinin/auxin ratio promotes shoot induction. In contrast, a high ratio of auxin/cytokinin ratio encourages root production (Skoog and Miller, 1957). Base on results in Table 6**,** multiple shoot induction at 100% was recorded in all treatments. The greatest mean number of shoots/single shoot (14.93 ± 0.97 shoots) and root formation percentage (82.71 ± 3.03) was observed in liquidified MS medium supplemented with 0.5 mg/L NAA and 1 mg/L BA after 4 weeks of culture. By contrast, PGR–free MS medium gave the lowest results in all parameters.

The present study showed that the combination of 0.5 mg/L NAA and 1 mg/L Kn gave the lower mean number of shoots/single shoot (10.53 ± 0.98 shoots) and the percentage root formation (65.75 ± 5.59) than the combination of BA with NAA. However, there was not significant difference when liquidified MS medium in the presence of both 0.5 mg/ L BA and 0.5 or 1.0 mg/L Kn was used. Several reports showed that the most suitable PGRs for plantlet regeneration in rice cv. Chiniguri was obtained on MS medium supplemented with 0.05 mg/L NAA and 5 mg/L BA (Werner *et al.*, 2003; Sikder *et al.*, 2006).

High number of multiple shoot induction of *indica* rice variety Jaya was achieved on liquidified MS medium with 5 mg/L BAP, 1% (w/ v) mannitol and 3% sucrose (Sandhu *et al.*, 1995). Similarly, in cv. MR219, liquidified MS medium containing 0.1 mg/ L Kn gave the higher shoot induction efficiency than solidified MS medium. Liquidified medium supplies good aeration and enhances the capacity for dissolved nutrient composition uptake by whole surface of explant (Lavanya *et al.*, 2012). In this study, the combination of 0.5 mg/L NAA and 1 mg/L BA containing liquidified MS medium was the most effective for multiple shoot formation and root induction in rice cv. Sangyod.

Table 6 Effect of PGRs containing liquidified MS medium on multiple shoot and root formation from culturing single shoot-derived plantlets of rice cv. Sangyod after 4 weeks of culture

PGRs (mg/ L)			Mean number of shoots/explant	Root induction frequency (%)
NAA	BA	Kn		
0	0	0	4.67 ± 0.40[c]	0.00 ± 0.00[c]
0.5	0.0	1.0	10.53 ± 0.98[b]	65.75 ± 5.59[ab]
0.5	1.0	0.0	14.93 ± 0.97[a]	82.71 ± 3.03[a]
0.5	0.5	0.5	9.67 ± 1.00[b]	59.52 ± 5.87[b]
0.5	0.5	1.0	11.07 ± 1.06[b]	53.63 ± 5.28[b]
F-test			**	**
CV(%)			8.86	6.37

** Significant difference at $p \leq 0.01$ level

Values are means of 8 replicates ± SD. Means followed by different letters within column are significantly different by DMRT.

Conclusion

In the present study, efficient of embryogenic callus induction and plantlet regeneration protocol for *indica* rice cv. Sangyod was established using mature seeds. The results revealed that CH, L-proline and PGRs were key factors promoting callus induction and plantlet regeneration. The highest frequency of callus induction (73.08 ± 2.65%) and mean callus fresh weight (67.5 ± 7.4%) were obtained on MS medium supplemented with 2 mg/L 2,4–D, 750 mg/L CH and 200 mg/L L-proline.

The combination of 1 mg/L BA, 0.5 mg/L Kn and 0. 5 mg/L NAA containing solidified MS medium gave the maximum mean fresh weight of callus (938.9 ± 44 mg), the highest percentage of green spot formation (64.17 ± 7.08%), maximum shoot induction frequency (66.25 ± 6.80%) and mean number of shoots/explant (6.12 ± 0.36 shoots). Furthermore, the greatest mean number of shoots/explant (14.93 ± 0.97

shoots) and root formation percentage (82.71 ± 3.03%) was observed in liquidified MS medium supplemented with 0.5 mg/L NAA and 1 mg/L BA. Improvement of callus formation and regeneration efficiency can be used for genetic engineering to create new varieties with desirable traits in the future.

CHAPTER 3

Effect of Culture Media and Plant Growth Regulators on Callus Induction and Regeneration of *Indica* rice (*Oryza sativa* L.) Cultivar Sangyod

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Introduction

Rice (*Oryza sativa* L.) is one of the most strategical important cereal crop in Asia and a fundamental food source for over half of the world's population. Aromatic rice is very popular in southeast Asia and known as fragrance rice or scented rice. It has built up larger approval in the Western and Middle East nations recently (Sarhadi *et al*., 2008; Myint *et al*., 2009; Hashemi *et al*., 2012). Rice grain of Sangyod is regarded as one of the economically important traits for rice grain quality with dark-red color dehusk seed, soft and aromatic of cooked, grown in Patthalung province, Thailand for hundred years. Sangyod grain contains more minerals (iron), vitamins, bioactive compounds (anthocyanin, flavonoid, phenolic compounds). In addition, to meet its demand for healthier rice products are being increasing globally. Emphasizing a need for the research and development of efficient mass propagation tools for Sangyod rice variety in the future is required.

Rice improvement via tissue culture technique base on effective protocols through callus formation and suitable plantlet regeneration under *in vitro* conditions (Vega *et al*., 2009; Khatun *et al*., 2012; Siddique *et al*., 2014). *In vitro* rice plantlet regeneration can be established via organogenesis (shoot, leaf, root) and somatic embryogenesis (somatic embryos). However, somatic embryogenesis is one of the most auspicious methods for expeditious propagation due to induction of large- scale of plantlets and for the utilization of genetic transformation to resist to biotic and abiotic stresses (Zuraida *et al*., 2011). Successful achievement of somatic embryogenesis and plantlet regeneration of rice are highly depended on genotype, kinds of culture media, plant growth regulators (PGRs), gelling agent and carbon sources (Deo *et al*., 2009). Many basal culture media such as MS, NN, N_6 and ARDA usually used for in vitro propagation of rice. Auxin (2,4-D or NAA) plays critical role for cell growth and development (Su *et al*., 2009; Rademacher *et al*., 2012). In rice, it

prompts somatic embryogenesis and plantlet regeneration on cultured callus (Vega *et al*., 2009). Auxin was recognized as a key component of somatic embryogenesis that impacts carbohydrate metabolism on shoot regeneration and osmotic requirement (Lee and Huang, 2014). Huang *et al*. (2012) reported that endogenous auxin stimulate shoot regeneration in rice callus. PGRs is key factor controlling many growth processes including organ regeneration from varied tissues (Rueb *et al.*, 1994).

Osmotic water promising influences by kinds of media, concentrations of gelling agent and carbon sources (Hadeler *et al*., 1995; Klimaszewska *et al*., 1997; Triqui *et al*., 2008). Hence, choice of suitable culture medium in combination with PGRs are vital for optimum callus induction and subsequent plantlet regeneration in order to apply genetic transformation of rice. The additions of casein hydrolysate and proline have been reported to enhance callusing reaction (Lin and Zhang, 2005). The optimum conditions for efficient callus induction and regeneration from mature seeds of *indica* rice cultivars is one of prerequisites for the successful application of genetic transformation in crops.

The objectives of this study were to find out the most efficient culture medium and concentrations of PGRs (NAA, BAP, TDZ, Kn) in order to optimize productive protocol for callus induction and plantlet regeneration in Thai rice cultivar Sangyod.

Materials and methods

Plant material and sterilization

Mature seeds of *indica* rice cultivar Sangyod were used as explant source. They were obtained from Department Plant Science, Faculty Natural Resources, Prince of Songkla University, Thailand. The seeds were dehusked, washed with running tap water for 20 minutes, then surface sterilized with 70% (v/v) ethanol for 2 minutes, followed by immersion in 20% (v/v) Clorox (commercial bleach containing 0.05-0.1 ml of a wetting agent"Tween-20" per 100 ml Clorox solution) on orbital shaker at 100 rpm for 10 minutes. Finally, the seeds were rinsed five times with sterile distilled water in laminar air flow hood before blotted dry on autoclaved tissue paper to remove excess water. Sterile seeds were then cultured on callus induction medium (CIM).

Callus induction and proliferation

The experiments were carried out to optimize culture medium (MS, NN and ARDA) by culturing disinfedted seeds on three types of CIM, MS (Murashige and Skoog, 1962), NN (Nitsch and Nitsch, 1969) and ARDA (Yinxia and Te-chato, 2013). All culture media were supplemented with 2 mg/L 2,4-D, 750 mg/L of casein hydrolysate (CH) and 200 mg/L L-Proline. Each medium was added with 3% (w/v) sucrose, adjusted to pH 5.7, solidified with 0.75% (w/v) agar, and autoclaved at 121^0 C, 1.07 kg/ cm^2 for 20 min. All cultures were maintained at 26 ± 2^0C in the culture room under 14 hours photoperiod with irradiance of 25 μmol/m^2/s provided by cool white fluorescent tubes. After four weeks of culture frequency of callus induction, number of days to callus initiation and mean callus fresh weight were recorded and statistical compared.

Effect of subculture on callus growth

Four-week-old calluses obtained from the most suitable medium of previous experiment at 0.1 gram fresh weight were excised and subcultured to the same medium component at weekly intervals for 8 weeks to proliferate and growth index (GI) and fresh biomass (FW) measured. The preparation of culture media and culture conditions were the same as previous experiments.

The average of GI, FW, browning callus frequency and morphological characteristics were evaluated every two-week of culture until the fourth times of subculture (8 weeks). GI was calculated as described by Chan *et al.* (2008) according to the following formula:

$$GI = (\text{Final FW} - \text{Initial FW}) / \text{Initial FW}$$

Effect of PGRs on plantlet regeneration

The calluses from the best subculture stage were transferred to regeneration medium (RM) which was MS supplemented with various concentrations of PGRs (NAA, TDZ, BAP and Kn) as shown in Table 8. The medium was added with 3% (w/v) sucrose and adjusted to pH 5.7 prior to addition with 0.75% (w/v) agar. The cultures were maintained at the same conditions as mentioned in previous experiments. At three weeks of culture the percentage of green spot (GS) formation, percentage of plantlet regeneration and mean number of plantlets per callus were recorded and statistically compared after six weeks of culture.

Statistical analysis

All the tissue culture experiments of callus induction, callus growth, and plantlet regeneration frequency were arranged in completely randomized design (CRD) with 6 replicates per treatment. Data were tested by using one–way analysis of variance (ANOVA) and the significant differences among means were separated by Duncan's multiple range test (DMRT) ($p \leq 0.01$) using program R statistical package version 2.14.

Results and discussions

Influence of different culture medium on callus induction

The results showed that different culture media were significant influence on time required for callus initiation, frequency of callus induction and mean FW (Table 7). After 17 days of culture on ARDA medium, callus was initiated from the scutellum region under 14 hour photoperiod. The highest frequency of callus induction (75.63 ± 5.28%) and FW (68.05 ± 20.04 mg) were observed on ARDA medium supplemented with 2 mg/L 2,4 – D, 750 mg/L CH and 200 mg/L L-proline after 4 weeks of culture whereas there was not significantly different ($p < 0.01$) with MS medium (Figure 7). Various morphological calluses were found in different culture media. However, embryogenic callus with good characteristics such as yellow or white color with globular structure was observed on MS and ARDA medium.

Mature seeds cultured on NN medium in combination with 2 mg/L 2,4 – D, 750 mg/L CH and 200 mg/L L-proline gave the longest time for callus initiation after 20 days of inoculation, lowest frequency of callus induction at 50.22 ± 6.51% and callus FW at 44.39 ± 25.36 mg. The earliest period time for callus appearance was obtained on MS medium after 10 days. Some researchers reported that the positive influence of NN medium on rice callus induction in some varieties such as Khao Dawk Mali 105, Chai Nat 1, Supanburi 1 (Rattana *et al*., 2012). Furthermore, the highest percentage of callus induction (100%) and mean FW of callus (341.2 mg) was obtained on NN medium suplemented with 1 mg/L 2,4 –D in Nam Roo rice (Poeaim *et al*., 2016). Recently, Yinxia and Te-chato (2013) reported that ARDA medium gave the best result for the percentage of green spot callus formation (61.3%) in rice cv. Hom Kra Dang Ngah. However, the effect of ARDA medium on callus induction has not been reported.

Nitrogen could apply to culture media as organic or inorganic sources. Nitrogen in form of organic source may be apply as amino acid and vitamins. The inorganic nitrogen is commonly provided through the structure of ammonium or nitrate ions (Kaushal *et al*., 2014a). The ratio of NO_3^-/NH_4^+ is known to be the main key for the successful efficient of callus induction in rice (Grimes and Hodges, 1990). MS and ARDA medium contain higher level of NH_4NO_3 than that of NN medium. Beside, ARDA medium also contains K_2SO_4 while the others two culture media (MS or NN medium) do not consist this component.

It may be one of reason to improve embryogenic callus incuction. Minyaka *et al.* (2008) reported the positive effect of K_2SO_4 on induction a high frequency of somatic embryo formation in cacao genotypes IMC67 and Sca6 on DKW medium (Driver and Kuniyuki, 1984). Zinc and Copper are two crucial micronutrients for plant cells which effect on embryogenesis (Silva, 2012). Copper plays important roles in respiration process which is the functional components of oxidative enzymes in plant tissue (Sumner and Somers, 1953).

Similarly, it has also reported that the copper enhanced the *in vitro* response in *japonica* rice cultivars (Yang *et al*., 1999). Furthermore, zinc is essential co-factors for the process of Krebs' and glycolysis cycle (Macelroy and Nason, 1954). Casein

hydrolysate was known as organic nitrogen which provides calcium sources, vitamins, several micronutrient and amino acids supplements on culture media which have been useful for callus induction. The addition of L-proline in the medium acts as stress condition due to the reduction of water potential, thus, enhancing the development of embryogenic callus through the accumulation of nutritional items in cells (Mallick *et al.*, 2013).

Base on our results, ARDA medium was the most suitable for callus induction and proliferation in Sangyod rice and this culture medium was used for next experiments. The composition of basal salts, strength of micronutrients and amino acids supplements in culture medium are the main factors influencing callus induction frequency and fresh weight of callus. In rice, callus formation depend on the kinds of culture medium used (Zhao, 1999). Therefore, it is necessary to optimize culture medium for each genotype before establishment of genetic transformation experiment.

Table 7 Influence of different culture media supplemented with 2 mg/ L 2,4-D, 750 mg/ L CH and 200 mg/ L L-proline on Sangyod callus induction after 4 weeks of culture

Culture media	Days to callus initiation	Frequency of callus induction (%)	Mean callus FW (mg)
MS	10	72.75 ± 4.67^{a}	66.51 ± 14.70^{a}
ARDA	17	75.63 ± 5.28^{a}	68.05 ± 20.04^{a}
NN	20	50.22 ± 6.51^{b}	44.39 ± 25.36^{b}
F-test	-	**	**
C.V. (%)	-	8.79	36.23

** Significant difference at $p \leq 0.01$ level

Values are means of 6 replicates ± SD. Means followed by different letters within column are significantly different by DMRT.

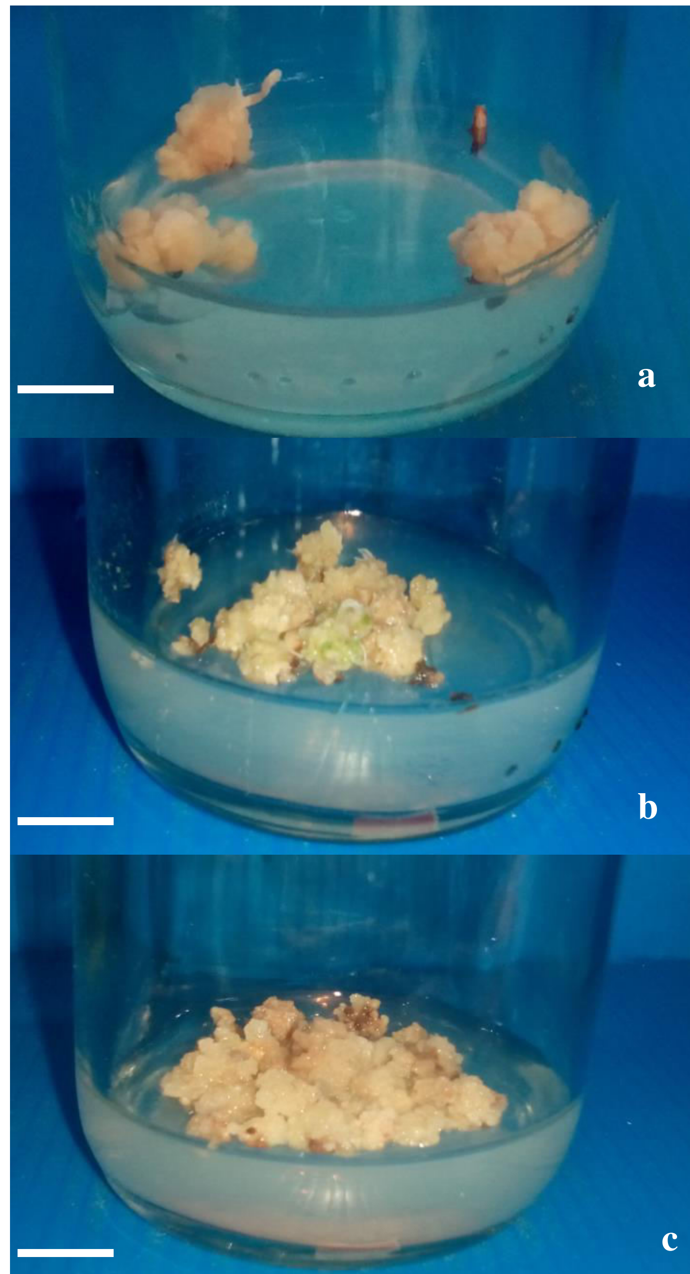

Figure 7 Characteristics of callus induction and callus proliferation in different subculture stages of Sangyod rice (bar = 1 cm)

(a): Callus induction on ARDA medium supplemented with 2 mg/ L 2,4- D, 750 mg/ L CH and 200 mg/ L L-proline after 4 weeks of culture.

(b):Callus proliferation after 35 days of subculture

(c):Callus proliferation after 56 days of subculture

Influence of repeated subculturing on callus growth

Based on the results in Figure 8 and 9, GI and mean FW of callus increased exponentially from day 7 to day 35 after subculture. After this period growth of callus was slow and enter stationary stage. Model of the callus growth curve is one of main parameters that determine the period for maximum callus induction and growth rate (Hussein *et al*., 2016). Four–week-old or 28-day-old callus were suitable period for routine subculture to ARDA medium supplemented with 2 mg/L 2,4–D, 750 mg/L CH and 200 mg/L L-proline. This peroiod showed significant response on mean fresh weight of callus and GI for 56 consecutive days of culture (Figure 8). The callus growth curves of Sangyod rice revealed according to a sigmoidal shape that the GI and FW were initially slow during first 7 days of culture (lag phase).

After this period growth of callus increased dramatically different ($p \leq 0.01$) until 35 days (log phase). The maximum mean FW at 1272.83 ± 48.63 mg was obtained at day 35 of culture. GI was also the highest at 11.73 folds of the initial FW (100 mg) at this date of culture (Figure 8). In case of browning of callus, increase in times of culture promoted the increment of callus browning. After the 5th time of culture browning of callus was severely increased, significantly different ($p \leq 0.01$) with the 1st to 4th time of culture (Figure 9). The highest browning callus at 69.17 ± 10.56 % was observed by day 56 after culture whereas the greatest mean FW of callus (1423 ± 37.59 mg) and GI (13.23) were obtained.

Therefore, the good quality and high GI of Sangyod callus were at 35 days after subculture which was in log phase and should be the most suitable for producing plant regeneration and genetic transformation experiment. Calluses are known as unorganized cell masses that could be produced from one differentiatiated cell. The quality of the callus in rice is one of the key factors to define the growth rate of regeneration (Amarasinghe, 2010). Callus growth, alike bacterial growth, is connected procedure that exposes unique phases with the specific GI. There were three main stages for callus growth (Figure 8). Commonly, the cycle of callus growth of rice in first stage could be maintained until the callus attain stationary phase.

It has been reported that the second subculture (42 successive days of culture) gave the best subculture period for the highest proliferation rate of callus and good quality such as nodular, compact, yellow or white color in ten rice varieties, especially, the maximum GI of callus in varieties Pachcha Perumal (4.993) and Taipei 309 (6.187) (Amarasinghe, 2010). Additionally, the callus growth rate of seven rice genotypes (IR24, Zhenshan 97, IRBB13, IRBB 4, IRBB 10, Minghui 63 and 93-11) on S medium were evalutated by Ge *et al.* (2006). After 20 days of subculturing, the GI of callus increased, particularly, Minghui 63 genotype showed the highest GI (8.13) among the other 7 genotypes.

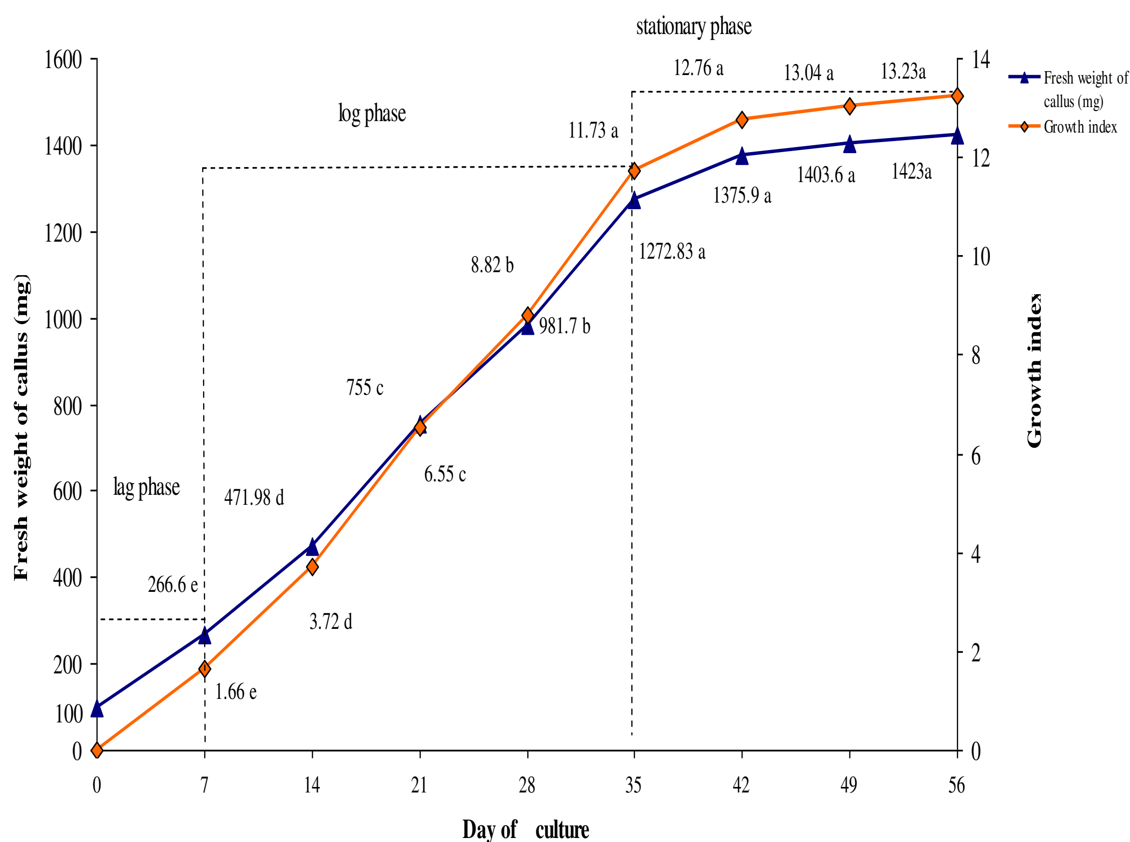

Figure 8 The effect of successive subcultures on mean fresh weight of callus and growth index of Sangyod rice on ARDA medium supplemented with 2 mg/ L 2,4 – D, 750 mg/ L CH and 200 mg/ L L-proline. The cultures were routinely subcultured 56 days intervals

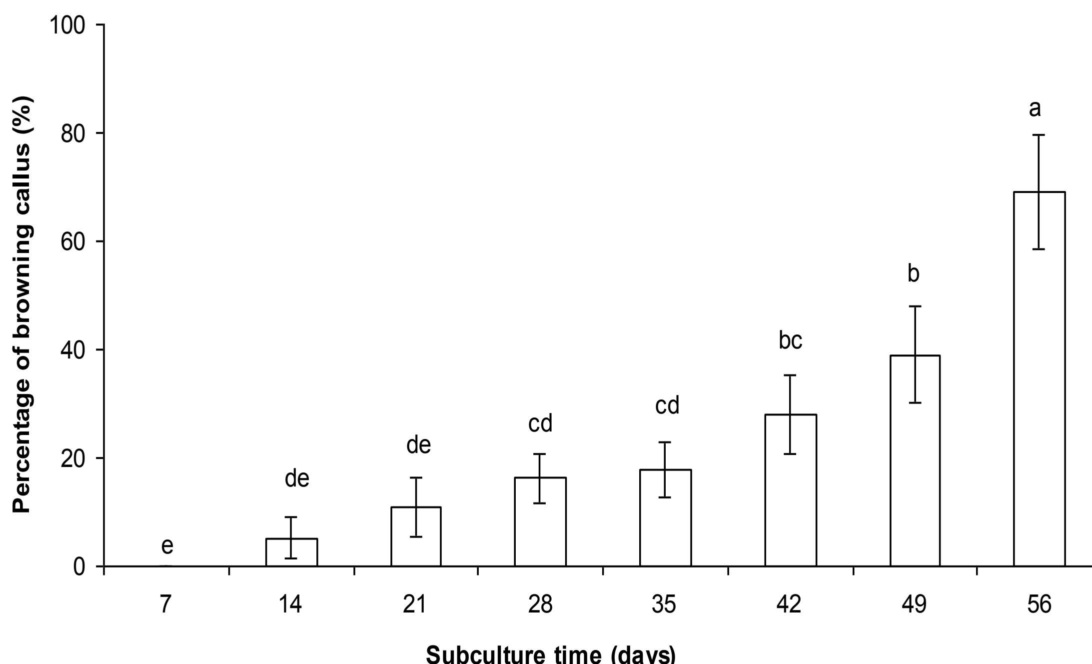

Figure 9 Influence of days after culture on the percentage of browning of Sangyod callus for 56 days. The callus was cultured on ARDA medium supplemented with 2 mg/ L 2,4 – D, 750 mg/ L CH and 200 mg/ L L-proline

Influence of various concentrations of PGRs on plantlet regeneration

Different kinds and concentrations of PGRs gave significant influence on green spot callus and plantlet regeneration. The combination of 0.5 mg/ L BAP, 0.5 mg/L TDZ, 1.0 mg/L Kn and 0. 5 mg/L NAA gave the maximum percentage of green spot (GS) formation (72.34 ± 8.75%), plantlet regeneration frequency (67.25 ± 6.14%) and mean number of plantlets/callus (6.63 ± 0.47 plantlets) which was statistically different ($p \leq 0.01$) with the other concentrations of PGRs (Table 8, Figure 10). Similarly, Din *et al.* (2016) revealed that the correct concentration of PGRs together (NAA, BAP, Kn and TDZ) was significantly increased regeneration frequency in Malaysian upland rice cv. Panderas, specifically, the optimum regeneration frequency (100%) was observed on MS medium containing containing 0.5 mg/L BAP , 0.5 mg/L NAA, 1.5 mg/L Kn, 0.5 mg/L TDZ. Plantlet regeneration frequency varied according to the combination of cytokinin and auxin. Kyungsoon *et al.* (2002) reported that the

greatest regeneration frequencies were 68, and 77% for cultivars Nak-Dong, Dong-Jin on MS medium containing 2.0 mg/L NAA and 2.0 and 4.0 mg/L Kn, respectively. Moreover, the most suitable for plantlet regeneration efficiency of rice cv. BRRI dhan 29 was 80% on MS medium supplemented with 1.0 mg/L NAA, 1.5 mg/L Kn and 2.0 mg/L BAP (Islam *et al*., 2014).

The action of TDZ could induce more shoot regeneration than BAP in rice cv. Jaumala (Gairi and Rashid, 2004). Raghavendra *et al* (2010) reported that MS medium supplemented with 4 mg/L TDZ gave the highest plant regeneration frequency (93.33%) and number of shoots/ explant (10.67 shoots) in rice cv. Rasi. The different responses of variety might be due to recalcitrance and genotype-dependence in *indica* rice. Therefore, it is essential to modify combination of PGRs base on different genotypes to increase regeneration efficiency.

Plant growth regulators play important role in plant tissue culture, where a high the ratio of auxin/cytokinin consistently used for producing embryogenic callus. In contract, a low ratio was used for the plantlets regeneration (Ge *et al*., 2006**)**. In earlier studies, it has been reported that the levels of endogenous auxin in rice callus could promote shoot regeneration (Huang *et al*., 2012). However, the action of endogenous auxin on the effective of the formation of callus subsequent to shoot induction still unknown. Skoog and Miller (1957) reported that organogenesis in callus and tissue culture occurred by the intake of PGRs (cytokinin, auxin) in medium in the initial phases of culture.

At present, the knowledge of mechanisms of auxin and cytokinin in synthesis sites and their molecular level that provides via the plant causes large process of endogenous auxin and cytokinin levels. Auxin may be recognized as a main factor that impacts osmotic prerequisite, phytohormone signal and carbohydrate metabolism on shoot regeneration (Lee and Huang, 2014). The ratio of plant growth regulators (cytokinin/ auxin) have significant role in growth and differeniation of cells in callus. Cytokinin can promote the growth rate of pro-embryonic masses (Kommamine *et al*., 1992). Some auxins such as 2,4-D or NAA in combination with kinetin at the specified concentrations could improve plantlet regeneration in rice. Using the

combination of PGRs promotes more efficient callus induction and plantlet regeneration than using PGR alone (Ge *et al*., 2006).

Table 8 Effects of different kinds and concentrations of PGRs containing ARDA medium on plantlet regeneration from callus of Sangyod rice after 6 weeks of culture

Concentrations of PGRs (mg/ L)				Green spot formation Frequency (%)	Plantlet regeneration frequency (%)	Mean number of plantlets/ callus
NAA	TDZ	BAP	Kn			
0.5	1.5	0.0	0.0	36.66 ± 7.04^{de}	31.3 ± 5.15^{c}	2.54 ± 0.21^{cd}
0.5	0.0	1.5	0.0	25.12 ± 10.31^{e}	25.54 ± 4.87^{c}	2.17 ± 0.44^{cd}
0.5	0.0	0.0	1.5	41.45 ± 6.50^{cd}	26.81 ± 5.23^{c}	1.80 ± 0.35^{d}
0.5	0.5	0.5	1.0	72.34 ± 8.75^{a}	67.25 ± 6.14^{a}	6.63 ± 0.47^{a}
1.0	1.5	0.0	0.5	64.50 ± 9.26^{ab}	54.75 ± 5.60^{ab}	4.06 ± 0.55^{b}
1.0	0.0	1.5	0.5	53.84 ± 6.78^{bc}	41.49 ± 7.08^{bc}	4.35 ± 0.28^{b}
1.0	0.5	0.5	1.5	39.26 ± 9.33^{d}	30.17 ± 4.22^{c}	3.33 ± 0.39^{bc}
F- test				**	**	**
C.V. (%)				19.82	13.98	12.39

**Significant difference at $p \leq 0.01$ level

Values are means of 6 replicates ± SD. Means followed by different letters within column are significantly different by DMRT.

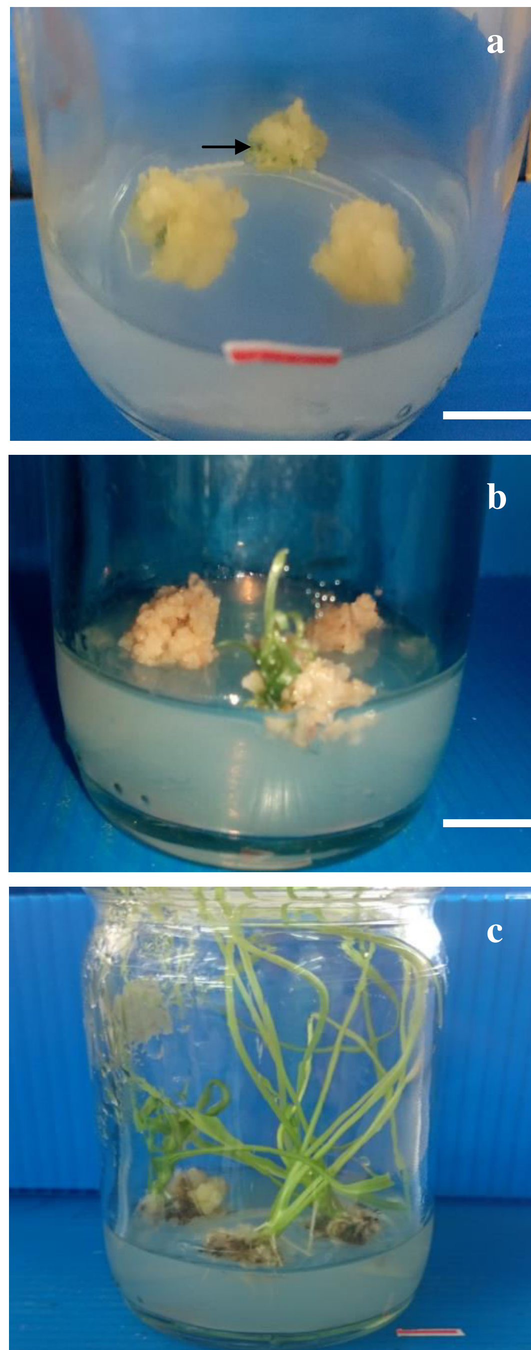

Figure 10 Plantlet regeneration from mature seed-derived callus of Sangyod rice on ARDA medium with 0.5 mg/ L BAP, 0.5 mg/ L TDZ, 1.0 mg/ L Kn and 0. 5 mg/ L NAA (bar = 1 cm)

(a): Green spot formation after 4 weeks of culture

(b): Shoot induction after 6 weeks of culture

(c): Plantlet regeneration after 8 weeks of culture

Conclusion

The results revealed that the culture medium and PGRs play a significant role in callus induction, callus growth index (CGI) and plantlet regeneration protocol of Sangyod from culturing mature seeds. The highest percentage of callus induction (75.63 ± 5.28%) and fresh weight (FW) (68.05 ± 20.04 mg) were achieved on ARDA medium supplemented with 2 mg/L 2,4 – D, 750 mg/L CH and 200 mg/L L-proline after 4 weeks of culture.

The optimum mean FW (1272.83 ± 48.63 mg) and the highest CGI (11.73-fold) were obtained on day 35 of culture. The combination of 0.5 mg/L BAP, 0.5 mg/L TDZ, 1.0 mg/L Kn and 0. 5 mg/L NAA gave the maximum percentage of green spot formation (72.34 ± 8.75%), plantlet regeneration frequency (67.25 ± 6.14%) and the mean number of plantlets/callus (6.63 ± 0.47 plantlets). Improvement of callus formation and regeneration efficiency can be used for genetic engineering to create new varieties with desirable traits in the future.

CHAPTER 4

Establishment of *Agrobacterium*–mediated Transformation Systems in the *Indica* Rice Cultivar Sangyod

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Introduction

Rice (*Oryza sativa* L.), which belongs to the Poaceae family, is one of the most strategically important cereal crops in Asia, and a fundamental staple food for over half of the world's population. Among the three subspecies, the *indica* group of rice varieties accounts for 90% of rice production and consumption in the Asian regions. Recently, rice yields and quality have been impacted by global climate changes, and biotic and abiotic stresses, and conventional breeding alone cannot satisfy the growing demand for rice with the desirable agronomic traits necessary to meet the needs of the global population. This emphasizes the need for research, and the development of tissue culture and genetic engineering techniques to be applied to rice to improve existing varieties in the future.

The development of new strains of rice may be initiated through different explant types such as leaf bases, roots, immature embryos and mature embryos (Abe and Futsuhara, 1984; Seraj *et al*., 1997; Ramesh and Gupta, 2006; Li *et al*., 2007; Ramesh *et al*., 2009). Calluses derived from immature or mature embryos have generally been used for the genetic transformation of cereal crops through *Agrobacterium tumefaciens* (*A.tumefaciens*) (Cheng *et al*., 1997). The efficient identification of callus induction and subsequent plant regeneration protocols are a prerequisite for rice improvement including by genetic transformation (Raemakers *et al*., 1997). Rice improvement has been achieved via protoplast fusion, particle gun bombardment, sexual hybridization and *Agrobacterium*–mediated gene transformation.

Tissue culture techniques, such as mass production, have become popular improvement tools used as alternative means of plant vegetative propagation and genetic engineering. Techniques for the transformation of rice are a suitable target for

development in cultivars (Rao *et al*., 2009) and *Agrobacterium*-mediated gene transformation is the favored mode which has emerged as being the most effective technique because it is a relatively accurate mode of DNA transfer, leading to plant regeneration with fewer transgene copies, higher transformation efficiency and lower costs, and is a simple procedure with decreased gene silencing (Hiei *et al*., 1994).

It is commonly used for many dicotyledonous plants and several monocots including rice (Mohanty *et al*., 1999). The success of *Agrobacterium*-mediated transformation has been reported in some *Japanica* and *Indica* rice cultivars such as IR 64, IR 72 (Kumar *et al*., 2005) and BRS Primavera (Bevitori *et al*., 2014). However, many *indica* rice cultivars often perform poorly in response to in vitro regeneration and produce low levels of transformation when compared to *Japanica* sub-species (Zhang *et al*., 1998; Khanna and Raina, 1998; Kumar *et al*., 2005). Several reports have focused on the assessment of the totipotency and transgenic capacity of *indica* genotypes (Lin and Zang, 2005).

There are many factors which affect the efficiency of transformation of rice by *Agrobacterium* such as explant types, the strain and density of bacteria, the expression vector-plasmid, the inoculation period, the elimination of *Agrobacterium* infections after co-cultivation, selection agents and culture medium conditions (Cheng *et al*., 2004; Tie *et al*., 2012). The genotype of explants has been regarded as the main factor that influences transformation efficiency (Tie *et al*., 2012). Gene transformation is dependent on genotype so, the development of the *Agrobacterium* host range and increasing the transformation frequency is the primary objective in research in higher plants with the aim of decreasing costs and the resources required (Cheng *et al*., 2004).

Transient ß-glucuronidase (GUS) expression has been performed to screen the source of explants, which is essential for rapid and cost-effective gene integration analysis through stable transformation (Wroblewski *et al*., 2005; Yasmin and Debener, 2010). Genetic engineering can alter conventional breeding to create desirable agronomic characteristics efficiently and rapidly (Arokiaraj *et al*., 2002). Herbicide resistance is one of the most important traits amongst genetic improvement strategies pursued in many crops (James, 2013) and significant numbers of glyphosate

(*N-phosphonomethyl glycine*)–resistant crops are grown in the USA and South America, particularly, cotton and soybean. Thus, the development of glyphosate-resistant rice would greatly facilitate effective weed control systems, while ensuring low toxicity and minimizing the risk of environmental effects as well as reducing production costs. Currently, glyphosate is rarely directly applied in paddy fields due to its toxicity to rice and it has been reported that the 5-enolpyruvylshikimate-3-phosphate synthase gene (*CP4-EPSPS*), which is responsible for glyphosphate resistance, only functions efficiently in soybean (Clemente *et al.,* 2000) and maize (Russell and Fromm, 1997).

The*CP4-EPSPS* gene has been studied in the rice cultivar *Hom kra Dang Ngah* (Yinxia and Te-Chato, 2012) and in the rubber tree *Hevea brasiliensis* (Kalawong *et al*., 2014). In addition, using glyphosate selection as a selectable marker has some benefits rather than other markers. In producing glyphosate-resistance, the CP4-EPSPS gene is associated with a single T-DNA (Monsanto, 2003). Furthermore, there have been several reports investigating the efficiency of glyphosate-resistant rice which have identified mutations of the *CP4-EPSPS* gene *known as the MdEPSPS* , *VvEPSPS* and *OsEPSPS* mutants (Zhao *et al*., 2011; Tian *et al*., 2013, 2015; Chandrasekhar *et al*., 2014; Deng *et al*., 2014; Chhapekar *et al*., 2015; Yi *et al*., 2016)

At present, the *indica* rice cultivar Sangyod is one of the most important commercial rice cultivars in Thailand. However, a review of the literature was unable to trace any published research considering the improvement by gene transformation of *Sangyod* using *Agrobacterium* tumefaciens. Therefore, the objectives of this study were to investigate the main factors affecting the efficiency of the gene transformation process and to establish an *Agrobacterium*–mediated transformation system for Sangyod rice to be applied in transformations involving various essential commercial genes in the future.

Material and methods

Plant material and sterilization

Healthy mature seeds of the indica rice cultivar Sangyod were used as the explant source, which were obtained from the Phatthalung Rice Research Center, Phatthalung province, Thailand. The seeds were dehusked, washed with running tap water for 20 minutes, then surface sterilized with 70% (v/v) ethanol for 2 minutes, followed by immersion in 20% (v/v) Clorox (a commercial bleach) containing 2-3 drops of a wetting agent "Tween-20" on an orbital shaker at 100 rpm for 10 minutes. Finally, the seeds were rinsed five times with sterile distilled water in a laminar air flow hood before being blotted dry on autoclaved tissue paper to remove excess water. The sterile seeds were then cultured on a callus induction medium.

Bacterial plasmid

A.tumefaciens strain EHA105 harboring the binary vector pCAMBIA1304-EPSPs and the β-glucuronidase (GUS) as well as the glyphosate resistance gene *CP4-EPSPS* was used in these experiments as shown in Figure 11.

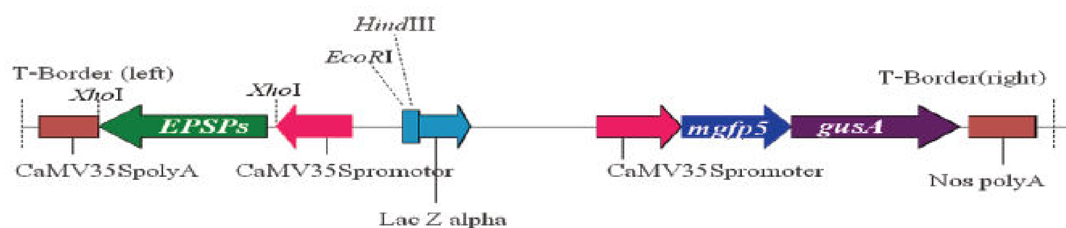

Figure 11 Construction of T-DNA region of pCAMBIA1304-EPSPS carrying the *gusA* gene as a reporter gene and the *EPSPS* gene for glyphosate (*N-phosphonomethyl glycine*) resistance

Callus induction

Mature seeds were cultured on the ARDA medium (CIM) supplemented with 2 mg/L 2,4-D, 750 mg/L CH and 200 mg/L L-Proline, 3% sucrose and the pH was adjusted to 5.7 prior to adding 0.75 % agar and autoclaving at 1.07 kg/cm^2, at 121

ºC for 20 minutes. The cultures were maintained at 26 ± 2 ºC in the culture room with a 14 hours photoperiod with irradiance of 25 µmol/m^2/s provided by cool white fluorescent tubes during 6 weeks.

Effect of the sensitive of glyphosate on the callus

Embryogenic calluses (6 week-old) were placed on ARDA medium supplemented with various concentrations of glyphosate (0, 0.2, 0.4, 0.6 and 1.0 mM). All the cultures were maintained at 26 ± 2ºC in the culture room with a 14-hour photoperiod with irradiance of 25 µmol/ m^2/ s provided by cool white fluorescent tubes. After three weeks of culture, the percentage of surviving calluses was recorded and statistically compared.

Preparation of *Agrobacterium* cells

Loopfuls of bacteria stock were streaked on solidified LB medium (Luria-Bertani) containing 5 g/L NaCl, 10 g/L tryptone and 5 g/L bacto yeast extract. The pH of the medium was adjusted to 7.0 and 0.75% agar in combination with 50 mg/L kanamycin. The cultures were incubated at 26ºC for 3 days in darkness. Then, one single colony of this *Agrobacterium* was selected and suspended in liquid LB medium (25 ml) containing 50 mg/L kanamycin and 200 µM acetosyringone that incubated on a rotary shaker at 120 rpm in the darkness at 26°C. After the *Agrobacterium* cells had proliferated in the liquid LB medium overnight, they were collected and adjusted to three optical densities (OD_{600}) at 0.4, 0.6 and 0.8 using a spectrophotometer.

Inoculation and selection of transgenic calluses

Embryogenic calluses were induced at six weeks old were cut into pieces of approximately 0.1 grams and submerged in 25 ml of the bacterial solution at various ODs as mentioned above for 15, 30 and 45 minutes at 26ºC on a rotary shaker at 120 rpm. The calluses were blotted on sterilized tissue paper to remove excess *A. tumefaciens*. The explants were cultured on a co–cultivation medium (ARDA) containing 0, 100, 150, 200 and 250 µM acetosyringone in combination with 100 mg/L myo–inositol and kept in the dark at 26ºC for 2 days. After co-cultivation, the

calluses were washed with liquefied ARDA medium supplemented with 100 mg/ L myo- inositol and different concentrations of cefotaxime (50, 100, 200, 300, 400 or 500 mg/L) for 20 minutes to remove excess *Agrobacteria*. The inoculated calluses were placed on selective medium (SM) containing ARDA medium supplemented with various concentration of cefotaxime as same as mention above, 100 mg/ L myo–inositol and minimum concentration of glyphosate that callus could survival (0.6 mM)

GUS expression (%) was tested after a 1 week transformation and early screening of the transformed calluses was conducted after 3 weeks. The calluses were keep in darkness for 1 weeks after that they were maintainted at 26 ± 2°C in the culture room with a 14-hour photoperiod with irradiance of 25 μmol/m^{2}/s provided by cool white fluorescent tubes to induce shoots and regenerated plants.

Histo-chemical GUS expression analysis

Histo-chemical GUS assay was conducted following the method described by Jefferson *et al.* (1987). The calluses were examined for GUS activity at 4 days after being transferred to the SM. The inoculated calluses were immersed in X-gluc buffer including 1 mM X-gluc (5-bromo-4-chloro-3-indolyl-glucuronide), 120 μl of Triton X-100 and 50 mM sodium phosphate buffer (pH 7.0). The percentage of calluses which exhibited blue spots (*gus* expression) was evaluated as indicating a positive transformation under a microscope. The explants were incubated overnight at 37°C in darkness and washed twice with 70% methanol. The frequency of the GUS gene transient expression (%) was calculated based on the number of blue spots on the calluses divided by the total the number calluses checked x 100. As a control treatment, GUS activity was recorded on non-transformed calluses without infection with *Agrobacterium*.

$$\text{GUS expression (\%)} = \frac{\text{Number of blue spots on calluses x 100}}{\text{Total number of calluses checked}}$$

Polymerase chain reaction (PCR) analysis

To confirm the presence of the transgene in the putative transformed rice calluses, PCR analysis was carried out using genomic DNA from transformed and non- transformed Sangyod rice calluses after 3 weeks of culture on SM, which were extracted according to the method described by Te-chato (2000). DNA samples were prepared for PCR amplification using *gusA* primers including plasmid DNA (positive control) and non–transformed DNA (negative control). The primer sequences were established. The forward and reverse primer 5'-ATCCACCGCTCGAGCGGTCATCAGGCAGCCTTCG sequences for the *gusA* gene amplification were 5'-CTGCGACGCTCACACCGATAC-3' and 5'-TCACCGAAGTTCATGCCAGTCCAG-3', respectively.

The PCR reaction volume was 20 µl containing 0.5 µL of each primer, PCR buffer (2µl), 10x *Taq* DNA polymerase (0.1 µl) , dNTP (4 µl) and 1 µl of genomic DNA (50 ng). The PCR amplification program was conducted as described by Yinxia (2013) under the following conditions: predenature at 94 ^{o}C for 5 minutes, followed by 30 cycles denaturation at 94 ^{o}C for 40 seconds; annealing at 60 ^{o}C for 30 seconds and extension at 72 ^{o}C for 1 minute with a final extension at 72 ^{o}C for 5 minutes. The amplified PCR products were analyzed using 1.5 % agarose gel electrophoresis, stained with ethidium bromide and visualized by gel documentation at 260 nm of UV.

Statistical analysis

The experiment was conducted using a completely randomized design (CRD) and the data were tested in a one–way analysis of variance (ANOVA) with the significant differences among means separated by Duncan's multiple range test (DMRT) ($p \leq 0.01$) using the R statistical package version 2.14.

Results and Discussion

The effect of concentrations of glyphosate on the calluses

An experiment was conducted to establish the effect of different concentrations of glyphosate on untransformed calluses in order to determine the concentration to be used to test the transformed calluses. The results are shown on Figure 12 and 13. The different responses of calluses to various concentrations of glyphosate were noted. Increasing concentrations of glyphosate induced browning, necrosis, a slowing of growth and eventually lead to death. The highest rate of survival of calluses (100%) was observed in the control treatment (0 mM glyphosate) whereas, there were not any survival calluses at 1 mM glyphosate.

The lowest survival rate of calluses (25.04 ± 3.13%) was obtained at 0.6 mM of glyphosate with 75 % of the calluses unable to grow and dead after 3 weeks of culturing. Thus, a culture medium containing 0.6 mM glyphosate was chosen to test the effect of the gene transformation of Sangyod rice as lower concentration to inhibit effective callus growth. It has previously been reported that 0.5 mM was selected as the optimum concentration of glyphosphate necessary to inhibit the growth of the rice, cv. *Hom Kra Dang Ngah* in gene transformation experiments (Yinxia and Te-chato, 2013). In addition, 0.5 mM glyphosate was also used for *Agrobacterium* mediated gene transformation in rubber tree (Kalawong *et al*., 2014).

Calluses derived from mature seeds have been widely used as explant sources for targeting by *Agrobacterium*-mediated and biolistic transformation in rice (Hiei *et al*., 1997; Kumar *et al*., 2005). Several studies have reported the positive influence of callus age on *gus* activity (Liu *et al*., 2005; Yinxia and Te-chato, 2013). Yinxia and Te-chato (2012) also suggested that 6 week-old calluses are the most suitable for infection by *Agrobacterium* because the calluses can divide vigorously leading to the production of mitogenetic cells which promote T-DNA to combine with genomic DNA. The breaking of calluses after six weeks may cause browning and the production of phenolics which decrease the frequency of somatic embryogenesis and callus proliferation (Kumar *et al*., 2005). Thus, 6 week-old calluses were used in the

experiments described as explant source for gene transformation mediated by *A. tumefaciens* in Sangyod rice.

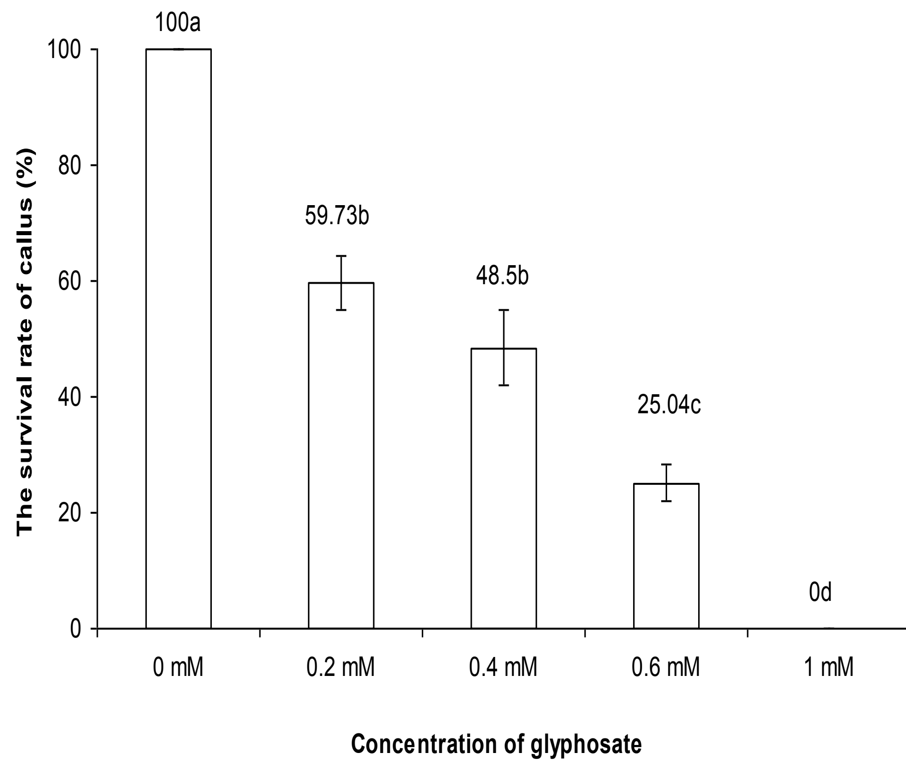

Figure 12 The effect of concentrations of glyphosate on the survival rate of callus (%) in Sangyod rice after 3 weeks of culture

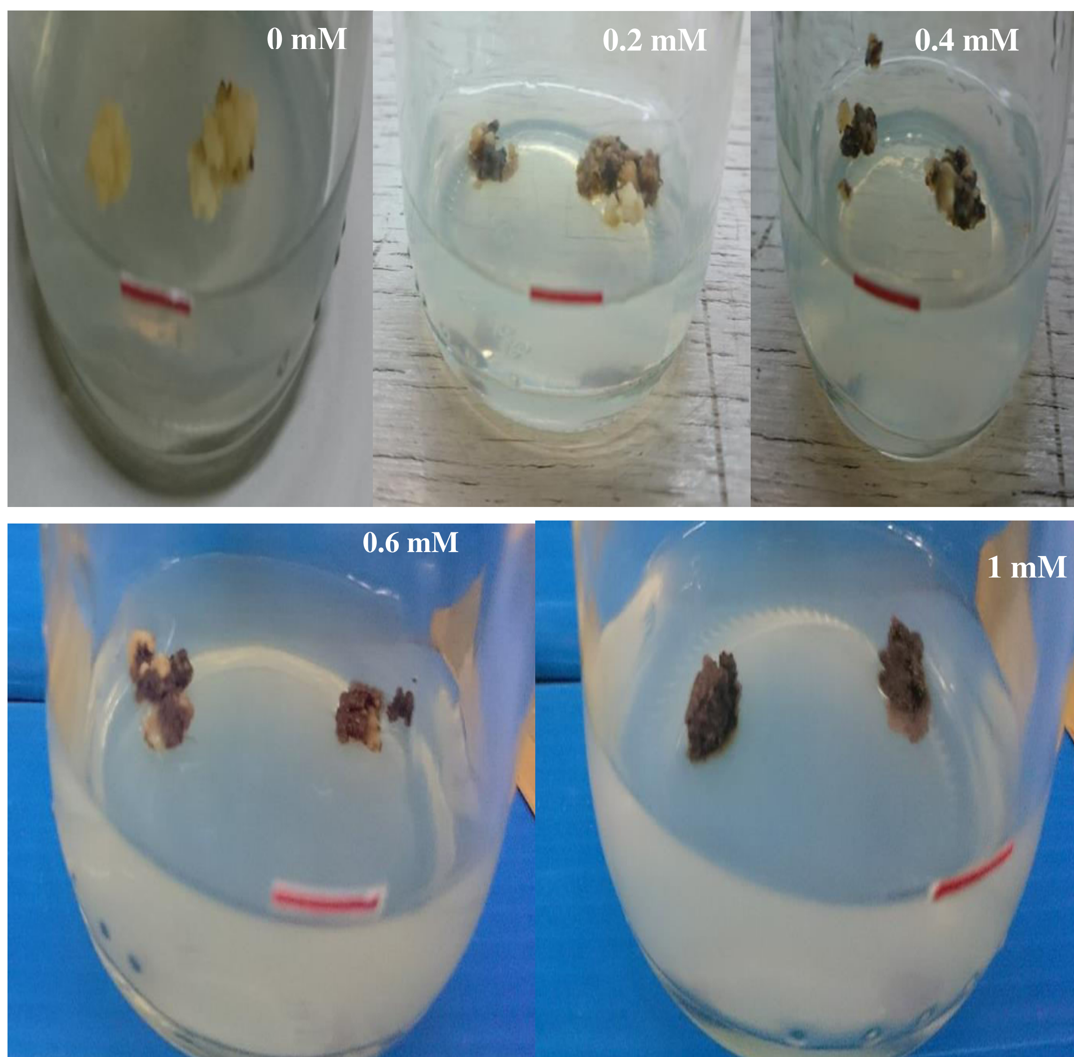

Figure 13 Growth status of callus of *indica* rice cv. Sangyod on ARDA medium suplemented with different concentrations of glyphosate (0, 0.2, 0.4, 0.6 and 1 mM) from left –right, respectively after 3 weeks (bar =1 cm)

Effect of bacteria density (OD_{600}) and inoculation time on transformation efficiency

In the present experiment, the transformation efficiency was tested for various bacteria densities (OD = 0.4, 0.6, 0.8) and inoculation times (15, 30 and 45 minutes). The transient GUS expression percentage was recorded after calluses were transferred to selection medium for 1 week. The results are shown in Table 9 and 10. There were significant differences in the effect on the percentage of GUS expression when the

calluses were immersed in *Agrobacterium* suspension with various bacterial cell densities (OD_{600}) and inoculation times. The highest transformation efficiency with a transient GUS expression of 66.67 ± 4.55% was observed at an OD of 0.6 for 30 minutes. This is similar to the result of Yinxia and Te-chato (2013) who reported that an OD of *Agrobacterium* of 0.6 for 20 minutes gave the greatest response of GUS expression (83.5%) in the rice cv *Hom Kra Dang Ngah*. It has also been reported that the maximum GUS activity in calluses (99.05%) was observed at an OD of 0.4 for 10 minutes with effective *Agrobacterium*-mediated transformation in the rice, cv.IR36. the effective of *Agrobacterium* transformation is impacted by many factors such as the co–cultivation temperature, inoculation methods, bacterial cell density and the inoculation period.

However, the bacterial cell density and inoculation period are the two key factors affecting the success of *Agrobacterium*–mediated transformation. An optical density of 600 nm in a range of 0.3 -1 has been reported as reasonable for transformation (Aananthi *et al*., 2010). Bacterial density has an impact on the early stage of transformation when bacterial infection is not inhibited by an overgrowth of *Agrobacterium*. Moreover, the efficient transfer of T-DNA to plant cells depends on the inoculation period. If the inoculation period is too long this may negatively influence the survival rate of calluses.

A slight decline in transformation efficiency was observed with an increase in bacterial density and inoculation time. The maximum percentage of callus survival (35.41 ± 7.46%) was found at an OD of *Agrobacterium* at 0.6 and an inoculation time of 30 minutes whereas the lowest callus survival rate (8.67 ± 3.67 %) was observed at an OD of 0.4 and an inoculation time of 15 minutes, after 3 weeks of transformation. Increasing the inoculation time leads to an overgrowth of *Agrobacterium*, which may cause hypertonic conditions, decreasing the frequency of transformation and could also be lethal to calluses (Rao *et al*., 2009). GUS expression was not found in any non-transformed calluses (data not shown). The use of GUS histochemical assay as a means of detecting transformation is a simple and rapid method in comparison to other forms of assay. It is often used to assess both stable and transient effective transformation by *Agrobacterium* by measuring either the quantity or quality of GUS expression and the frequency of GUS explants (Wydro *et al*., 2006). GUS histo-

chemical assay does not confuse the influences of chromosomal sequences contiguous to the sites of integration and almost all of the transferred DNA remains extra-chromosomal leading to a low frequency of false positive readings (Dekeyser *et al*., 1990; Werr and Lorz, 1986).

Some previous reports have revealed that a high optical density of *Agrobacterium* resulted in poor transient GUS expression (Yasmin and Debener, 2010) because the explants can be almost entirely colonized by the *Agrobacterium* and the elimination stage will thus become more difficult (Jha *et al*., 2011). Transformation with *Agrobacterium* produces a dispersed light blue stain over almost the whole transformed callus (Figure 14). The callus surface will then show bright dark blue spots. A dark blue stain over the whole callus indicates full transformation whereas a partial dark blue callus indicates the chimerical nature of the transformants.

Table 9 Effect of different bacteria densities (OD_{600}) and inoculation times on the efficiency of GUS expression after 1 week of transformation

Bacteria density	Gus expression (%) inoculation time			Mean bacteria density
	15 min	30 min	45 min	
OD_{600} = 0.4	8.33 ± 4.17^{cd}	12.5 ± 7.22^{cd}	37.45 ± 14.43^{abc}	19.43 ± 8.61^{B}
OD_{600} = 0.6	29.14 ± 11.02^{bcd}	66.67 ± 4.55^{a}	52.38 ± 7.59^{ab}	49.40 ± 7.72^{A}
OD_{600} =0.8	25 ± 6.87^{bcd}	62.28 ± 5.81^{a}	45.87 ± 8.33^{ab}	44.41 ± 7.0^{A}
Mean inoculation time	20.82 ± 7.35^{B}	47.15 ± 5.86^{A}	45.23 ± 10.12^{A}	**

**Significant difference at $p \leq 0.01$ level

Values are means of 3 replicates ± SD. Means followed by different letters within a column are significantly different ($p \leq 0.01$) by DMRT.

Means followed by capital letters are significantly different among factors and among treatment ($p \leq 0.01$) by DMRT.

Table 10 Effect of different bacteria densities (OD_{600}) and inoculation times on the survival rate of callus after 3 weeks of transformation

Bacteria density	Callus survival (%) inoculation times			Mean bacteria density
	15 min	30 min	45 min	
OD_{600} = 0.4	8.67 ± 3.67^{c}	10.46 ± 6.55^{c}	18.4 ± 4.12^{abc}	12.51 ± 4.78^{B}
OD_{600} = 0.6	14.52 ± 7.84^{bc}	35.41 ± 7.46^{a}	20.08 ± 5.97^{abc}	23.34 ± 7.09^{A}
OD_{600} = 0.8	18.34 ± 8.16^{abc}	26.97 ± 6.04^{ab}	17.75 ± 5.23^{abc}	21.02 ± 6.48^{A}
Mean inoculation time	13.84 ± 6.56^{B}	24.28 ± 6.68^{A}	18.74 ± 5.11^{AB}	**

** Significant difference at $p \leq 0.01$ level

Values are means of 3 replicates ± SD. Means followed by different letters within a column are significantly different ($p \leq 0.01$) by DMRT.

Means followed by capital letters are significantly different among factors and among treatment ($p \leq 0.01$) by DMRT.

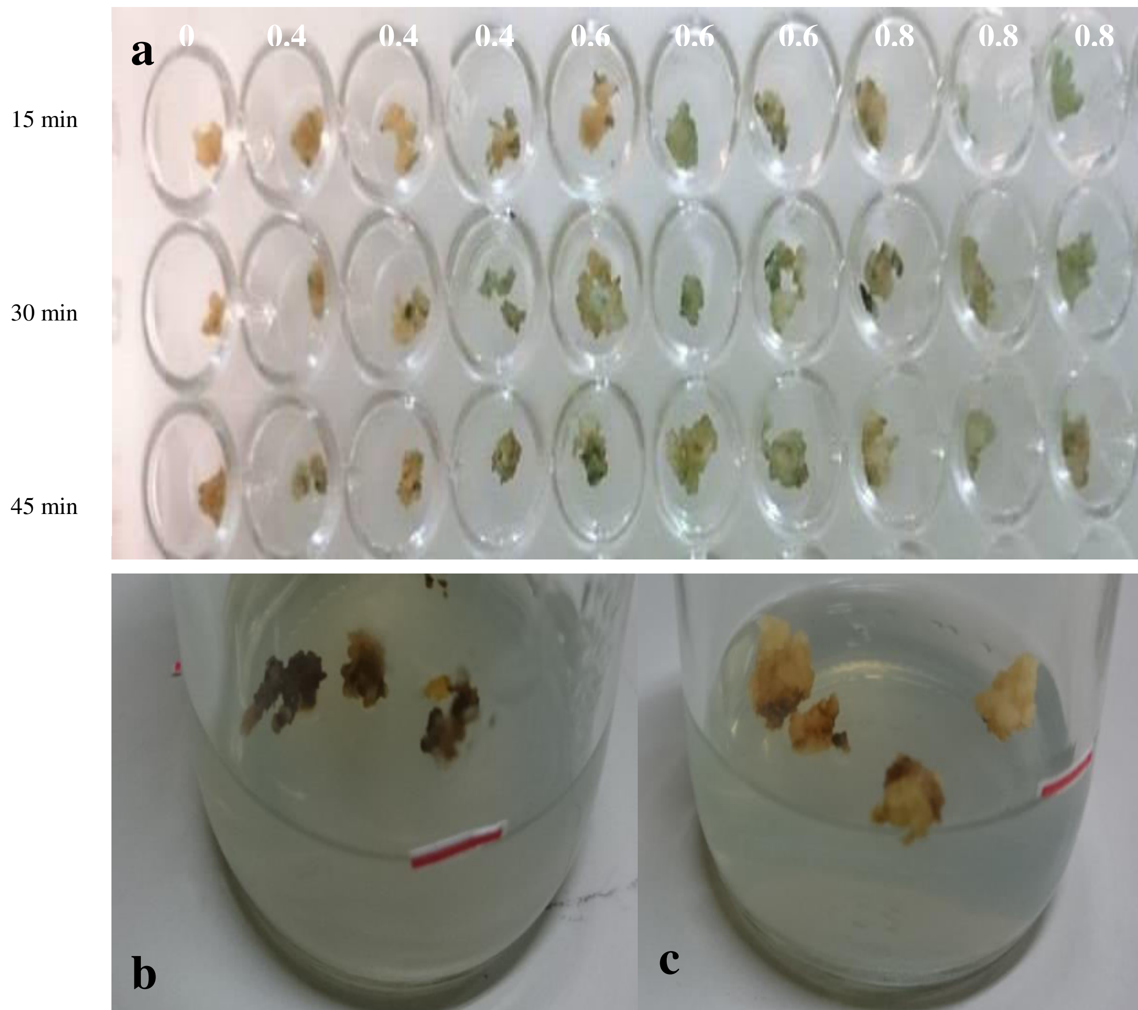

Figure 14 GUS histochemical assay and transformed- callus of *indica* rice cv. Sangyod at various OD and inoculation time.

(a): GUS expression non-transformed callus and transformed callus with various OD (0, 0.4, 0.6 and 0.8) and different inoculation time (15, 30 and 45 min) respectively after 1 week selection (left -right)

(b): non -transformed- callus after 3 weeks selection (bar =1 cm)

(c): transformed- callus at OD=0.6 and inoculation time for 30 min after 3 weeks selection (bar =1 cm)

The effect of different concentrations of acetosyringone on transformation efficiency

The presence of acetosyringone (AS) is considered to be an important factor affecting the success of the transformation protocol as compared to other factors (Mohanty *et al*., 1999; Gao *et al*., 2009). An experiment was therefore conducted to find the optimum concentration of AS in the co–cultivation stage and different concentrations of AS (0, 100, 150, 200 and 250 μM) were tested separately. The results are shown in Figure 15 and 16, the analysis revealed that there were significant differences in transient GUS expression and the survival rate of calluses based on the concentration of AS. The maximum GUS expression (70.43 ± 5.26 %) were observed at 150 μM AS whereas, the highest callus survival rate (41.02 ± 5.58%) were obtained at 200 μM AS.

The absence of AS produced the lowest GUS expression and callus transformation efficiency at 9.38 ± 5.98 % and 6.14 ± 4.80 % respectively. Based on this result, 200 μM AS was considered to be the optimum concentration for transformation in Sangyod rice with the maximum survival rate of callus. In a previous study, a concentration of AS (200 μM) was concluded to be the most efficient for transformation in the rice, cv. *Hom Kra Dang Ngah* (Yinxia and Te-chato, 2013). In addition, previous studies have reported that a concentration of AS of 100 μM was suitable for transformation in the rice varieties, cv. ADT 43 and IR 36 (Karthikeyan *et al*., 2012; Krishnan *et al*., 2013).

Thus, each genotype requires a suitable concentration of AS to promote gene transformation. Antioxidants, phytohormones and phenolic compounds can enhance the optimum *Agrobacterium*-mediated transformation. AS is one of the phenolic compounds that plays a vital role in the induction of virulence in genes and has been found to be essential for transformation in other rice varieties and other *Agrobacterium* strains (Godwin *et al*., 1992; Hiei *et al*., 1994; Tyagi *et al*., 2007). The T-DNA transfer system initiates when *Agrobacterium* detects certain phenolic compounds from wounded plant cells. The absence of AS results in a low efficiency transformation (Hoque *et al*., 2005).

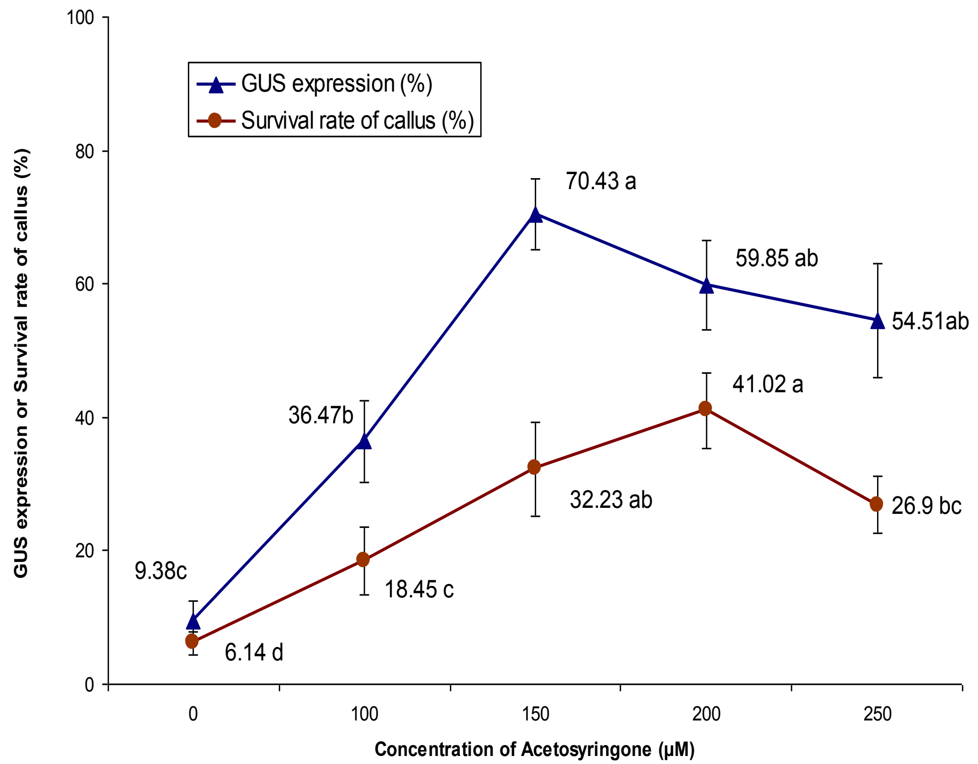

Figure 15 Effect of different concentrations of acetosyringone on transient GUS expression (%) and survival rate of callus (%)

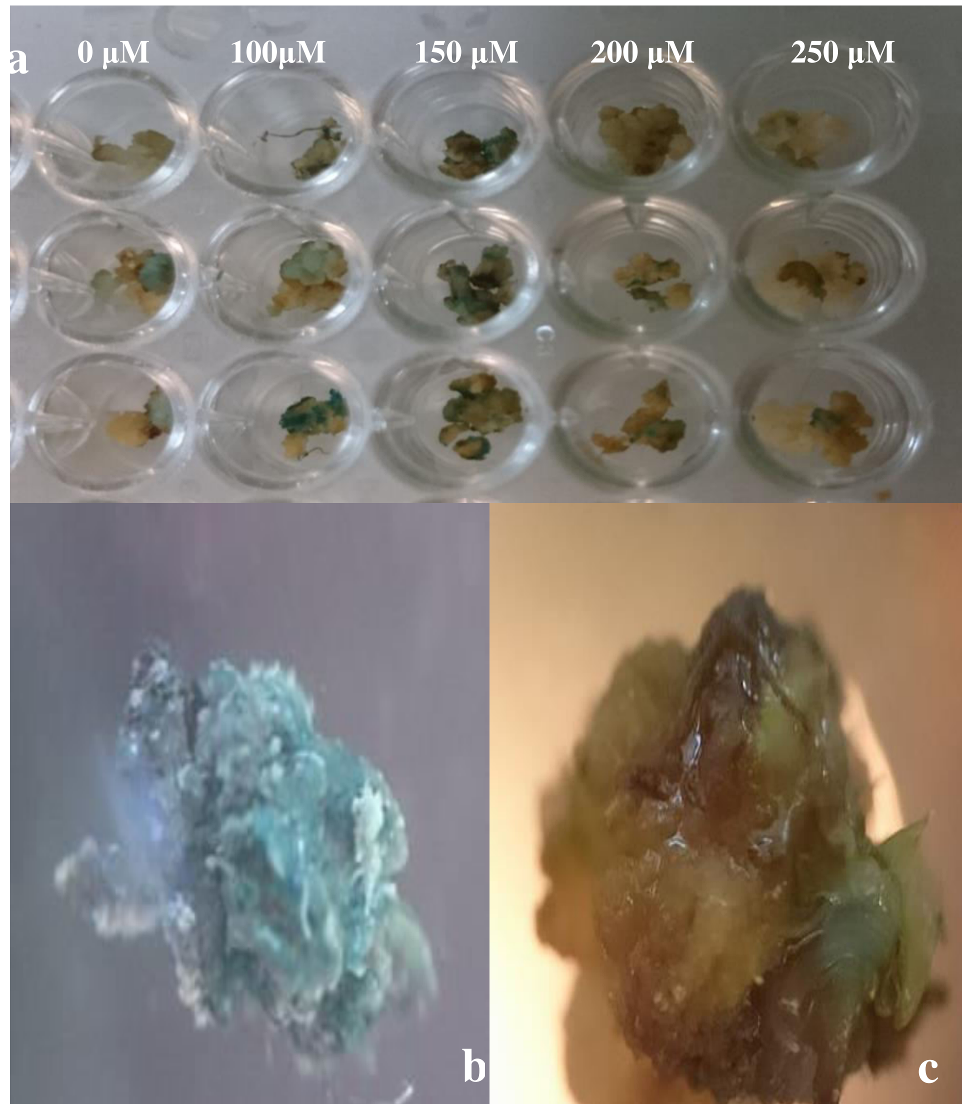

Figure 16 GUS histochemical assay and morphological of transformed- callus of *indica* rice cv. Sangyod.

(a): transformed callus at different concentration of acetosyringone (0, 100, 150, 200 and 250 µM) after 1 week selection (left – right)

(b): GUS expression of transformed transgenic callus with entire blue zone at AS = 150 µM under microscope (40X) after 1 week selection

(c): morphological of transformed callus under microscope (40X) after 3 weeks selection

Effect of different concentrations of antibiotic (cefotaxime) on transformation efficiency

The ability of the antibiotic, cefotaxime to inhibit the growth of *A. tumefaciens* was evaluated during the transformation of Sangyod rice. Different concentrations of cefotaxime (50, 100, 200, 300, 400 and 500 mg/L) were added to the washing liquidified medium and the solidified selection medium. Based on the results shown in Figure 17 and 18, the maximum callus survival rate (43.5%) were observed at 300 mg/L cefotaxime whereas, 50 mg /L of cefotaxime was found to produce the lowest callus survival rate (3.58%). The rate of callus browning were noted at concentrations of above 300 mg/L of cefotaxime. Overgrowth of *Agrobacterium* occurred on lower concentration cefotaxime (under 300 mg/L) after 2 week. Thus, the optimum concentration of cefotaxime for the elimination *Agrobacterium* was 300 mg/ L. Several antibiotics have commonly been used for the effective elimination of *Agrobacterium* which is necessary for the efficient recovery of transgenic calluses. These include carbenicillin, cefotaxime and timentin.

Cefotaxime is a cephalosporin antibiotic with a broad spectrum of activity against a wide variety of gram-negative bacteria, effective at low doses and is toxic to eukaryotes (Mathias and Boyd, 1986). Several previous studies have reported that it was necessary to eliminate excess *Agrobacterium* in calluses after transformation to decrease their negative influence on growth and regeneration and to inhibit the release of genetically modified microorganisms (Matzk *et al*., 1996). The impact of cefotaxime is similar to that of polyamines which promote the growth of transgenic calluses (Bais *et al*., 1999). The positive influence of cefotaxime on callus growth and *Agrobacterium*-mediated transformation has been reported in the rice, cv. IR 64 (Mohana *et al.,* 2012).

A common phenomenon which develops after co-cultivation during transformation is necrosis of calluses (Ozawa, 2009), by which the transformation efficiency may be limited. This problem can be solved by adding 100 mg/L myo–inositol in the co–cultivation medium and selection medium to induce and promote callus recovery.

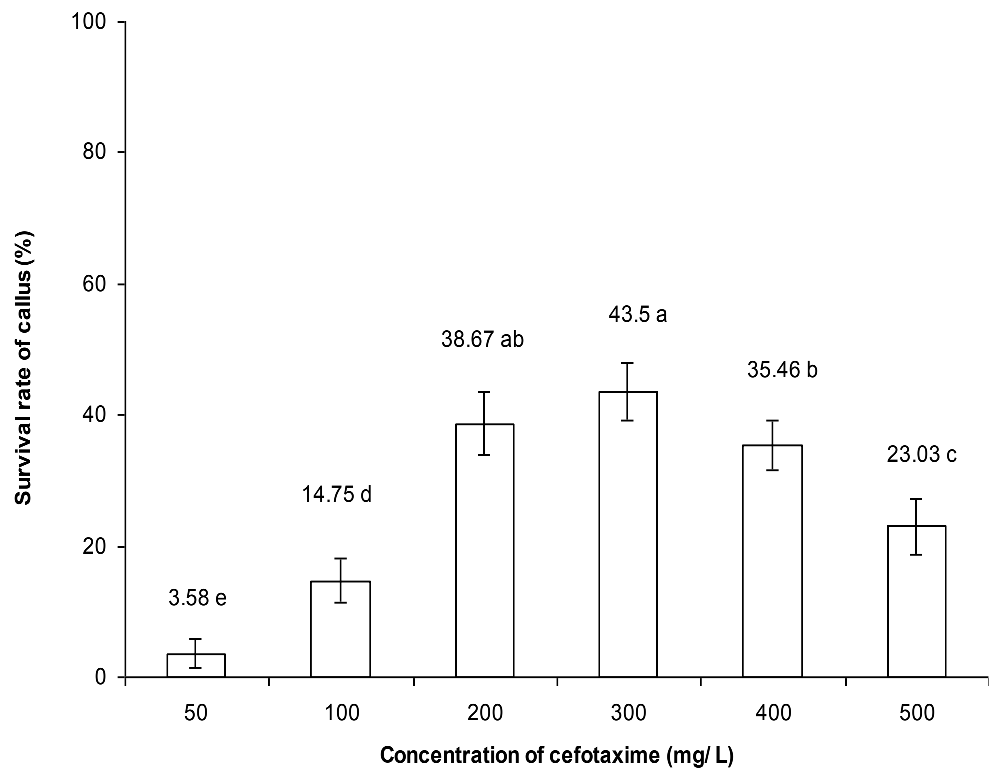

Figure 17 Effect of different concentrations of cefotaxime on the efficiency of survival rate of callus (%) after 3 weeks transformation.

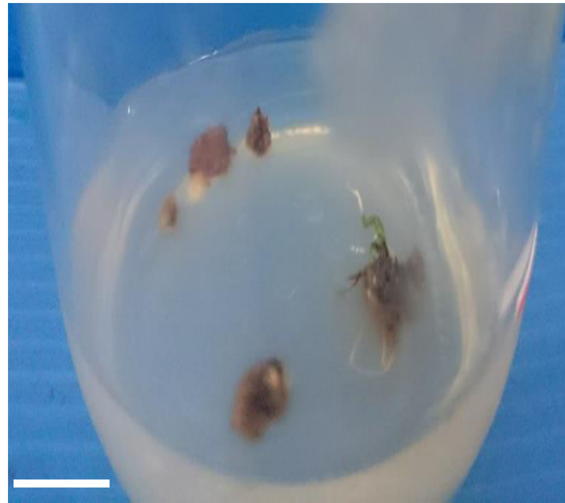

Figure 18 Induction of transformed shoot on selective medium ARDA supplemented with 100 mg/ L myo-inositol, 300 mg/ L cefotaxime and 0.6 m M glyphosate after 8 weeks (bar =1 cm)

Polymerase chain reaction (PCR) analysis

PCR analysis confirmed at a molecular level that calluses with the GUS gene had been transformed. The presence of *GUS* genes was established in eight transformed calluses and was absent in one non–transformed callus used as a control. DNA samples were amplified with GUS primers. All eight putative transformed calluses were found to contain the transgene with the expected band size of 495 bp, whereas the non–transformed callus did not produce the band size of 495 bp (Figure 19). Based on these results it was confirmed that *A.tumefaciens* was able to transfer T-DNA into calluses of Sangyod rice and integrate the transgene into the genomic DNA.

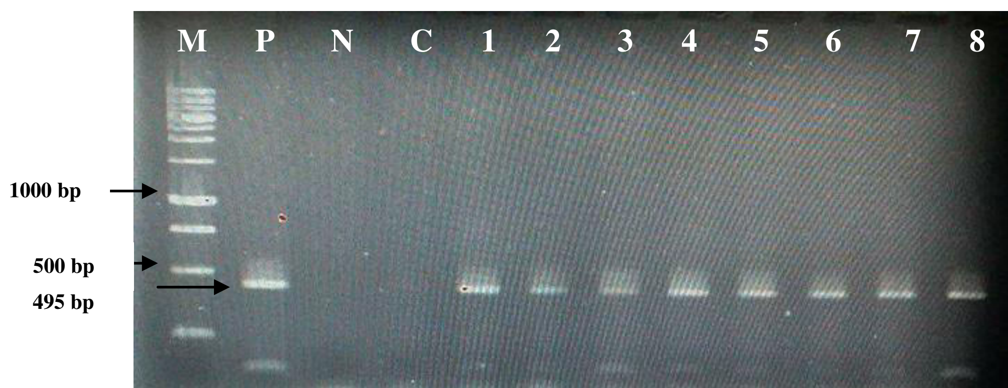

Figure 19 PCR analysis of genomic DNA of the putative transgenic callus to detect the presence of the GUS gene. The red arrow indicates the expected PCR product of the GUS gene. Lane M: marker 1kb size, P: positive control, N: negative control, 1-8: lines of transformed callus

Conclusion

In this study, an efficient transformation system for the *indica* rice cultivar Sangyod was established using an *Agrobacterium*-mediated technique to create a transgenic rice containing the *CP4-EPSPS gene which* is known to create resistant-glyphosate. 6-week–old Sangyod. calluses derived from mature seeds were inoculated at an OD_{600} of 0.6 for 30 minutes, Then the calluses were cultured on co–cultivation medium containing ARDA medium supplemented with 100 mg/ L myo-inositol and 200 µM AS for 2 days. After co-cultivation, the explants were washed with liquid CIM1 containing 300 mg/L cefotaxime for 20 minutes. Finally, the inoculated calluses were placed on selection medium (ARDA medium containing, 300 mg/L cefotaxime, 100 mg/L myo–inositol and 0.6 mM glyphosate) which had been found in this study to have the highest efficient transformation of *indica* rice cv. Sangyod.

Based on the results, the highest transformation efficiency detected by measuring transient GUS expression (70.43%) and callus survival rate (43.5%) was achieved with the foregoing conditions. Furthermore, the stable transfer of the GUS gene were confirmed by PCR analysis.

Therefore this study found that the *A. tumefaciens* strain EHA 105 successfully transferred the binary vector pCAMBIA1304-EPSPS into callus of rice cultivar Sangyod and the methods used in this study could be applied for the transformation of this cultivar by various commercially important genes as well as being applied with other genotypes in the future.

CHAPTER 5
CONCLUDING REMARKS

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Concluding Remarks

The *indica* rice cultivar Sangyod is one of the most important commercial rice cultivars in Thailand which contains high nutrition component. However, a review of the literature was unable to trace any published research considering the improvement by tissue culture methods and gene transformation of *Sangyod* using *Agrobacterium* tumefaciens. Therefore, the present study aimed to establish protocol for callus induction and regeneration systems as well as using *Agrobacterium* tumefaciens for efficient gene transformation of *indica* rice cv. Sangyod. These results were achieved through this research including:

The first, the protocol of callus induction and plantlet regeneration system of *indica* rice cv. Sangyod were established which the highest frequency of callus induction (73.08 ± 2.65%) and mean callus fresh weight (67.5 ± 7.4 mg) were obtained on MS medium supplemented with 2 mg/L 2,4–D, 750 mg/L CH and 200 mg/L L-proline. The combination of 1 mg/L BA, 0.5 mg/L Kn and 0. 5 mg/L NAA containing solidified MS medium gave the maximum mean fresh weight of callus (938.9 ± 44 mg), the highest percentage of green spot formation (64.17 ± 7.08%), maximum shoot induction frequency (66.25 ± 6.80 %) and mean number of shoots/explant (6.12 ± 0.36 shoots). Furthermore, the greatest mean number of shoots/explant (14.93 ± 0.97 shoots) and root formation percentage (82.71 ± 3.03%) was observed in liquidified MS medium supplemented with 0.5 mg/L NAA and 1 mg/L BA.

The second, the culture medium and PGRs play a significant role in callus induction, fresh weight of callus, the callus growth index and the plantlet regeneration protocol of mature rice seeds. The highest percentage of callus induction (75.63 ± 5.28 %) and fresh weight of callus (68.05 ± 20.04 mg) were achieved on ARDA medium supplemented with 2 mg/ L 2, 4-dichlorophenoxyacetic acid, 750 mg/ L casein hydrolysate and 200 mg/ L L-proline after 4 weeks of culture. The optimum mean fresh weight of callus (1272.83 ± 48.63 mg) and the highest callus growth index

(11.73-fold) were obtained on day 35 of sub-culture. The combination of 0.5 mg/ L 6-benzylaminopurine, 0.5 mg/ L Thidiazuron, 1.0 mg/ L Kinetin and 0.5 mg/ L α-naphthaleneacetic acid gave the maximum percentage of green spot formation (72.34 ± 8.75 %), plantlet regeneration frequency (67.25 ± 6.14 %) and the mean number of plantlets/ callus (6.63 ± 0.47 plantlets).

Finally, an efficient transformation system for the *indica* rice cultivar Sangyod was established using an *Agrobacterium*-mediated technique to create a transgenic rice containing the *CP4-EPSPS gene which* is known to create resistant-glyphosate. 6-week–old Sangyod calluses derived from mature seeds were inoculated at an OD_{600} of 0.6 for 30 minutes, Then the calluses were cultured on co–cultivation medium containing ARDA medium supplemented with 100 mg/ L myo-inositol and 200 μM AS for 2 days. After co-cultivation, the explants were washed with liquid CIM1 containing 300 mg/ L cefotaxime for 20 minutes. Finally, the inoculated calluses were placed on selection medium (ARDA medium containing, 300 mg/L cefotaxime, 100 mg/L Myo–inositol and 0.6 mM glyphosate). The highest transformation efficiency detected by measuring transient GUS expression (70.43 %) and callus survival rate (43.5 %) was achieved with the foregoing conditions, the stable transfer of the GUS gene were confirmed by PCR analysis.

Base on these results of this study, the optimum conditions and improved factors affecting the efficency of tissue culture process as well as gene transformation system were obtained for *indica* rice cv. Sangyod which is one of the most important commercial rice cultivars in Thailand. Therefore, the improvement regeneration system and gene transformation protocols can help applied in transformations similar genotype involving various essential commercial genes in the future. Although this research has not yet been successful to create transgenic plants from transformed callus, the high response in transient GUS expression indicates that it might be potential in the future.

References

Aananthi, N., Anandakumar, C. R., Ushakumari, R., and Shanthi, P. 2010. Regeneration study of some indica rice cultivars followed by *Agrobacterium*-mediated transformation of highly regenerable cultivar, Pusa Basmati 1. Electronic Journal of Plant Breeding 1: 1249-1256.

Abbasi, F. M., Ahmad, H., Perveen, F., Inamullah., Sajid, M. and Brar D. S. 2010. Assesment of genomic relationship between *Oryza sativa* and *Oryza australiensis.* African Journal Biotechnology 9: 1312-1316.

Abe, T., and Futsuhara, Y. 1984. Varietal difference of plant regeneration from root callus tissues in rice. Japanese Journal of Breeding 34:147–155.

Adelina, T., Madsen, S., Olesen, A. 2001. *Agrobacterium*-mediated transgene delivery and integration into barley under a range of *in vitro* culture conditions. Plant Science 161: 871-880.

Afolabi, A. S., Oyebanji, O., Odusanya, O., Abo, M. E., Misra, M. and Ogbadu, G. H. 2008. Regeneration of plants from rice caryopsis derived callus culture of Nigerian local cv. Suakoko 8 and a nerice cv. Faro 55. African Journal of Plant Science 2: 109-112.

Ageel, S. and Elmeer, K. 2011. Effects of Casein Hydrolysates and glutamine on callus and somatic embryogenesis of date palm (*Phoenix dactylifera* L.). New York Science Journal 4: 121-125.

Alibhai, M. F. and Stallings, W. C. 2001. Closing down on glyphosate inhibition - with a new structure for drug discovery. Proceedings of the National Academy of Sciences 98: 2944-2946.

Altpeter, F., Baisakh, N., Beachy, R., Bock, R., Capell, T., Christou, P. and Fauquet, C. 2005. Particle bombardment and the genetic enhancement of crops: myths and realities. Molecular Breeding 15: 305-327.

Amarasinghe, A. A. Y. 2010. Effects of copper sulphate and cobalt chloride on in vitro performances of traditional indica rice (*Oryza sativa* L.) varieties in Sri Lanka. Journal of Agricultural Sciences 4: 132– 141.

Amoah, B. K., Wang, H., Sparks, C., Jones, H. D. 2001. Factors influencing *Agrobacterium*-mediated transient expression of *uidA* in wheat inflorescence tissue. Journal of Experimental Botany 52: 1135-1142.

Arencibia, A. D., Carmona, E. R. C., Tellez, P., Chan, M. T., Yu, S. M., Trujillo, L. E. and Oramas, P. 1998. An efficient protocol for sugarcane (*Saccharum* spp. L.)

transformation mediated by *Agrobacterium tumefaciens*. Transgenic Research **7**: 213-222.

Arokiaraj, P., Rueker, F., Obermayr, E., Shamsul Bahri, A. R., Hafsah, J., Carter, D. C., Yeang, H. Y. 2002. Expression of human serum albumin in transgenic *Hevea brasiliensis.* Journal Rubber Research 5: 157-166.

Arezki, O., Boxus, P., Kevers, C., Gaspar, T. 2001. Changes in peroxidase activity, and level of phenolic compounds during lightinduced plantlet regeneration from *Eucalyptus camaldulensis* Dehn nodes *in vitro*. Plant Growth Regulation 33: 215-219.

Ayres, N. M., and Park, W. D. 1994. Genetic transformation of rice, Critical Review Plant Science 13: 219-239.

Bais, H. P., George, J. and Ravishankar, G. A. 1999. Influence of polyamines on growth of hairy root cultures of witloof chicory (*Cichorium intybus* L. cv. Lucknow Local) and formation of coumarins. Journal of Plant Growth Regulation 18: 33–37.

Bechtold, N., Ellis, J. and Pelletier, G. 1993. In planta *Agrobacterium* mediated gene transfer by infiltration of adult *Arabidopsis thaliana* plants. Comptes rendus de l'Académie des Sciences. Série 3, Sciences de la vie 316 : 1194-1199.

Bevitori, R., Popielarska-Konieczna, M., Dos Santos, E. M., Grossi-de-Sá, M. F., and Petrofeza, S. 2014. Morpho-anatomical characterization of mature embryo-derived callus of rice (*Oryzasativa* L.) suitable for transformation. Protoplasma 251: 545-554.

Bhausaheb, P., Prashant, K. A. L. E., Bahurupe, J., Jadhav, A., Anil, K. A. L. E. and Pawar, S. 2015. Proline and glutamine improve *in vitro* callus induction and subsequent shooting in rice. Rice Science 22: 283-289.

Binns, A. N., and Thomashow, M. F. 1988. Cell biology of *Agrobacterium* infection and transformation of plants. Annual Reviews in Microbiology 42: 575-606.

Braun, A. 1954. The physiology of plant tumors. Annual Review Plant Physiology 5: 133-192.

Caboche, M. 1990. Liposome mediated transfer of nucleic acids in plant protoplasts. Physiologia Plantarum 79: 173-176.

Braun, A. C. 1958. A physiological basis for autonomous growth of the crown- gall tumor cell. Proceedings of the National Academy of Sciences USA 44: 344-349.

Caseley, J. C. and Coupland, D. 1985. Environmental and plant factors affecting glyphosate uptake, movement and activity. *In*: Grossbard, E. and Atkinson (eds), The Herbicide Glyphosate. D. Butterworth and Co., London, UK, pp. 92–123.

Century K, Reuber TL, Ratcliffe O. 2008. Regulating the regulators: the future prospects for transcription-factor-based agricultural biotechnology products. Plant Physiology 147: 20–29.

Chan, M.T., Lee, T.M. and Chang, H.H. 1992.Transformation of *indica* rice (*Oryza sativa* L.) mediated by *Agrobacterium* tumefaciens. Plant Cell Physiology 33:577-583.

Chan, L.K., See, K. S., Hoon, L. P., Lim, B. P. 2008. Effect of plant growth regulators and subculture frequency on callus culture and the establishment of *Melastoma malabathricum* cell suspension culture for the production of pigments. Biotechnology 7: 678-685.

Chandrasekhar, K., Reddy, G. M., Singh, J., Vani, K., Vijayalakshmi, M. and Kaul, T. 2014. Development of transgenic rice harbouring mutated rice 5-enolpyruvylshikimate 3-phosphate synthase (*Os-mEPSPS*) and *Allium sativum* leaf agglutinin (*ASAL*) genes conferring tolerance to herbicides and sap-sucking insects. Plant Molecular Biology Reporter 32: 1146–1157.

Chateau, S., Sangwan, R. S., Sangwan-Norreel, B. S. 2000. Competence of *Arabidopsis thaliana* genotypes and mutants for *Agrobacterium tumefaciens*-mediated gene transfer: role of phytohormones. Journal of Experimental Botany 51: 1961-1968.

Chabaud, M., Passiatore, J. E., Cannon, F., Buchanan-Wollaston, V. 1988. Parameters affecting the frequency of kanamycin-resistant alfalfa obtained by *Agrobacterium tumefaciens*-mediated transformation. Plant Cell Reports 7: 512-516.

Chabaud, M., de Carvalho-Niebel, F., Barker, D. G. 2003. Efficient transformation of *Medicago truncatula* cv. Jemalong using the hypervirulent *Agrobacterium tumefaciens* strain AGL1. Plant Cell Reports 22: 46-51.

Che Radziah, C. M. Z., Siti Nurkhalida, A. K., Zamri, Z. and Ismanizan, I. 2012. Effect of illumination, casein hydrolysate and proline on callus induction of *Oryza sativa* L. var. MR219. Malaysian Applied Biology 41: 37-41.

Chen, C. C., Tsay, H. S. and Huang, C. R. 1991. Factors affecting androgenesis in rice (*Oryza sativa* L.). *In*: Bajaj Y. P. S (eds.). Biotechnology in Agriculture and Forestry. Berlin, Germany: Springer Berlin Heidelberg, pp: 193-215.

Chen, H. and Qing, R.Z.H. 2007. Rereview of research about the effect factors on the anther culture of rice. China Agricultural Science and Technology 9: 52-56.

Cheng, M., Fry, J. E., Pang, S., Zhou, H., Hironaka, C. M., Duncan, D. R and Wan, Y. 1997. Genetic transformation of wheat mediated by *Agrobacterium* tumefaciens. Plant Physiology 115: 971-980.

Cheng, M., Lowe, B. A., Michael Spencer, T., Ye, X., and Armstrong, C. L. 2004. Invited review: factors influencing *Agrobacterium*-mediated transformation of monocotyledonous species. *In Vitro* Cellular and Developmental Biology Plant 40: 31-45.

Cheng, M., Fry, J. E., Pang, S., Zhou, H., Hironaka, C. M., Duncan, D. R. and Wan, Y. 1997. Genetic transformation of wheat mediated by *Agrobacterium tumefaciens*. Plant Physiology 115: 971-980.

Chhapekar, S., Raghavendrarao, S., Pavan, G., Ramakrishna, C., Singh, V. K. and Phanindra, M. L. 2015. Transgenic rice expressing a codon-modified synthetic *CP4-EPSPS* confers tolerance to broad-spectrum herbicide, glyphosate. Plant Cell Report 34: 721–731.

Chowdhry, C. N., Tyagi, A. K., Maheshwari, N. and Maheshwari, S. C. 1993. Effect of L-proline and L-tryptophan on somatic embryogenesis and plantlet regeneration of rice (*Oryza sativa* L. cv. Pusa 169). Plant Cell, Tissue and Organ Culture 32: 357-361.

Chu, C. C., Wang, C. C., Sun, C. S., Hsu, C., Chu, C. Y., Bin, F. Y. 1975. Establishment of an efficient medium for anther culture of rice through comparative experiment on the nitrogen sources. Scientia Sinica 18: 659-668.

Chee, P. P., Fober, K. A. and Slightom, J. L. 1989. Transformation of soybean (*Glycine max*) by infecting germinating seeds with *Agrobacterium tumefacients*. Plant Physiology 91: 1212-1218.

Clemente, T. E., LaValle, B. J., Howe, A. R., Conner-Ward, D., Rozman, R. J., Hunter, P. E., Broyles, D. L., Kasten, D. S. and Hinchee, M. A. 2000. Progeny analysis of glyphosate selected transgenic soybeans derived from *Agrobacterium-* mediated transformation. Crop Science 40: 797–803.

Clercq, J. D., Zambre, M., Van Montagu, M., Dillen, W., Angenon, G. 2002. An optimized *Agrobacterium*-mediated transformation procedure for *Phaseolus acutifolius* A. Gray. Plant Cell Reports 21: 333-340.

Coenen, C. and Lomax, T. L. 1997. Auxin-cytokinin interactions in higher plants: old problems and new tools. Trends in Plant Science 2: 351-356.

Dafny-Yelin, M. and Tzfira T. 2007. Delivery of multiple transgenes to plant cells. Plant Physiology 145: 1118-1128.

Dekeyser R.A., Claes B., Riet M.U., Rycke D., Habets M.E., Montagu M.C.V., Capla A.B. 1990. Transient gene expression in intact and organized rice tissues. The Plant Cell 2: 591–602.

Dillen, W., De Clercq, J., Kapila, J., Zambre, M., Van Montagu, M., Angenon, G. 1997. The effect of temperature on *Agrobacterium tumefaciens*-mediated gene transfer to plants. Plant Journal 12: 1459-1463.

Deng, L. H., Weng, L. S. and Xiao, G. Y. 2014. Optimization of EPSPS gene and development of double herbicide tolerant transgenic PGMS rice. Journal Agricultural Science Technology 16: 217–228.

Deo, P. C., Harding, R. H., Taylor, M., Tyagi, A. P and Becker, D. K. 2009. Somatic embryogenesis, organogenesis and plant regeneration in taro (*Colocasia esculenta* var. esculenta). Plant Cell Tissue Organ Culture 99: 61-71.

Devine, M., Duke, S. O., Fedtke, E. 1993. Physiology of herbicide action. *In:* Inhibition of Amino Acid Biosynthesis, Englewood Cliffs, NJ: PTR Prentice Hall pp. 441.

de Kathen, A. and Jacobsen, H. J. 1995. Cell competence for *Agrobacterium*-mediated DNA transfer in *Pisum sativum* L. Transgenic Research 4: 184-191.

Dixon, R. A. and Paiva, N. L. 1995. Stress-induced phenylpropanoid metabolism. Plant Cell 7: 1085-1097.

Din, A. R. J. M., Ahmad, F. I., Wagiran, A., Samad, A. A., Rahmat, Z. and Sarmidi, M. R. 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: 69-77.

Driver, J. A. and Kuniyuki, A. H. 1984. *In vitro* propagation of *Paradox walnut* root stock. Horticultural Science 19:507–509.

Duke, S. O. 1988. Glyphosate in herbicides. *In*: Kearney, P. C and Kaufman, D. D. Marcel Dekker (eds), Chemistry, Degradation, and Mode of Action. New York, USA, vol 3, pp. 1–70.

Duke, S. O. and Powles, S. B. 2008. Glyphosate: a once –in- a- century herbicide. Pest Management Science 64: 319-325.

Eapen, S. and George, L. 1990. Influence of phytohormones, carbohydrates, amino acids, growth supplements and antibiotics on somatic embryo-genesis and plant differentiation in figer millet. Plant Cell, Tissue Organ Culture 22: 87-95.

Enríquez-Obregón, G. A., Vázquez-Padrón, R. I., Prieto-Samsónov, D. L., Pérez, M. and Selman-Housein, G. 1997. Genetic transformation of sugarcane by *Agrobacterium tumefaciens* using antioxidant compounds. Biotecnologia Aplicada 14: 169-174.

Escudero, J. and Hohn, B. 1997. Transfer and integration of T-DNA without cell injury in the host plant. Plant Cell 9: 2135-2142.

Estopa, M., Marfa, V., Mele, E. and Messeguer, J. 2001. Study of different antibiotic combinations for use in the elimination of *Agrobacterium* with kanamycin selection in carnation. Plant Cell, Tissue and Organ Culture 65: 211-220.

Evans, L T. 1998. Feeding the ten billion plants and population growth. London, UK: Cambridge University Press, pp: 247.

FAO. 2002. Issues and challenges in rice technological development for sustainable food security. In Proceedings of the 20th Session of the International Rice Commission Bangkok, Thailand, 23-26 July 2002.

Feng, X., Zhao, P., Hao, J., Hu, J., Kang, D. and Wang, H. 2011. Effects of sorbitol on expression of genes involved in regeneration of upland rice (*Oryza sativa* L.). Plant Cell, Tissue and Organ Culture 106: 455-463.

Frame, B. R., Drayton, P. R., Bagnall, S. V., Lewnau, C. J., Bullock, W. P., Wilson, H. M. and Wang, K. 1994. Production of fertile transgenic maize plants by silicon carbide whisker mediated transformation. The Plant Journal 6: 941-948.

Frame, B. R., Shou, H., Chikwamba, R. K., Zhang, Z., Xiang, C., Fonger, T. M., Pegg, S. E. K., Li, B., Nettleton, D. S., Pei, D., Wang, K. 2002. *Agrobacterium tumefaciens*-mediated transformation of maize embryos using a standard binary vector system. Plant Physiology 129: 13-22.

Franz, J.E., Mao, M.K. and Sikorski, J.A. 1997. Glyphosate. A Unique Global Herbicide, American Chemical Society, Washington DC, USA, pp 653.

Gairi, A. and Rashid, A. 2004. TDZ-induced somatic embryogenesis in non-responsive caryopses of rice using a short treatment with 2, 4- D. Plant Cell, Tissue and Organ Culture 76: 29–33.

Gao, N., Shen, W., Cao, Y., Su, Y. and Shi, W. 2009. Influence of bacterial density during preculture on *Agrobacterium*-mediated transformation of tomato. Plant Cell Tissue Organ Culture 98: 321–330.

Ge, X., Chu, Z., Lin, Y. and Wang, S. 2006. A tissue culture system for different germplasms of *indica* rice. Plant Cell Reported 25: 392-402.

Gelvin, S. B. 2000. *Agrobacterium* and plant genes involved in T-DNA transfer and integration. Annual Review of Plant Physiology and Plant Molecular Biology 51: 223-256.

Gelvin, S. B. 2003. *Agrobacterium*-mediated plant transformation: the biology behind the "Gene-Jockeying" tool. Microbiology and Molecular Biology Reviews 67: 16-37.

Ghanti, S. K., Sujata, K. G., Rao, S., Udayakumar, M. and Kishor, P. K. 2009. Role of enzymes and identification of stage-specific proteins in developing somatic embryos of chickpea (*Cicer arietinum* L.). *In Vitro* Cellular and Developmental Biology Plant 45: 667 -672.

Godfray HCJ, Beddington JR, Crute IR, Haddad L, Lawrence D, Muir JF, Pretty J, Robinson S, Thomas, Godwin I.D., Ford-Lloyd B.V. and Newbury H.J. 1992. *In vitro* approaches to extending the host-range of *Agrobacterium* for plant transformation. Australian Journal of Botany 40: 751–763.

Godfray, H. C. J., Beddington, J. R., Crute, I. R., Haddad, L., Lawrence, D., Muir, J. F. and Toulmin, C. 2010. Food security: the challenge of feeding 9 billion people. Science 327: 812-818.

Godwin, I. D., Fordlloyd, B. V., and Newbury, H. J. 1992. *In vitro* approaches to extending the host-range of *Agrobacterium* for plant transformation. Australian Journal of Botany 40: 751-763.

Gordon-Kamm, W., Dilkes, B. P., Lowe, K., Hoerster, G., Sun, X., Oss, M., Church, L., Bunde, C., Farrell, J., Maddock, S., Synder, J., Ykes, L., Li, Z., Woo, Y. M., Idney, D., Larkins, B. A. 2002. Stimulation of the cell cycle and maize trans- formation by disruption of the plant retinoblastoma pathway. Proceedings of the National Academy of Sciences USA 99: 11975-11980.

Goodner, B., Hinkle, G., Gattung, S., Miller, N., Blanchard, M., Qurollo, B. and Mullin, L. 2001. Genome sequence of the plant pathogen and biotechnology agent *Agrobacterium tumefaciens* C58. Science Journal 294: 2323-2328.

Grimes, H. D. and Hodges, T. K. 1990. The inorganic NO_3: NH_4 ratio influence plant regeneration and auxin sensitivity in primary callus derived from immature embryos of indica rice (*Oryza sativa* L.). Journal of Plant Physiology 136: 362-367.

Ghorbel, R., Domínguez, A., Navarro, L., Peña, L. 2000. High efficiency genetic transformation of sour orange (*Citrus aurantium*) and production of transgenic trees containing the coat protein gene of *Citrus tristeza virus*. Tree Physiology 20: 1183-1189.

Grossbard, E., and Atkinson, D. 1985. The herbicide glyphosate. Butterworth and Co., London, UK, pp 490.

Guyer D, Tuttle A, Rouse S, Volrath S, Johnson M, Potter S, Gorlach J, Goff S, Cross land L, Ward, E. 1998. Activation of latent transgenes in Arabidopsis using hybrid transcription factor. Genetics 149: 633-639.

Hadeler, B., Scholtz, S. and Reski, R. 1995. Gelrite and agar differently influence cytokinin-sensitivity of a moss. Journal Plant Physiol 146: 369-371.

Hansen, G. and Wright, M. S. 1999. Recent advances in the transformation of plants. Trends in Plant Science 4: 226-231.

Huang, X. and Wei, Z. 2005. Successful *Agrobacterium*-mediated genetic transfor- mation of maize elite inbred lines. Plant Cell, Tissue and Organ Culture 83: 187-200.

Hashemi, F. S. G., Rafi, M. Y., Ismail, M. R., Mohamed, M. T. M., Rahim, H A., Huang, W. L., C. H. Lee and Y. R. Chen. 2012. Levels of endogenous abscisic acid and indole-3-acetic acid influence shoot organogenesis in callus cultures of rice subjected to osmotic stress. Plant Cell Tissuse Organ Culture 108: 257-263.

He, G. Y., Lazzeri, P. A. and Cannell, M. E. 2001. Fertile transgenic plants obtained from tritordeum inflorescences by tissue electroporation. Plant Cell Reports 20: 67-72.

Hetherington, P. R., Reynolds, T. L., Marshall, G. and Kirkwood, R. C. 1999. The absorption, translocation and distribution of the herbicide glyphosate in maize expressing the CP-4 transgene. Journal of Experimental Botany 50: 1567-1576.

Herath, S. P., Suzuki, T. and Hattori, K. 2005. Factors influencing *Agrobacterium* mediated genetic transformation of kenaf. Plant Cell, Tissue Organ Culture 82: 201-206.

Hiei, Y., Ohta, S., Komari, T., and Kumashiro, T. 1994. Efficient transformation of rice (*Oryza sativa* L.) mediated by *Agrobacterium* and sequence analysis of the boundaries of the T-DNA. Plant Journal 6: 271-282.

Hiei, Y., Komari, T., and Kubo, T. 1997. Transformation of rice mediated by *Agrobacterium* tumefaciens. Plant Molecular Biology 35 : 205-218.

Hiei, Y., Komari, T. 2006. Improved protocols for transformation of indica rice mediated by *Agrobacterium tumefaciens*. Plant Cell, Tissue Organ Culture 85: 271-283.

Hiei, Y. and Komari, T. 2008. *Agrobacterium*-mediated transformation of rice using immature embryos or calli induced from mature seed. Nature Protocols 3: 824-834.

Hood, E. E., Fraley, R. T., Chilton, M. D. 1987. Virulence of *Agrobacterium tumefaciens* strain A281 on legumes. Plant Physiology 83: 529-534.

Hood, E. E., Helmer, G. L., Fraley, R. T., Chilton, M. D. 1986. The hypervirulence of *Agrobacterium tumefaciens* A281 is encoded in a region of pTiBo542 outside of TDNA. Journal of Bacteriology 168: 1291-1301.

Hood, E. E., Gelvin, A. B., Melchers, L. S., Hoekema, A. 1993. New *Agrobacterium* helper plasmids for gene transfer to plants. Transgenic Research 2: 208-218.

Holford, P. and Newbury, H. J. 1992. The effects of antibiotics and their breakdown products on the *in vitro* growth of *Antirrhinum majus*. Plant Cell Reports 11: 93-96.

Hoque, M. E. and Mansfield, J. W. 2004. Effect of genotype and explant age on callus induction and subsequent plant regeneration from root-derived callus of Indica rice genotypes. Plant Cell, Tissue and Organ Culture 78: 217-223.

Hoque, M. N., John, W., Mansfield, B. M. H. 2005. *Agrobacterium*-mediated transformation of indica rice genotypes an assessment of factors affecting the transformation efficiency. Plant Cell Tissue Organ Culture 82: 45–55.

Huang, W. L., Lee, C. H. and Chen, Y. R. 2012. Levels of endogenous abscisic acid and indole-3-acetic acid influence shoot organogenesis in callus cultures of rice subjected to osmotic stress. Plant Cell, Tissue and Organ Culture 108: 257-263.

Hussain, A., Qarshi, I. A., Nazir, H. and Ullah, I. 2012. Plant tissue culture: current status and opportunities. *In:* Recent Advances in Plant *In Vitro* Culture. Intechopen, pp. 4-8.

Hussein, S., Halmi, M. I. E. and Kiong, A. L. P. 2016. Modelling the growth kinetics of callus cultures from the seedling of *Jatropha curcas* L. according to the modified Gompertz model. Journal of Biochemistry, Microbiology and Biotechnology 4: 20-23.

Iglesias, V. A., Moscone, E. A., Papp, I., Neuhuber, F., Michalowski, S., Phelan, T. and Matzke, A. J. 1997. Molecular and cytogenetic analyses of stably and unstably expressed transgene loci in tobacco. The Plant Cell 9: 1251-1264.

Inoue, M. and Maeda, E. 1982. Control of organ formation in rice callus using two-step culture method. *In* Plant Tissue Culture Proceeding, 5th International Congress of Plant Tissue and Cell Culture, Tokyo and Lake Yamanake, Japan, July 11-16.

Ishida, Y., Spiker, S., Saito, H., Ohta, S., Hiei, Y., Komari, T. and Kumashiro, T. 1996. High efficiency transformation of maize (*Zea mays* L.) mediated by *Agrobacterium tumefaciens*. Nature Biotechnology 14: 745-750.

Islam, M. M., Haque, M. E., Islam, M. A., Sikdar, B. and Khalekuzzaman, M. 2014. Establishment of efficient protocol for *in vitro* callus induction and regeneration system using mature embryo in elite rice (*Oryza sativa* L.) cultivars. Research in Plant Biology 4: 09-20.

Islam, M. M. and Khalekuzzaman M. 2015. Development of Transgenic Rice (*Oryza sativa* L.) Plant Using Cadmium Tolerance Gene (YCFI) through *Agrobacterium* Mediated Transformation for Phytoremediation. Asian Journal Agriculture Research 9: 139-154.

Jain, R. K., Davey, M. R., Cocking, E. C. and Wu, R. 1997. Carbohydrate and osmotic requirements for high-frequency plant regeneration from protoplast-derived colonies of indica and japonica rice varieties. Journal of Experimental Botany 48: 751-758.

James, C. 2002. Global status of commercialized transgenic crops. ISAAA Briefs No. 27: Preview. The International Service for the Acquisition of Agri-biotech Applications, c/o IRRI, DAPO Box 7777, Metro Manila, Philippines.

Jaseela, F., Sumitha, V. R. and Nair, G. M. 2009. Somatic embryogenesis and plantlet regeneration in an agronomically important wild rice species *Oryza nivara*. Asian Journal of Biotechnology 1: 74-78.

Jefferson, R. 1987. Assaying chimeric genes in plants: the GUS gene fusion system. Plant Molecular Biology Reporter 5: 387-405.

Jha, P., Shashi, R. A., Agnihotri, P. K., Kulkarni, V. M. and Bhat, V. 2011. Efficient *Agrobacterium*-mediated transformation of *Pennisetum glaucum* (L.) R. Br. using shoot apices as explant source. Plant Cell, Tissue Organ Culture 107:501–512.

Joyia, F. A. and Khan, M. S. 2012. Reproducible and expedient rice regeneration system using in vitro grown plants. African Journal of Biotechnology 11: 138-44.

Joyia, F. A. and Khan, M. S. 2013. Scutellum-derived callus-based efficient and reproducible regeneration system for elite varieties of indica rice in Pakistan. International Journal Agriculture Biology 15: 27-33.

Joubert, P., Beaupere, D., Lelie`vre, P., Wadouachi, A., Sangwan, R. S., Sangwan- Norreel, B. S. 2002. Effects of phenolic compounds on *Agrobacterium vir* genes and gene transfer induction a plausible molecular mechanism of phenol binding protein activation. Plant Science 162: 733-743.

Jubair, T. A., Salma, U., Haque, N., Akter, F., Mukti, I. J., Haque, A. K. M. F. and Ali, M. R. 2008. Callus induction and regeneration of local rice (*Oryza sativa* L.) variety Topa. Asian Journal of Plant Sciences 7: 514-517.

Kalawong, S., Srichuay, W. and Te-chato, S. 2014. The effect of *Agrobacterium* densities and inoculation times on gene transformation efficiency in rubber tree. African Journal of Biotechnology 13: 2321-2329.

Karami, O. 2008. Factors affecting *Agrobacterium*-mediated transformation of plants. Transgenic Plant Journal 2: 127-137.

Karthikeyan, A., Pandian, S. K. and Ramesh, M. 2011. *Agrobacterium*-mediated transformation of leaf base derived callus tissues of popular indica rice (*Oryza sativa* L. sub sp. indica cv. ADT 43). Plant Science 181: 258-268.

Karthikeyan, A., Shilpha, J., Pandian, S.K., Ramesh, M. 2012. *Agrobacterium*-mediated transformation of indica rice cv. ADT 43. Plant Cell Tissue Organ Culture 109: 153–165.

Kaushal, L., Balachandran, S. M., Ulaganathan, K. and Shenoy, V. 2014a. Effect of media on improving anther culture response of rice (*Oryza sativa* L.). International Journal for Agriculture Innovations and Research 3: 218-224.

Kaushal, L., Sharma, R., Balachandran, S. M., Ulaganathan, K. and Shenoy, V. 2014b. Effect of cold pretreatment on improving anther culture response of rice (*Oryza sativa* L.). Journal of Experimental Biology and Agricultural Sciences 2: 233-242.

Khaleda, L. and Al-Forkan, M. 2006. Stimulatory effects of casein hydrolysate and proline in in vitro callus induction and plant regeneration from five deep water rice (*Oryza sativa* L.). Biotechnology 5: 379-384.

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

Khanna, H. K., Daggard, G. E. 2003. *Agrobacterium tumefaciens*-mediated transformation of wheat using a superbinary vector and a polyamine supplemented regeneration medium. Plant Cell Reports 21: 429-436.

Khatun, R., . Islam, S. M. S., Ara, I., Tuteja, N. and Bari, M. A. 2012. Effect of cold pretreatment and different media in improving anther culture response in rice (*Oryza sativa* L.) in Bangladesh. Indian Journal Biotechnology 11: 458-463.

Khush, G. S. 2005. What it will take to feed 5.0 billion rice consumers in 2030. Plant Molecular Biology 59: 1-6.

Kishore, G., Shah, D., Padgette, S., Dells-Cioppa, G., Gasser, C., Re, D., Hironak, C., Taylor, M., Wibbenmeyer, J., Eichholtz, D., Hayford, M., Hoffmann, N., Delannay, X., Horsch, R., Klee, H., Rogers, S., Rochester, D., Brundage, L., Sanders, P. and Fraley R.T. 1988. 5-Enolpyruvylshikimate 3-Phosphate Synthase from biochemistry to genetic engineering of glyphosate tolerance. *In*: Hedin, P. A., Menn, J. J., and

Hollingworth, R. M. (eds.), Biotechnology for Crop Protection. American Chemical Society, Series No. 379, Wahington, USA pp. 37 - 48.

Klimaszewska, K., Devantier, Y., Lachance, D., Lelu, M. A. and Charest, J.P. 1997. *Larix laricina* (tamarack) somatic embryogenesis and genetic transformation. Canadian Journal of Forest Research 27: 538-550.

Kohli, A., Twyman, R. M., Abranches, R., Wegel, E., Stoger, E. and Christou, P. 2003. Transgene integration, organization and interaction in plants. Plant Molecular Biology 52: 247-258.

Komamine, A., Kawahara, R., Matsumoto, M., Sunabori, S., Toya, T., Fujiwara, A., Tsukahara, M., Smith, J., Ito, M., Fukuda, H., Nomura, K. and Fujimura, T. 1992. Mechanisms of somatic embryogenesis in cell cultures: physiology, biochemistry, and molecular biology. *In Vitro* Cellular and Developmental Biology 28: 11–14.

Kondo, T., Hasegawa, H. and Suzuki, M. 2000. Transformation and regeneration of garlic (*Allium sativum* L.) by *Agrobacterium*-mediated gene transfer. Plant Cell Reports 19: 989-993.

Krishnan, S. R., Priya, A. M. and Ramesh, M. 2013. Rapid regeneration and ploidy stability of ‘cv IR36’indica rice (*Oryza Sativa*. L) confers efficient protocol for *in vitro* callus organogenesis and *Agrobacterium* tumefaciens mediated transformation. Botanical Studies 54: 47.

Kudirka, D. T., Colburn, S. M., Hinchee, M. A. and Wright, M. S. 1986. Interactions of *Agrobacterium tumefaciens* with soybean (*Glycine max* (L.) Merr) leaf explants in tissue culture. Canadian Journal of Genetics and Cytology 28: 808- 817.

Kumria, R., Waie, B., Rajam, M. V. 2001. Plant regeneration from transformed embryogenic callus of elite indica rice via *Agrobacterium*. Plant Cell, tissue Organ Culture 67: 63-71.

Kumar, K. K., Maruthasalam, S., Loganathan, M., Sudhakar, D. and Balasubramanian, P. 2005. An improved *Agrobacterium*-mediated transformation protocol for recalcitrant elite indica rice cultivars. Plant Molecular Biology Reporter 23: 67–73.

Kumlehn, J., Serazetdinora, L., Hensel, G., Becker, D., Loerz, H. 2006. Genetic transformation of barley (*Hordeum vulgare* L.) via infection of androgenetic pollen culture with *Agrobacterum tumefaciens*. Plant Biotechnology Journal 4: 251-258.

Kyungsoon, L., Hysung, J. and Kim, M. 2002. Optimazation of a mature embryo-based in vitro culture system for high frequency somatic embryogenic callus induction and

plant regeneration from japonica rice cultivars. Plant Cell Tissue and Organ Culture 71: 237-244.

Lavanya, S., Rosimah, N. and Faridah, Q. Z. 2012. Effects of plant growth regulators on in vitro regeneration of Malaysian Indica rice (*Oryza sativa* L.) cv. MR219 by shoot apical meristem. Asian Journal Agriculture Researchs 6: 180-7.

Latif, M. A. and Aslani, M. A. 2015. The genetic and molecular origin of natural variation for the fragrance trait in an elite Malaysian aromatic rice through quantitative trait loci mapping using SSR and gene-based markers. Gene 555: 101-107.

Lee, S. T. and Huang, W. L. 2014. Osmotic stress stimulates shoot organogenesis in callus of rice (*Oryza sativa* L.) *via* auxin signaling and carbohydrate metabolism regulation. Plant Growth Regulation 73: 193-204.

Li, L., R. Qu, A. De Kochko, C. Fauquet and R.N. Beachy. 1993. An improved rice transformation method using the biolistic method. Plant Cell Report 12: 250-255.

Li, Z. N., Fang, F., Liu, G. F., Bao, M. Z. 2007. Stable *Agrobacterium* mediated genetic transformation of London plane tree *(Platanus acerifolia* Willd.). Plant Cell Report 26: 641–650.

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

Lin, Y. J. and Zhang, Q. 2005. Optimising the tissue culture conditions for high efficiency transformation of indica rice. Plant Cell Reports 23: 540-547.

Lippincott, B. B., Whatley, M. H. and Lippincott, J. A. 1977. Tumor induction by *Agrobacterium* involves attachment of bacterium to a site on host plant cell wall. Plant Physiology 59: 388-390.

Liu, C., Moon, K., Honda, H. and Kobayashi, T. 2001. Enhanced regeneration of rice (*Oryza sativa* L.) embryogenic callus by light irradiation in growth phase. Journal of Bioscience and Bioengineering 91: 319-321.

Liu, P. and Nester, E. W. 2006. Indoleacetic acid, a product of transferred DNA, inhibits vir gene expression and growth of *Agrobacterium tumefaciens* C58. Proceedings of the National Academy of Sciences, USA 103: 4658-4662.

Lowe, B. A. and Krul, W. R. 1991. Physical, chemical, developmental, and genetic factors that modulate the *Agrobacterium-Vitis* interaction. Plant Physiology 96: 121-129.

Lu, B.R. 1999. Taxonomy of the genus *Oryza* (Poaceae): historical perspective and current status. International Rice Research Notes 24: 4-8.

Mallick, R. S., Divya, G. and Gyana, R. R. 2013. *In vitro* somatic embryogenesis of high yielding varieties of rice (*Oryza sativa* L.). African Journal of Biotechnology 12: 6113-6118.

Matzk, A., Mantell, S. and Schiemann, J. 1996. Localization of persisting *Agrobacterium* in transgenic tobacco plants. Molecular Plant Microbe Interactions 9: 373-381.

Mathias, R. J. and Boyd, L. A. 1986. Cefotaxime stimulates callus growth embryogenesis and regeneration in hexaploid bread wheat (*Triticum aestivum* L.E.M.Thell). Plant Science 46 : 217-223.

Matsuta, N. and Hirabayashi, T. 1989. Embryogenic cell lines from somatic embryos of grape (*Vitis vinifera* L.). Plant Cell Reports 7: 684-687.

Matzk, A., Mantell, S. and Schiemann, J. 1996. Localization of persisting agrobacteria in transgenic tobacco plants. Molecular Plant Microbe Interactions 9: 373-381.

McCullen, C.A., and Binns, A. N. 2006. *Agrobacterium tumefaciens* and plant cell interactions and activities required for interkingdom macromolecular transfer. Annual Review of Cell and Developmental Biology 22: 101–127.

Mcelroy, W. D., and A. Nason. 1954. Mechanism of action of micronutrient elements in enzyme systems. Annual Review of Plant Physiology 5: 1-30.

Meneses, A., Flores, D., Muñoz, M., Arrieta, G. and Espinoza, A. M. 2005. Effect of 2, 4-D, hydric stress and light on indica rice (*Oryza sativa*) somatic embryogenesis. Revista de Biología Tropical 53: 361-68.

Minyaka, E., Niemenak, N., Sangare, A. and Omokolo, D. N. 2008. Effect of $MgSO_4$ and K_2SO_4 on somatic embryo differentiation in *Theobroma cacao* L. Plant Cell Tissue and Organ Culture 94: 149 -157.

Mondal, T. K., Bhattacharya, A., Ahuja, P. S., Chand, P. K. 2001. Transgenic tea (*Camellia sinensis* (L) O. Kuntze cv. Kangra Jat) plants obtained by *Agrobacterium* mediated transformation of somatic embryos. Plant Cell Reports 20: 712-720.

Mohd Din, A. R. J., Ahmad, F. L., Wagiran, A., Samad, A. A., Rahmat, Z. and Sarmidi, M. R. 2016. Improvement of efficient in vitro regeneration potential of mature callus induced from Malaysian upland rice seed (*Oryza sativa* cv. Panderas). Saudi Journal Biology Science 23: 69-77.

Montoro, P., Teinseree, N., Rattana, W., Kongsawadworakul, P., Michaux-Ferriere, N. 2000. Effect of exogenous calcium on *Agrobacterium tumefaciens*- mediated gene transfer in *Hevea brasiliensis* (rubber tree) friable calli. Plant Cell Reports 19: 851-855.

Moghaddam, B. E., Mesbah, M. and Yavari, N. 2000. The effect of in planta TIBA and proline treatment on somatic embryogenesis of sugar beet (*Beta vulgaris* L.). Euphytica 112: 151-156.

Mohanty, A., Sarma, N.P., Tyagi, A. K. 1999. *Agrobacterium*-mediated high frequency transformation of an elite indica rice variety Pusa Basmati 1 and transmission of the transgenes to R2 progeny. Plant Science 27:137–147.

Monsanto. 2003. Safety assessment of Roundup Ready canola event GT73. http://www.monsanto.com/monsanto/content/ our pledge/roundupcanola product.pdf. html.

Murashige, T. and Skoog, F. 1962. A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiologia Plantarum 15: 473-497.

Muthukrishan, S., Liang, G. H., Trick, H. N., Grill, B. S. 2001. Pathogenesis related proteins and their genes in cereals. Plant Cell, Tissue Organ Culture 64: 93- 114.

Myint, K. K. M., Yasui, H., Takagi, M. and Matsumura. M. 2009. Virulence of long-term laboratory populations of the brown planthopper, *Nilaparvata lugens* (Stal), and *Whitebacked planthopper*, *Sogatella furcifera* (Horvath) (Homoptera: Delphacidae), on rice differential varieties. Applied Entomology Zoology 44: 149 -153.

Nam, S. H., Choi, S. P., Kang, M. Y., Koh, H. J., Kozukue, N. and Friedman, M. 2006. Antioxidative activities of bran extracts from twenty one pigmented rice cultivars. Food Chemistry 94: 613-620.

Nam, J., Matthysse, A. G. and Gelvin, S. B. 1997. Differences in susceptibility of *Arabidopsis* ecotypes to crown gall disease may result from a deficiency in T-DNA integration. Plant Cell 9: 317-333.

Nandakumar, R., Li, C., Suzanne, M. D. 2004. Factors affecting the *Agrobacterium*-mediated transient transformation of the wetland monocot, *Typha latifolia*. Plant Cell, Tissue Organ Culture 79: 31-38.

Nan, G. L., Tang, C. S., Kuehnle, A. R., Kado, C. I. 1997. *Dendrobium* orchids contain an inducer of *Agrobacterium* virulence genes. Physiological and Molecular Plant Pathology 51: 391-399.

Negrutiu, I., Shillito, R., Potrykus, I., Biasini, G. and Sala, F. 1987. Hybrid genes in the analysis of transformation conditions. Plant Molecular Biology 8:363–373.

Nelson, G. C., Rosegrant, M. W., Palazzo, A., Gray, I., Ingersoll, C., Robertson, R. and Msangi, S. 2010. Food security, farming, and climate change to 2050: Scenarios, results, policy options. International Food Policy Research Institute 172: 1-10.

Nitsch, J. P. and Nitsch, C. 1969. Haploid plants from pollen grains. Science 163: 85-87.

Oinam, G. S., and Kothari, S. L. 1993. Genotypic differences in embryogenic callus formation and plant regeneration in indica rice. International Rice Research Notes 20:11.

Owens, L. D. and Cress, D. E. 1985. Genotypic variability of soybean response to *Agrobacterium* strains harboring the Ti or Ri plasmids. Plant Physiology 77: 87-94.

Ozawa, K., Kawahigashi, H., Kayano, T. and Ohkawa, Y. 2004. Enhancement of regeneration of rice (*Oryza sativa* L.) calli by integration of the gene involved in regeneration ability of the callus. Plant Science 165:395-402.

Ozawa, K. 2009. Establishment of a high efficiency *Agrobacterium*-mediated transformation system of rice (*Oryza sativa* L.). Plant Science 176: 522–527.

Olhoft, P. M., Flagel, L. E., Donovan, C. M., Somers, D. A. 2003. Efficient soybean transformation using hygromycin B selection in the cotyledonarynode method. Planta 216: 723-735

Pawar, B., Prashant, K. A. L. E., Bahurupe, J., Jadhav, A., Anil, K. A. L. E. and Pawar, S. 2015. Proline and glutamine improve in vitro callus induction and subsequent shooting in rice. Rice Science 22: 283-289.

Parrott, D. L., Anderson, A. J. and Carman, J. G. 2002. *Agrobacterium* induces plant cell death in wheat (*Triticum estivum* L.). Physiological and Molecular Plant Pathology 60: 59-69.

Pathak, M. R. and Hamzah, R. Y. 2008. An effective method of sonication-assisted *Agrobacterium*-mediated transformation of chickpeas. Plant Cell, Tissue and Organ Culture 93: 65-71.

Pipatpanukul, T., Bunnag, S., Theerakulpisut, P., and Kosittrakul, M. 2004. Transformation of indica rice (*Oryza sativa* L.) cv. RD6 mediated by Agrobacterium tumefaciens. Songklanakarin Journal Science Technology 26: 1-13.

Poeaim, A., Poeaim, S., Poraha, R., Pongjaroenkit, S. and Pongthongkam, P. 2016. Optimization for callus induction and plant regeneration from mature seeds of Thai rice variety: Nam Roo (*Oryza sativa L.*). Bioengineering and Bioscience 4: 95-99.

Potrykus, I. 1990. Gene transfer to cereal: an assessment. Nature Biotechnology 8: 535-542.

Priya, A. M., Pandian, S. K. and Manikandan, R. 2012. The efect of dfferent atibiotics on the eimination. Czech Journal Genetics Plant Breeding 48: 120-130.

Rademacher, E., Lokerse, A., Schlereth. A., Llavata-Peris, C., Bayer, M., Kientz, M., Freire Rios, A., Borst, J., Lukowitz, W., Jürgens, G. and Weijers. D. 2012. Different auxin

response machineries control distinct cell fates in the early plant embryo. Developmental Cell Journal 22: 211-222.

Raemakers, C. J. J. M., Sofiari, E., Jacobsen, E. and Visser, R. G. F. 1997. Regeneration and transformation of *cassava*. Euphytica 96: 153–161.

Raghavendra, G., Kumaraswamy, G. K., Ramya, B., Sandesh, H. S., Yogendra, K. N., Deepak, N. and Gowda, P. H. R. 2010. Direct multiple shoot regeneration of indica rice (*Oryza sativa* L.) var.'Rasi'. Asian and Australasian Journal of Plant Science and Biotechnology 4: 71-73.

Rajesh, S., Krishnaveni, S., Sudhakar, D., Raveendran, M., Sivakumar, P., Gnanam, R., et al. 2008. *Agrobacterium*-mediated transformation of indica rice (*Oryza sativa* L.), IR64 with mung bean LEA protein gene for water-stress tolerance. American Journal of Plant Physiology. 3: 101–110.

Ramesh, M. and Gupta, A. K. 2006. Genetic transformation of embryogenic callus cultures of indica rice using particle bombardment and selection of stably transformed hygromycin resistant plants. Asian Journal Microbiology Biotechnology Environmental Science 8: 617–622.

Ramesh, M., Murugiah, V.and Gupta, A. K. 2009. Efficient *in vitro* plant regeneration via leaf base segments of indica rice (*Oryza sativa* L.)*.* Indian Journal of Experimental Biology 47: 68-74.

Rao, A. Q., Bakhsh, A., Kiani, S., Shahzad, K., Shahid, A. A., Husnain, T. and Riazuddin, S. 2009. The myth of plant transformation. Biotechnology Advances 27: 753-763.

Rashid, H., Yokoi, S., Toriyama, K., Hinata, K. 1996. Transgenic plant production mediated by *Agrobacterium* in indica rice. Plant Cell Reports 15: 727- 730.

Rashid, H., Abbasi, F. M. and Quraishi, A. 2003. Plant regeneration from seed derived callus of three varieties of Basmati rice. Plant Tissue Culture 13: 75-79.

Rashid, H., Toriyama, A., Qurashi, K., Hinta and Malik, KA. 2000. An improved method for shoot regeneration from calli of indica rice. Pakistan Journal of Biological Sciences 3: 2229-2231.

Rattana, K., Theerakulpisut, P. and Bunnag, S. 2012. The effect of plant growth regulators and organic supplements on callus induction and plant regeneration in rice (*Oryza sativa* L.). Asian Journal of Plant Sciences 11, 182-189.

Raval, M. I. H. A. L. I. and Chattoo, B. B. 1993. Role of media constituents and proline in callus growth, somatic embryogenesis and regeneration of *Oryza sativa* cv Indica. Indian Journal of Experimental Biology 31: 600-603.

Ritchie, S. W., Lui, C. N., Sellmer, J. C., Kononowicz, H., Hodges, T. K. and Gelvin, S. B. 1993. *Agrobacterium tumefaciens*-mediated expression of *gusA* in maize tissues. Transgenic Research 2: 252-265.

Rueb, S., Leneman, M., Schilperoort, R. A. and Hensgens, L. A. M. 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.

Ruff, T., Eichholtz, D., Re, D., Padgette, S. and Kishore, G. 1991. Effects of amino acid substitutions on glyphosate tolerance and activity of EPSPS. Plant Physiology 96.

Russell, D. A. and Fromm, M. E. 1997. Tissue-specific expression in transgenic maize of four endosperm promoters from maize and rice. Translational Research 6: 157–168.

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

Sales, M. G., Park, S. H., Srivatanakul, A., Smith, R. H. 2001. Temperature influence on stable T-DNA integration in plant cells. Plant Cell Reports 20: 701-705.

Santarem, E. R., Trick, H. N., Essig, J. S., Finer, J. J. 1998. Sonication-assisted *Agrobacterium*-mediated transformation of soybean immature cotyledons: optimization of transient expression. Plant Cell Reports 17: 752-759.

Sangwan, R. S., Bourgeois, Y., Brown, S., Vasseur, G., Sangwan-Norreel, B. S. 1992. Characterization of competent cells and early events of *Agrobacterium*-mediated genetic transformation in *Arabidopsis thaliana*. Planta 188: 439-456.

Sandhu, J. S., Gosal, S. S., Gill, M. S. and Dhaliwal, H. S. 1995. Micropropagation of Indica rice through proliferation of axillary shoots. Euphytica 81: 139-142.

Sarhadi, W. A., Hien,N. L., Zanjani, M., Yosofzai, W., Yoshihashi, T. and Hirata, Y. 2008. Comparative analysis for aroma and agronomic traits of native rice cultivars from central Asia. Journal Crop Science Biotechnology 11: 17-22.

Seraj, Z. I., Islam, Z., Faruque, M. O., Devi, T., Ahmed, S. 1997. Identification of the regeneration potential of embryo derived calluses from various indica rice varieties. Plant Cell Tissue Organ Culture 48: 9- 13.

Servaites, J. C., Tucci, M. A. and Geiger, D. R. 1987. Glyphosate effects on carbon assimilation, ribulose bisphosphate carboxylase activity, and metabolite levels in sugar beet leaves. Plant Physiology 85:370–374.

Shahsavari, E., Maheran, A. A., Akmar, A. S. N. and Hanafi, M. M. 2010. The effect of plant growth regulators on optimization of tissue culture system in Malaysian upland rice. African Journal of Biotechnology 9: 2089-2094.

Shou, H., Frame, B. R., Whitham, S. A. and Wang, K. 2004. Assessment of transgenic maize events produced by particle bombardment or *Agrobacterium*-mediated transformation. Molecular Breeding 13: 201-208.

Shackelford, N. J. and Chlan, C. A. 1996. Identification of antibiotics that are effective in eliminating *Agrobacterium tumefaciens*. Plant Molecular Biology Reporter 14: 50-57.

Shrawat, A. K. and Lörz, H. 2006. *Agrobacterium* mediated transformation of cereals: a promising approach crossing barriers. Plant Biotechnology Journal 4: 575-603.

Sidorov, V. A., Kasten, D., Pang, S. Z., Hajdukiewicz, P. T. J., Staub, J. M. and Nehra, N. S. 1999. Stable chloroplast transformation in potato use of green fluorescent protein as a plastid marker. Plant Journal 19: 209–216.

Siddique, A. B., Ara, I., Islam, S. S. and Tuteja, N. 2014. Effect of air desiccation and salt stress factors on in vitro regeneration of rice (*Oryza sativa* L.). Plant Signaling and Behavior 9: 209 -213.

Siddique, A. B., Ara, I., Islam, S. M. S. and Tuteja, N. 2014. Effect of air desiccation and salt stress factors on in vitro regeneration of rice (*Oryza sativa* L.). Plant Signaling and Behavior 9:12 -19.

Siehl, D. L. 1997. Inhibitors of EPSP synthase, glutamine synthase and histidine synthesis. *In:* Roe R. M., Burton, J. D and Kuhr, R. J (eds.), Herbicide Activity: Toxicology, Biochemistry and Molecular Biology. IOS Press, Amsterdam, The Netherlands, pp. 37–67.

Sikder, M. B. H., Sen, P. K., Mamun, M. A., Ali, M. R. and Rahman, S. M. 2006. In vitro regeneration of aromatic rice (*Oryza sativa* L.). International Journal of Agriculture and Biology 8: 759 -762.

Silva, T. D. 2012. Microspore embryogenesis. *In:* Ken-Ichi Sato (ed.), Embryogenesis. Intech Open Access Publisher. Available at www. Intechopen.com/books/embryogenesis/ microspore-embryogenesis. html.

Silvarajan, L., Nulit, R. and Zaman, F. Q. 2012. Effect of plant growth regulators on in vitro regeneration of Malaysian indica rice (*Oryza sativa* L.) cv. MR219 by shoot apical meristem. Asian Journal of Agriculture Research 6: 180-87.

Skoog, F. and Miller, C. O. 1957. Chemical regulation of growth and organ formation in plant tissues cultured. *In Vitro* Symposia Society Experimental Biology 11: 118-131.

Song, J. 2003. Sustaining food security. Rice science: innovations and impact for livelihood, Proceedings of the International Rice Research Conference, Beijing, China, pp: 5-7.

Srivatanakul, M., Park, S. H., Salas, M. G., Smith, R. H. 2001. Transformation para- meters enhancing T-DNA expression in kenaf (*Hibiscus cannabinus*). Journal of Plant Physiology 158: 255-260.

Stachel, S. E., Nester, E. W., Zambryski, P. C. 1986. A plant cell factor induces *Agrobacterium tumefaciens vir* gene expression. Proceedings of the National Academy of Sciences *USA* 83: 379-383.

Steinrücken, H. C. and Amrhein, N. 1980. The herbicide glyphosate is a potent inhibitor of 5-enolpyruvylshikimic acid-3-phosphate synthase. Biochemical and Biophysical Research Communications 94: 1207-1212.

Su, Y. H., Zhao, X. Y., Liu.,Y. B., Zhang, C. L., O'Neill, S. D. and Zhang, X. S. 2009. Auxin-induced WUS expression is essential for embryonic stem cell renewal during somatic embryogenesis in Arabidopsis. Plant Journal 59: 448-460.

Subhadra, R. M., Divya, G and Gyana, R. R. 2013. *In vitro* somatic embryogenesis of high yielding varieties of rice (*Oryza sativa* L.). African Journal Biotechnology 12: 6113-6118.

Summart, J., Panichajakul, S., Prathepha, P. and Thanonkeo, P. 2008. Callus induction and influence of culture condition and culture medium on growth of Thai Aromatic rice, Khao Dawk Mali 105, cell culture. World Applied Sciences Journal *5*: 246-251.

Sumner, J.B. and G.F. Somers, 1953. Chemistry and methods of enzymes. Academic Press, New York, USA, pp 166.

Suzuki, S. and Nakano, M. 2002. *Agrobacterium*-mediated production of transgenic plants of *Muscari armeniacum* Leichtl, Ex Bak. Plant Cell Reports 20: 835- 841.

Tariq, M., Ali, G., Hadi, F., Ahmad, S., Ali, N., and Shah, A. A. 2008. Callus induction and in vitro plant regeneration of rice (*Oryza sativa* L.). Pakistan Journal of Biological Sciences 11: 255-259.

Tang, H., Ren, Z. and Krczal, G. 2000. An evaluation of antibiotics for the elimination of *Agrobacterium tumefaciens* from walnut somatic embryos and for the effects on the proliferation of somatic embryos and regeneration of transgenic plants. Plant Cell Reports 19: 881-887.

Tian, W. Z. 1994. Study on enhancing the regeneration capacity of callus from indica rice. Acta Genetica Sinica 21:215–221.

Tian, Y. S., Xu, J., Peng, R. H., Xiong, A. S., Xu, H. and Zhao, W. 2013. Mutation by DNA shuffling of 5-enolpyruvylshikimate-3-phosphate synthase from *Malus domestica* for improved glyphosate resistance. Plant Biotechnology Journal 11: 829–838.

Tian, Y. S., Xu, J., Xing, X. J., Zhao, W., Fu, X. Y. and Peng, R. H. 2015. Improved glyphosate resistance of 5-enolpyruvylshikimate-3-phosphate synthase from *Vitis vinifera* in transgenic *Arabidopsis* and rice by DNA shuffling. Molecular Breeding 35:148.

Tie, W., Zhou, F., Wang, L., Xie, W., Chen, H., Li, X. and Lin, Y. 2012. Reasons for lower transformation efficiency in indica rice using *Agrobacterium tumefaciens*-mediated transformation: lessons from transformation assays and genome-wide expression profiling. Plant Molecular Biology 78: 1-18.

Trifonova, A., Sten, M., Annette, O. 2001. *Agrobacterium*-mediated transgene delivery and integration into barley under a range of *in vitro* culture condi- tions. Plant Science 161: 871-880.

Triqui, Z., Guedira, A., Chlyah, A., Souvannavong, V., Haicour , R. and Sihachakr, D. 2008. Effect of genotype, gelling agent, and auxin on the induction of somatic embryogenesis in sweet potato (*Ipomoea batatas* Lam.). Comptes Rendus Biologies 331: 198-205.

Tyagi, H., Rajasubramaniam, S. and Dasgupta, I. 2007. Regeneration and *Agrobacterium*-mediated transformation of a popular indica rice variety ADT39. Current Science 93: 678-683.

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

Urushibara, S., Tozawa, Y., Kawagishi-Kobayashi, M. and Wakasa, K. 2001. Efficient transformation of suspension-cultured rice cells mediated by *Agrobacterium tumefaciens*. Breed Science 5: 33-38.

Vega, R., N., Espinoza, A. M., Gatica, A. M. and Melara, M. V. 2009. Histology of somatic embryogenesis in rice (*Oryza sativa* cv. 5272). Revíta de Biologia Tropical 57: 141-150.

Verma, D., Joshi, R., Shukla, A. and Kumar, P. 2011. Protocol for in vitro somatic embryogenesis and regeneration of rice (*Oryza sativa* L.) Indian Journal of Experimental Biology 49: 958-963.

Veena, J. H., Doerge, R. W., Gelvin, S. B**.** 2003. Transfer of T-DNA and Vir proteins to plant cells by *Agrobacterium tumefaciens* induces expression of host genes involved in mediating transformation and suppresses host defense gene expression. Plant Journal 35: 219-226.

Watson, B., Currier, T. C., Gordon, M. P., Chilton, M. D., Nester, E. W. 1975. Plasmid required for virulence of *Agrobacterium tumefaciens*. Journal of Bacteriology 123: 255-264.

Werner, T., Motyka, V., Laucou, V., Smets, R., Van Onckelen, H. and Schmülling, T. 2003. Cytokinin-deficient transgenic Arabidopsis plants show multiple developmental alterations indicating opposite functions of cytokinins in the regulation of shoot and root meristem activity. The Plant Cell 15: 2532- 2550.

Werr, W. and Lörz, H. 1986. Transient gene expression in a gramineae cell line. Molecular and General Genetics Journal 202: 471-475.

Wetherell, D. F. and Dougall, D. K. 1976. Sources of nitrogen supporting growth and embryogenesis in cultured wild carrot tissue. Physiologia Plantarum 37: 97-103.

Villemont, E., Dubois, F., Sangwan, R. S., Vasseur, G., Bourgeois, Y., Sangwan-Norreel, B. S. 1997. Role of the host cell cycle in the *Agrobacterium*-mediated genetic transformation of Petunia: evidence of an S-phase control mechanism for T-DNA transfer. Planta 201: 160-172.

Wroblewski, T., Tomczak, A. and Michelmore, R. 2005. Optimization of *Agrobacterium* mediated transient expression assays for lettuce, tomato and Arabidopsis. Plant Biotechnology Journal 3: 259–273.

Wu, H., Sparks, C., Amoah, B. and Jones, H. D. 2003. Factors influencing successful Agrobacterium-mediated genetic transformation of wheat. Plant Cell Reports 21: 659-668.

Wu, H., McCormac, A. C., Elliott, M. C., Chen, D. F. 1998. *Agrobacterium*-mediated stable transformation of suspension cultures of barley (*Hordeum vulgare* L.). Plant Cell, Tissue Organ Culture 54: 161-167.

Wydro, M., Kozubek, E. and Lehmann, P. 2006. Optimization of transient *Agrobacterium*-mediated gene expression system in leaves of *Nicotiana benthamiana*. Acta Biochimica Polonica 53: 289.

Xa, T. T. T. and Lang, N. T. 2011. Rice breeding for high grain quality through anther culture. Omonrice 18: 68-72.

Yang, Y. S., Jian, Y. Y. and Zheng, Y. D. 1999. Copper enhances plant regeneration in callus culture of rice. Chinese Journal Rice Science 13: 95-97.

Yasmin, A. and Debener, T. 2010. Transient gene expression in rose petals via *Agrobacterium* infiltration. Plant Cell, Tissue Organ Culture 102: 245–250.

Yatazawa, M., Furuhashi, K. and Shimizu, M. 1967. Growth of callus tissue from rice-root *in vitro*. Plant and Cell Physiology 8: 363-373.

Yi, S. Y., Cui, Y., Zhao, Y., Liu, Z. D., Lin, Y. Z. and Zhou, F. 2016. A novel naturally-occurring class I 5-enolpyruvylshikimate-3-phosphate synthase from *Janibacter* sp. confers high glyphosate tolerance to rice. Science Reports 6: 191 – 204.

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

Yinxia, Z. and Te-Chato, S. 2013. Improved plantlet regeneration systems in Indica rice (*Oryza sativa* L.) landrace Hom Kra Dang Ngah. Journal of Agricultural Technology 9: 1641-1654.

Yookongkaew, N., Srivatanakul, M. and Narangajavana, J. 2007. Development of genotype-independent regeneration system for transformation of rice (*Oryza sativa* ssp. *indica*). Journal of Plant Research 120: 237-245.

Yodmanee, S. Karrila, T. T. and Pakdeechanuan, P. 2011. Physical, chemical and antioxidant properties of pigmented rice grown in Southern Thailand. International Food Research Journal 18: 901–906.

Zaidi, M. A., Narayanan, M., Sardana, R., Taga, I., Postel, S., Johns, R., McNulty, M., Mottiar, Y., Mao, J., Loit, E. and Altossar, I. 2006. Optimizing tissue culture media for efficient transformation of different indica rice genotypes. Agronomy Research 4: 563–575.

Zambryski, P. 1988. Basic processes underlying *Agrobacterium*-mediated DNA transfer to plant cells. Annual Review of Genetics 22: 1-30.

Zambre, M., Terryn, N., De Clercq, J., De Buck, S., Dillen, W., Montagu, M. V., Straeten, D. V. D., Angenon, G. 2003. Light strongly promotes gene transfer from *Agrobacterium tumefaciens* to plant cells. Planta 216: 580-586.

Zhang, S., Song, W. Y., Chen, L., Ruan, D., Taylor, N., Ronald, P. and Fauquet, C. 1998. Transgenic elite Indica rice varieties, resistant to Xanthomonas oryzae pv. oryzae. Molecular Breeding 4 : 551-558.

Zhang, J., Boone, L., Kocz, R., Zhang, C., Binns, A. N. 2000. At the maize/*Agrobacterium* interface: natural factors limiting host transformation. Chemical Biology 7: 611-621.

Zhangsun, D., Luo, Su., Chen, R., Tang, K. 2007. Improved *Agrobacterium*-me- diated genetic transformation of GNA transgenic sugarcane. *Biologia (Bratislava)* 62: 386-393.

Zhao, J., Zhou, C. and Yang, H. Y. 1999. *In vitro* development of early proembryos and plant regeneration via microculture in *Oryza sativa*. Plant Cell, Tissue Organ Culture 55: 167–174.

Zhao, Z., Cai, T., Tagliani, L., Miller, M., Wang, N., Pang, H., Rudert, M., Schroeder, S., Hondred, D., Seltzer, J., Pierce, D. 2000. *Agrobacterium*-mediated sorghum transformation. Plant Molecular Biology 44: 789-798.

Zhao, Z., Gu, W., Cai, T., Tagliani, L., Hondred, D., Bond, D., Schroeder, S., Rudert, M., Pierce, D. 2001. High throughput genetic transformation mediated by *Agrobacterium tumefaciens* in maize. Molecular Breeding 8: 323-333

Zhao, T., Lin, C. and Shen, Z. 2011. Development of transgenic glyphosate-resistant rice with G6 gene encoding 5-enolpyruvylshikimate-3-phosphate synthase. Agricultural Sciences in China 10: 1307–1312.

Zhu, J. K. 2000. Genetic analysis of plant salt tolerance using *Arabidopsis*. Plant Physiology 124: 941-948.

Zhu, Y., Nam, J., Humara, J. M., Mysore, K. S., Lee, L. Y., Cao, H. 2003. Identification of *Arabidopsis rat* mutants. Plant Physiology 132: 494-505.

Zhuo, L. S., Si, H. M., Cheng, S. H. and Sun, Z. X. 1996. Phenylacetic acid stimulation of direct shoot formation in anther and somatic tissue cultures of rice (*Oryza saliva* L.). Plant Breeding 115: 295-300.

Zuraida, A. R., Naziah, B., Zamri, Z. and Sreeramanan, S. 2011. Efficient plant regeneration of Malaysian indica rice MR 219 and 232 *via* somatic embryogenesis system. Acta Physiologiae Plantarum 33: 1913-1921.

Zuker, A., Ahroni, A., Tzfira, T., Ben-Meir, H., Vainstein, A. 1999. Wounding by bombardment yields highly efficient *Agrobacterium*-mediated transformation of carnation (*Dianthus caryophyllus* L.). Molecular Breeding 5: 367-372.

APPENDICES

Appendix A

Preparation of solution buffers and regents

1. **DNA isolation by the modified Te-chato method**

TE buffer

500 µl of 20 mM Tris –HCl (pH 8.0)

200 µl of 0.1 M EDTA (pH 8.0)

Adjust the volume to 500 ml with distilled water and sterilize using an autoclave.

10% Sodium dodecyl sulfate (SDS)

5 gram of SDS

Adjust the volume to 50 ml with distilled water and sterilize using an autoclave

5 M Amonium acetate

38.54 gram amonium acetate

Adjust the volume to 50 ml with distilled water and sterilize using millipore filter

0.5 M Ethylenediaminetetraacetic (EDTA)

37.224 gram EDTA

Add about 700 ml H_2O and add 16-18 gram of NaOH pellets for adjust pH to 8.0 by with a few more pellets, EDTA won't dissolve until the pH is near 8.0. bring total volume to 1 litter with distilled water.

1.0 M Tris- HCl (pH 8.0)

121.1 gram of Tris –HCl

Dissolve in about 700 ml of H_2O, adjust pH decrease until to 8.0 by adding concentrated HCl 1N

TE buffer

500 µl of 1.0 M Tris – HCl (pH 7.5)

200 µl of 0.25 M Na_2EDTA (pH 7.0)

Adjust the volume to 500 ml with distilled water and sterilize using an autoclave.

TAE buffer

121.1 gram Tris Base

28.5 ml Acetic acid

50 ml of 0.5M Na_2EDTA (pH 8.0)

Adjust the volume to 500 ml with distilled water and sterilize using an autoclave

To make 1X TAE buffer before using.

TBE buffer

216 gram of Tris Base

110 gram of Boric acid

80 ml of 0.5 M Na_2EDTA (pH 8.0)

Adjust the volume to 4 litters with distilled water and sterilize using an autoclave. To make 0.5X TAE buffer before using.

2. **Histochemical detection of GUS gene**

   X- Gluc solution

0.2 M $NaHPO_4$, pH 7.0	100 ml
0.2 M Na_2HPO_4 $7H_2O$	62 ml (53.614 gram)
0.2 M NaH_2PO_4 H_2O	38 ml (27.598 gram)
DI H_2O	94 ml
0.1 M $K_3[Fe(CN)_6]$ $3H_2O$	1 ml (6.586 gram)
0.1 M $K_4[Fe(CN)_6]$ $3H_2O$	1 ml (8.448 gram)
0.5 M Na_2EDTA	4 ml (93.06 gram)
200 mg X-Gluc	
Sodium phosphate buffer	50 ml
Triton- X	20 µl

Kept in refrigerator at 4^0C

Appendix B

Table A Composition of nutrion of MS, ARDA and NN medium

Components	MS	ARDA	NN
		mg/L	
NH_4NO_3	1650	1025	720
KNO_3	1900	950	950
KH_2PO_4	170	170	68
$K_2 SO_4$	-	495	-
$MgSO_4.7H_2O$	370	185	185
$CaCl_2.2 H_2O$	440	268	219
H_3BO_3	6.2	6.2	10
$MnSO_4.4 H_2O$	16.90	16.9	25
$ZnSO_4.7 H_2O$	10.60	9.6	10
$Na_2MoO_4.2 H_2O$	0.25	0.25	0.25
KI	0.83	0.415	-
$CuSO_4.5 H_2O$	0.025	3.138	0.025
$CoCl_2.6 H_2O$	0.025	0.013	-
$FeSO_4.7 H_2O$	27.8	27.8	27.80
Na_2EDTA	37.3	37.3	37.30
Thiamine-HCl	0.1	0.55	0.50
Nicotinic acid	0.5	0.5	0.50
Pyridoxine-HCl	0.5	0.5	0.50
Glycine	2.00	2.00	5.00
Myo -inositol	100	100	2.00
Biotin	-	-	0.05
Folic acid	-	-	0.50
Sucrose	30,000	30,000	30,000

Appendix B (continued)

Table B Composition of nutrion of LB medium

Component	Volume (g/ L)
Trypone	10
Yeast extract	5
NaCl	5
pH	7.0
Agar	7.0

LIST OF PAPERS

Paper 1

Improved Callus Induction and Plantlet Regeneration Systems in *Indica* Rice (*Oryza sativa* L.) Cultivar Sangyod

Improved Callus Induction and Plantlet Regeneration Systems in *Indica* Rice (*Oryza sativa* L.) Cultivar Sangyod

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Abstract

This study was conducted to determine the optimum concentrations of 2, 4 –D, L-proline and casein hydrolysate (CH), plant growth regulator for efficient callus induction and plantlet regeneration from culturing mature embryos of Sangyod, an economically important *indica* rice variety in Thailand. The highest frequency of callus induction (68.56 ± 4.35%) was obtained on MS medium supplemented with 2 mg/L 2,4–D, 750 mg/L CH and 200 mg/L L-proline. The combination of 1 mg/L BA, 0.5 mg/L Kn and 0. 5 mg/L NAA containing solidified MS medium gave the maximum mean fresh weight of callus (938.9 ± 44 mg), the highest percentage of green spot formation (64.17 ± 7.08 %), maximum shoot induction frequency (66.25 ± 6.80 %) and mean number of shoots/explant (6.12 ± 0.36 shoots). Furthermore, the greatest mean number of shoots/explant (14.93 ± 0.97 shoots) and root formation percentage (82.71 ± 3.03%) was observed in liquidified MS medium supplemented with 0.5 mg/L NAA and 1 mg/L BA.

Keywords: *Indica* rice, Sangyod, Tissue culture, Mature embryos, Proline, Casein hydrolysate.

Abbreviations: 2, 4-D = 2, 4-dichlorophenoxyacetic acid, NAA =α-naphthaleneacetic acid, Kn =Kinetin, BA = 6-benzyladenine.

1. Introduction

Rice (*Oryza sativa* L.) belongs to the family Poaceae. It is a primary staple food for a half of the world's population. It is also the most important cereal crop in Asia regions where nearly 90% of global rice production and consumption (Evans, 1998). It accounts for 23% of world's total crop area, over 20% of global human daily calories intake (Song, 2003). Gobal rice demand is

projected to rise 26% in the next 25 years and achieve nearly 555 million tons in 2035. Driven by both population growth and climate changes traditional plant breeding cannot meet the rising demand of rice production. Among the two most popular sub-species (ssp.) of rice, the *indica* group are largely grown in hot climates of the tropical, south and southeast Asia such as India, Thailand, Vietnam.

Sangyod rice is one of special rice varieties with dark-red color dehusk seed, soft and aromatic of cooked, grown in Pattalung province, Thailand for hundred years. Red rice had more minerals (iron), vitamins, bioactive compounds (anthocyanin, flavonoid, phenolic compounds) than white rice. Pigmented rice also contains the highest antioxidant activity (Nam, 2006) that helps reduction in the risk of some chronic diseases for people such as diabetes, cancer and cardiovascular syndrom.

Currently, the demand for healthier rice products are being increasing globally. Providing specially rice varieties for market will rise economic profits to farmers and nutritional benefits to consumers. Highlighting a need for the research and development of efficient mass propagation tools for Sangyod rice variety in the future is required. Crop improvement through tissue culture techiques are being widely applied for large scale plant multiplication including rice. However, *indica* varieties are generally difficult to culture and requires a longer period as compared to *japonica* varieties (Bajaij *et al*, 1995). Most of *indica* subspecies are recalcitrant to *in vitro* response due to poor callus formation and regeneration capacity (Chen et al, 1991; Ramesh *et al*, 2009).

Successful of callus induction and regeneration *in vitro* commonly depend on many factors such as genotype, types of explants, culture media, plant growth regulator (PGRs), carbon sources and culture conditions (Ge *et al,* 2006; Saharan *et al*, 2004). Genotype and culture media are the two key factors that determine the fate of *in vitro* raised culture (Lin and Zhang, 2005). In rice, plant regeneration has been obtained from different type of explants such as immature seeds (Feng *et al*, 2011), mature seeds (Khanna and Raina, 1998), anther (Hiei and Komari, 2008), leaf (Islan *et al*, 2014) and root (Xa and Lang, 2011). Node or shoot tip has less potential for callogenesis than mature seeds (Karthikeyan *et al*, 2011). In rice, mature embryos are generally applied for callus formation and efficient plant regeneration system *in vitro* cultures (Yatazawa *et al*, 1967) as the best explant source for genetic transformation in comparison with other explants.

MS medium (Murashige and Skoog, 1962) is the most commonly used for the propagation of many plant species (Rashid *et al*, 2000). The plant hormones and the nitrogen source have profound impact on the response of the initial explant (Verma *et al,* 2011). PGRs play a crucial role in deciding of the improvement pathway of plant cells in culture medium. Amino acids also offered positive impacts on rice callus growth (Hussain *et al*, 2012) particularly proline and casein hydrolysate (CH) was reported by several researchers (Chowdhury *et al*, 1993; Shahsavari *et al*, 2010). Establishment of a highly efficient plant regeneration for *indica* varieties system are prerequisite for the application of genetic transformation technology of rice for high yield and quality improvement. Hence optimization of plant regeneration protocol for desired genotype is essential. To our best knowledge, protocol for high frequency plant regeneration is still lacking in the other pigmented rice cultivars in Thailand using embryogenic callus cultures. Moreover, establish an improvement callus induction and plant regeneration system for pigmented rice cv. Sangyod have not been reported earlier.

The objectives of this study were to define the optimum concentrations of 2,4 –D, L-proline and CH for callus induction from culturing mature seeds of *indica* rice variety Sangyod and establish high plantlet regeneration protocol for applying gene transformation.

2. Materials and methods

Plant material and sterilization

Mature seeds of indica rice cultivar Sangyod were used as explant source. They were obtained from Department Plant Science, Faculty Natural Resources, Prince of Songkla University, Thailand. The seeds were dehusked, washed with running tap water for 20 minutes, then surface sterilized with 70% (v/v) ethanol for 2 minutes, followed” by immersion in 20% (v/v) Clorox (commercial bleach) containing 2-3 drops of a wetting agent“Tween-20” on orbital shaker at 100 rpm for 6 minutes. Finally, the seeds were rinsed five times with sterile distilled water in laminar air flow hood before blotted dry on autoclaved tissue paper to remove excess water. Sterile seeds were then cultured on callus induction medium.

Callus induction

Three experiments were carried out to optimize concentration of 2,4-D, casein hydrolysate (CH) and L-proline in order to find out the best medum for callus induction of Sangyod rice.

Experiment 1: Disinfested seeds were cultured on callus induction medium (CIM) which was MS basal medium supplemented with different concentrations (0 – 4 mg/L) of 2,4–D in combination with 1000 mg/ L CH and, 100 mg/L L-proline.

Experiment 2: Disinfested explants were inoculated on CIM fortified with 2,4–D at the best concentration obtained from experiment 1, 100 mg/L L-proline and various concentrations (100, 250, 500, 1000 mg/L) of CH.

Experiment 3: Finally, the influence of different concentrations of L-proline on callus induction was investigated. Disinfested seeds were cultured on CIM with the best concentration of 2,4-D from experiment 1, CH from experiment 2 and different concentrations (0, 50, 100, 200, 300 mg/ L) of L-proline.

CIM from each experiment was added with 3% (w/v) sucrose and solidified with 0.75% (w/v) agar, the pH of the culture medium were adjusted to 5.7 before autoclaving at 121 ^{0}C, 1.07 kg/ cm^{2} for 20 min, culture bottles were sealed by Parafilm. All cultures were maintained at 26 ± 2 ^{0}C in the culture room under 14 hours photoperiod with irradiance of 25 μmol/m^{2}/s provied by cool white fluorescent tubes. After four weeks of culture frequency of callus induction, morphology of callus and mean fresh weight of callus were recorded and statistically compared.

Callus proliferation and shoot formation

The calluses (0.1 gram fresh weight) obtained from the most suitable medium of previous experiments were transferred to regeneration medium (RM) which was MS supplemented with various concentrations of PGRs (NAA, BA and Kn) as shown in Table 4. The medium was supplemented with 3% (w/v) sucrose and adjusted to pH 5.7 prior to addition with 0.75% (w/v) agar and autoclaving at 121 ^{0}C, 1.07 kg/cm^{2} for 20 min. The cultures were maintained at the same conditions as mentioned in previous experiments. The calluses were sub-cultured to fresh medium with the same composition every 2 weeks for 10 weeks. At the end of culture period mean fresh weight of callus, the percentage of green spots (GS) formation, the percentage of shoots formation and mean number of shoots per callus were determined and statistically compared.

Multiple shoot formation and root induction

Shoots tip at approximately 5 mm in length were transferred to liquidified RM with different concentrations (0.5, 1.0 mg/L) of BA or Kn alone or in combination with 0.5 mg/L NAA (Table 5). All PGRs containing RM were supplemented with 30 g/ L sucrose, adjusted to pH 5.7 prior to addition with 0.75% (w/v) agar and autoclaving at 121 ^{0}C, 1.07 kg/ cm^{2} for 20 min. All cultures were incubated on rotary shaker at 100 rpm under 14 hour photoperiod in the culture room in order to optimize plantlet regeneration. Mean number of shoots per cultured shoot tip and the percentage of roots induction were recorded and statistically compared after 4 weeks of culture.

Statistical analysis

All the tissue culture experiments of callus induction, shoot proliferation, and root induction were arranged in completely randomized design (CRD) with 8 replicates per treatment. Data were tested by using one–way analysis of variance (ANOVA) and the significant differences among means were separated by Duncan's multiple range test (DMRT) (p = 0.01) using program R statistical package version 2.14.

3. Results and discussions

3.1 Effect of different concentrations of 2,4–D on callus induction

For callus induction, CIM supplemented with various concentrations of 2,4-D (0 – 4 mg/ L) in combination with 1000 mg/ L CH and 100 mg/ L L-proline gave different frequency of callus formation and callus morphology. Mature seeds can be selected as explant source for callus induction and regeneration effectively due to they are accessible all the time of year. The seeds swelled and clearly observed from the scutellum region after 10 days of culture under 16 hour photoperiod. Based on results (Table 1), the percentage of callus induction was from 17.29 to 64.38% across various concentrations of 2, 4-D after 1 month of culture. No callus initiation was obtained on the CIM without 2, 4-D (control). Our result revealed that 2,4–D in the presence of CH and L-proline stimulated callus induction of Sangyod rice similar to those reported previously (Wetherell *et al,* 1976; Raval and Chatto, 1993). The concentration of 2, 4-D higher than 2.0 mg/ L caused the decrease in callus induction frequency. Different characteristics of callus were found in different concentrations of 2,4-D. CIM containing 2.0 mg/ L 2,4-D gave the best response in callus formation (64.38%) and desired morphology of embryogenic callus as white or yellowish color with globular structure (Table 1, Fig.1b & 1c). Our results are in agreement with Shahsavari

et al (2010) who reported that the highest percentage of callus induction was obtained on MS medium supplemented with 2.0 mg/ L 2,4-D in upland rice cultivars Selasi, Kusan, Siam and Lamsan, respectively. Several researchers showed that MS medium supplemented with 2.0 mg/ L 2,4-D was better for aromatic rice KDML105, Basmati 370 (Sara Ageel and Khaled Elmeer, 2011). Appearance of non - embryogenic callus consisting of brown color, necrosis and rhizogenesis was observed on high 2,4-D containing CIM. Besides, the decrease in frequency of callus formation was obtained on high concentration of 2, 4-D (3 - 4.0 mg/ L) similar to those reports from Libin *et al* (2012) and Mohd Din *et al* (2016). The choice and distinction of embyogenic callus is essential to obtain efficiency of plantlet regeneration (Jaseela *et al*, 2009; Summart *et al*, 2008). 2,4-D was known as a strong synthetic auxin and popular applied as growth regulator in plant tissue culture. It plays critical role for successful in callus initiation and sustainment in rice (Karthikeyan *et al*, 2011). 2,4-D alone was often used for callus induction. However, several researchers reported that supplementation of 2,4-D together with CH or L-proline enhanced the response of callus formation and proliferation rather than 2,4 – D alone (Yinxia and Te-Chato, 2012; Rattana *et al*, 2012). The combination of 300 mg/ L L-proline and 400 mg/ L CH in MS medium supplemented with 2.5 mg/ L 2, 4-D and 1.0 mg/ L Kn gave the highest callus induction frequency of rice cv. BRRI dhan32 (Khaleda and Al-Forkan, 2006). Each genotype of rice requires different hormonal composition. Therefore, CIM should be modified to suit for each variety of rice. The function of 2,4–D was reported to enhanced amount of callus through increasing cell division rates. Embryogenic callus initiation could be promoted through sufficient concentration of 2,4-D (Che Radziah *et al*, 2012). Our result also revealed that the combination of 2,4-D with amino acids (CH and L-proline) could be useful for promoting a high percentage of callus induction in Sangyod rice.

Table 1 Effect of concentrations of 2,4-D on callus induction from mature seed of Sangyod rice on CIM supplemented with 1000 mg/ L CH and 100 mg/ L L-proline after 4 weeks of culture

2,4 –D (mg/ L)	Percentage of callus formation (%)	Morphology of callus
0.0	0.00 ± 0.00^{e}	
1.0	30.62 ± 2.92^{c}	White, friable
1.5	45.79 ± 3.21^{b}	Yellow, friable
2.0	64.38 ± 2.87^{a}	White- Yellowish, nodular
3.0	17.29 ± 2.20^{d}	Yellowish-brown, compact, little rhizogenic
4.0	18.91 ± 2.97^{d}	Brown, compact, little necrosis

Values are means of 8 replicates ± SD. Means followed by different letters within column are significantly different ($p \leq 0.01$) by DMRT.

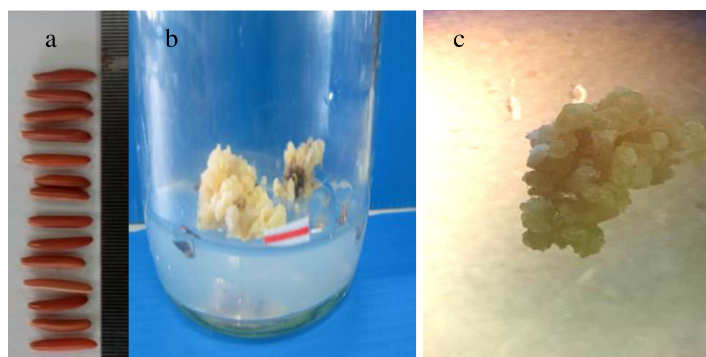

Figure 1 Morphological characteristic of callus induction of Sangyod rice on MS medium supplemented with 2 mg/ L 2,4 –D, 750 mg/L CH and 200 mg/ L L-proline after 4 weeks of culture

(a): Dehusked mature seeds of Sangyod rice

(b): Callus induction

(c): Characteristic of embryogenic callus with glubolar shape observed under microscope (X40)

3.2 Effect of different concentrations of CH on callus formation

The results in Table 2 revealed that increasing concentrations of CH promoted callus formation percentage from 25.37% (100 mg/ L) up to 68.56% (750 mg/ L) on MS medium supplemented with 2 mg/ L 2,4–D and 100 mg/ L L-proline. Callus induction frequency was significantly different ($p < 0.01$) among various concentrations of casein hydrolysate after 4 weeks of culture. The highest percentage of callus formation (68.56 ± 4.35 %) was obtained on MS medium supplemented with 750 mg/ L CH. However, the response of callus induction was slightly decreased to 61.67% when the concentration of CH was increased to 1000 mg/ L. Our results was different to those previous reports in rice cv. Selasi which found that the combination of, 300 or 600 mg/ L CH and 500 mg/ L L-proline increased callus induction percentage (Rashid *et al*, 2003). Growth of rice calluses in terms of size and quality were obtained in the presence of CH whereas the number of embryogenic calluses did not increase (Fig. 1). The result was_similar to the study reported by Raval & Chatto and Khaleda & Al-Forkan. They reported that high callus induction percentage (87%) was obtained from rice cv. HA-8 on MS medium with 2 mg/ L 2,4–D and 0.6 % (w/v) CH. Moreover, NN medium (Nitsch & Nitsch, 1969) containing 2 mg/ L 2,4–D and 300 mg/ L CH increased callus induction in rice cv. KDML 105 (Liu *et al*, 2001; Siddique *et al*, 2014). Although our results were in contrary to previous report of Che Radziah *et al* different concentrations of CH (300 -1000 mg/ L) did not show significant impact on callus formation percentage of rice cv. MR 219 (72.5 % to 97.5 %). It has been earlier studied that addition of amino acids such as L-proline, CH can stimulate callus formation and plantlet regeneration frequencies [10, 42]. CH is organic nitrogen source (Mohdin *et al*, 2016). It also provides several vitamins, micronutrient, calcium and particular a mixture of 18 amino acids and have been reported to improve callus growth in the culture medium (Afolabi *et al*, 2008). Base on this result, 750 mg/ L CH was selected due to the most suitable for induction of rice callus cv. Sangyod and this concentration was used for next experiment (Table 2).

Table 2 Effect of concentrations of CH on callus formation from mature seed of Sangyod rice on CIM supplemented with 2 mg/ L 2,4 -D and 100 mg/ L L-proline after 4 weeks of culture.

CH (mg/ L)	Frequency of callus formation (%)
100	25.37 ± 2.76^{c}
250	36.81 ± 3.63^{bc}
500	46.67 ± 1.69^{b}
750	$\mathbf{68.56 \pm 4.35^{a}}$
1000	61.67 ± 4.29^{a}

Values are means of 8 replicates ± SD. Means followed by different letters within column are significantly different ($p \leq 0.01$) by DMRT.

3.3 Effect of different concentrations of L-proline on callus formation

Based on data showed in Table 3, the percentage of callus initiation (34.38-73.08 %) and callus fresh weight (31.3-67.5 mg) were obtained on MS medium supplemented with various concentrations of proline, 2 mg/ L 2,4 – D and 750 mg/ L CH after 4 weeks of culture. Our result indicated that various concentrations of proline gave a significant effect on callus induction percentage and callus fresh weight. This finding is in approval with those earlier results reported by Chowdhury *et al* (1993), Che Radziah *et al* (2012) and Bhausaheb *et al* (2015). The absence of L-proline in culture medium (control treatment) showed the lowest frequency of callus formation and callus fresh weight at 34.38 % and 31.3 mg, respectively. The supplement of L-proline in the culture medium at 200 mg/ L yielded an optimum callus induction percentage (73.08 ± 2.65) and callus fresh weight (67.5 ± 7.4 mg) followed by 100 mg/ L (61.67 ± 3.52 and 66.1 ± 11.6 mg, respectively). The increment in concentration of L-proline up to 300 mg/ L decreased callus induction percentage (40.21 ± 2.77) and callus fresh weight (39.0 ± 3.7 mg) significantly. Similar results were also reported in callus growth of some rice varieties such as Udayagiri, Pratikhya and Khandagiri [45]. The positive effects of L-proline on the response of callus induction and regeneration have been demonstrated in rice cv. Hom Kra Dang Ngah (Yinxia and Te – Chato,

2012). However, the highest percentage callus formation (100) of cv. MR 219 was observed on MS medium supplemented with 3.0 mg/ L 2,4–D and 1000 mg/ L L-proline (Libin *et al*, 2012). In addition, NN medium supplemented with 1.5 mg/ L 2,4 –D, 300 mg/ L CH and 1000 mg/ L L-proline was reported to be the most suitable for increasing of callus formation in Supanburi 1 (Rattana *et al*, 2012) whereas the combination of 500 mg/ L CH and 500 mg/ L L-proline was suggested to have positive influence on callus formation in rice cv. HKR-46 and HKR-126 (Feng *et al*, 2011). L-proline is a type of amino acids and also known as organic nitrogen source supplied growth and development of plant cells. The addition of L-proline in the medium acts as stress condition due to the reduction of water potential, thus,enhancing the development of embryogenic callus through the accumulation of nutritional items in cells (Subhadra *et al*, 2013). Moreover, L-proline was recommended to act as an osmoticum, source of $NADP^+$ and a nitrogen storage pool, essential for rapid embryo growth (Matsuta and Hirabayashi, 1989). Raval and Chatto (1993) reported that L-proline assisted increasing embryogenic callus induction and callus growth as similar to our observation as shown in Fig. 2 The highest embryogenic callus induction percentage was also observed on MS medium supplemented with 3.0 mg/ L 2,4-D, 2.0 mg/ L, Kn and 200 mg/ L L-proline in cv. Pratikhya and Swarna (Moghaddan *et al*, 2000). L-proline could provide a readily available nitrogen source to promote callus growth (Bhausaheb *et al*, 2015). In the present study, we recommend that 200 mg/ L L-proline containing MS medium together with 2 mg/ L 2,4–D and 750 mg/ L CH was the most effective for callus induction frequency and growth in rice cv. Sangyod (Table 3).

Table 3 Effect of L-proline on callus formation from mature seed of Sangyod rice on MS medium supplemented with 2 mg/L 2,4-D and 750 mg/ L CH after 4 weeks of culture

L-Proline (mg/ L)	**Percentage of callus formation (%)**	**Mean callus fresh weight (mg)**
0	34.38 ± 2.31[b]	31.3 ± 5.4[b]
50	46.04 ± 4.82[b]	49.5 ± 8.1[ab]
100	61.67 ± 3.52[a]	66.1 ±11.6[a]
200	**73.08 ± 2.65[a]**	**67.5 ± 7.4[a]**
300	40.21 ± 2.77[b]	39.0 ± 3.7[ab]

Values are means of 8 replicates ± SD. Means followed by different letters within column are significantly different ($p \leq 0.01$) by DMRT

3.4 Effect of various concentrations of cytokinin and auxin on regeneration efficiency

The ratio of cytokinin (BA with Kn) to auxin (NAA) is essential for *in vitro* regeneration efficiency of rice cv. Sangyod. The combinations of NAA with BA and Kn are often used for plantlet regeneration in several rice varieties from embryonic callus (Ghanti *et al*, 2009). Both types of cytokinin and auxin affected cell cycles. Therefore, the ratio of cytokinin to auxin is key factor controlling many growth processes including organ regeneration from varied tissues (Rueb *et al*, 1994). Several kinds of interaction may be antagonistic, additive or synergistic (Joyia and Khan, 2013). Calluses transferred to those PGRs containing regeneration medium showed different response of development (Table 4). Green spots were observed from embryogenic callus after 8 weeks of cultured (Fig. 1). Shoot induction percentage and mean number of shoots/callus varied based on the combination of PGRs which performed 2 weeks later. Green spots were produced from callus by the process of photosynthesis of the callus when they were emerged to the light (Inoue and Maeda, 1982). In Poaceae, the appearance of green spots has been recognized as predictors of shoot induction capacity (Coenen and Lomax, 1997). Our data showed that different concentrations of PGRs gave significant influence on callus growth and shoot regeneration (Table 4). The combination of 1 mg/ L BA, 0.5 mg/ L Kn and 0. 5 mg/ L NAA gave the maximum mean fresh weight of callus (938.9 ± 44 mg), the highest percentage of green spot formation (64.17 ± 7.08), optimum shoot induction frequency (66.25 ± 6.80 %) and maximum

mean number of shoots/callus (6.12 ± 0.36 shoots) which was statistically different ($p < 0.01$) with the other concentrations of PGRs.

Decrease in callus fresh weight and shoot regeneration frequency in Sangyod rice were observed on high ratio of cytokinin (BA + Kn) to auxin (NAA) above 3:1 in Table 4. Conversely, in cv. Hom Kra Dang Ngah with high ratio of those PGRs (6:1) had the highest percentage green spot formation (75.5) and plantlets regeneraion (33.3) on MS medium supplemented with 0.5 mg/ L NAA, 1.0 mg/L BA and 2.0 mg/L Kn (Yinxia and Te-chato, 2013). Some authors reported that addition of 0.5 mg/ L NAA, 3 mg/ L BA and 0.5 mg/L Kn gave the most suitable for plantlet regeneration frequency at 80% and mean number of shoots/explant at 3.1 shoots/ callus in rice cv. Topa (Liu *et al,* 2001). Our results showed that the combination of NAA and BA or Kn alone also promoted callus growth (fresh weight) and shoot regeneration in rice cv. Sangyod. Similarly result was reported in rice cv. Super Basmati on MS medium supplemented with 1 mg/ L NAA and 3 mg/ L Kn which provided number of shoots/callus at 9.66 ± 2.0 shoots (Bhausaheb *et al*, 2015). The response of callus growth and shoots regeneration in rice cv. Sangyod *in vitro* was affected by various interactions and concentrations of PGRs (NAA, BA and Kn). Low ratio of cytokinin to auxin was suitable for this cultivar. The different responses of variety might be due to recalcitrance and genotype-dependence in *indica* rice. Therefore, it is essential to modify combination of PGRs base on different genotypes to inceasing regeneration efficiency.

Figure 2 Morphological of callus and plantlets regeneration of Sangyod rice

(a–c): Callus proliferation, geen spot formation and shoot formation on solidified MS medium supplemented with 0.5 mg/L NAA, 1 mg/L BA and 0.5 mg/L KN**.**

(d): Shoot multiplication and root formation in liquidified MS medium supplemented with 0.5 mg/L NAA and 1 mg/L BA.

Table 4. Effect of PGRs (NAA, BA, Kn) containing MS medium with 750 mg/L CH and 200 mg/L L-proline on regeneration efficiency from mature seed of Sangyod rice after 6 weeks of culture.

Concentrations of PGRs (mg/ L)			**Mean fresh weight of callus (mg)**	**Percentage of GS formation (%)**	**Shoot induction frequency (%)**	**Mean number of shoots/ callus**
NAA	**BA**	**Kn**				
0.5	2.0	0.0	526.0 ± 20.5[bc]	28.33 ± 5.74[ab]	42.50 ± 7.73[ab]	3.13 ± 0.28[b]
0.5	0.0	2.0	608.7 ± 18.2[bc]	37.50 ± 13.21[ab]	35.00 ± 9.45[b]	2.68 ± 0.65[b]
0.5	**1.0**	**0.5**	**938.9 ± 44.0[a]**	**64.17 ± 7.08[a]**	**66.25 ± 6.80[a]**	**6.12 ± 0.36[a]**
0.5	1.0	1.5	613.7 ± 16.2[bc]	22.50 ± 9.59[b]	36.25 ± 7.78[b]	2.55 ± 0.50[b]
0.5	1.0	2.0	653.0 ± 14.9[b]	16.67 ± 3.56[b]	21.25 ± 4.79[b]	3.05 ± 0.48[b]
1.0	1.5	1.0	571.7 ± 29.5[bc]	40.00 ± 6.55[ab]	38.75 ± 2.95[b]	4.03 ± 0.42[b]
1.0	1.5	2.0	563.3 ± 35.1[bc]	47.50 ± 13.83[ab]	32.50 ± 5.26[b]	3.18 ± 0.14[b]

Values are means of 8 replicates ± SD. Means followed by different letters within column are significantly different ($p \leq 0.01$) by DMRT.

3.5 Effect of different concentrations of PGRs (NAA, BA and Kn) on multiple shoot and root formation

In rice, plantlet regeneration efficiency affected by many factors such as genotype, physiological status of the explants, PGRs and culture environments (Menseses *et al*, 2005). Shoot tip is considered as good explant and obtained strongly the dividing of meristematic cells that might easily maintain *in vitro* regeneration (Jubair *et al*, 2008). A high cytokinin/auxin ratio promotes shoot induction. In contrast, a high ratio of auxin/cytokinin ratio encourages root production (Skoog and Miller, 1957). Base on results in Table 5**,** multiple shoot induction at 100% was recorded in all treatments. The greatest mean number of shoots/single shoot (14.93 ± 0.97 shoots) and root formation percentage (82.71 ± 3.03) was observed in liquidified MS medium supplemented with 0.5 mg/ L NAA and 1 mg/ L BA after 4 weeks of culture. By contrast, PGR–free MS medium gave the lowest results in all parameters. The present study showed that the combination of 0.5 mg/ L NAA and 1 mg/ L Kn gave the lower mean number of shoots/single shoot (10.53 ± 0.98 shoots) and the percentage root formation (65.75 ± 5.59) than the combination of BA with NAA. However, there was not significant difference when liquidified MS medium in the presence of both 0.5 mg/ L BA and 0.5 or 1.0 mg/ L Kn was used. Several reports showed that the most suitable PGRs for plantlet regeneration in rice cv. Chiniguri was obtained on MS medium supplemented with 0.05 mg/ L NAA and 5 mg/ L BA (Werner *et al*, 2003; Sikder *et al*, 2006). High number of multiple shoot induction of *indica* rice variety Jaya was achieved on liquidified MS medium with 5 mg/ L BAP, 1% (w/ v) mannitol and 3% sucrose (Sandhu *et al*, 1995). Similarly, in cv. MR219, liquidified MS medium containing 0.1 mg/ L Kn gave the higher shoot induction efficiency than solidified MS medium. Liquidified medium supplies good aeration and enhances the capacity for dissolved nutrient composition uptake by whole surface of explant (Lavanya *et al*, 2012). In this study, the combination of 0.5 mg/ L NAA and 1 mg/ L BA containing liquidified MS medium was the most effective for multiple shoot formation and root induction in rice cv. Sangyod.

Table 5 Effect of PGRs containing liquidified MS medium on multiple shoot and root formation from culturing single shoot-derived plantlets of rice cv. Sangyod after 4 weeks of culture

PGRs (mg/ L)			Mean number of shoots/explant	Root Induction frequency (%)
NAA	BA	Kn		
0	0	0	4.67 ± 0.40[c]	0.00 ± 0.00[c]
0.5	0.0	1.0	10.53 ± 0.98[b]	65.75 ± 5.59[ab]
0.5	**1.0**	**0.0**	**14.93 ± 0.97[a]**	**82.71 ± 3.03[a]**
0.5	0.5	0.5	9.67 ± 1.00[b]	59.52 ± 5.87[b]
0.5	0.5	1.0	11.07 ± 1.06[b]	53.63 ± 5.28[b]

Values are means of 8 replicates ± SD. Means followed by different letters within column are significantly different ($p \leq 0.01$) by DMRT.

4. Conclusion

In the present study, efficient of embryogenic callus induction and plantlet regeneration protocol for *indica* rice cv. Sangyod was established using mature seeds. The results revealed that CH, L-proline and PGRs were key factors promoting callus induction and plantlet regeneration. Improvement of callus formation and regeneration efficiency can be used for genetic engineering to create new varieties with desirable traits in the future.

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References

[1] LT Evans. *Feeding the Ten Billion: Plants and Population Growth*. Cambridge University Press, Cambridge, UK, 1998, p. 247.

[2] J Song. Sustaining food security. Rice science: innovations and impact for livelihood. *In*: Proceedings of the International Rice Research Conference, Beijing, China. 2003, p. 5-7.

[3] IRRI, AfricaRice & CIAT. Global Rice Science Partnership. 2010. Available at: http://www.africarice.org/publications/GRiSP.pdf, accessed December 2016.

[4] SH Nam, SP Choi, MY Kang, HJ Koh, N Kozukue and M Friedman. Antioxidative activities of bran extracts from twenty one pigmented rice cultivars. *Food Chem*. 2006; **94**, 613-20.

[5] S Bajaj and MV Rajan. Effcient plant regeneration from long term callus culture of rice by spermidice. *Plant Cell Rep.* 1995*;* **14***,* 717 – 20.

[6] CC Chen, HS Tsay and CR Huang. *Factors Affecting Androgenesis in Rice (Oryza sativa* L.). *In*: Bajaj YPS (ed.). Biotechnology in Agriculture and Forestry. Springer Berlin Heidelberg, 1991, p. 193-215.

[7] M Ramesh, V Murugiah and AK Gupta. Efficient *in vitro* plant regeneration via leaf base segments of indica rice (*Oryza sativa* L.). *Indian J. Exp. Biol*. 2009; **47**, 68-74.

[8] XJ Ge, ZH Chu, YJ Lin and SP Wang. A tissue culture system for different germplasms of indica rice. *Plant Cell Rep*. 2006; **25**, 392-402.

[9] V Saharan, RC Yadav, NR Yadav and BP Chapagain. High frequency plant regeneration from desiccated calli of indica rice (Oryza sativa L.). *Afr. J. Biotechnol*. 2004; **3**, 256-9.

[10] YJ Lin and QF Zhang. Optimizing the tissue culture conditions for high efficiency transformation of indica rice. *Plant Cell Rep*. 2005; **23**, 540–7.

[11] X Feng, P Zhao, J Hao, J Hu, D Kang and H Wang. Effects of sorbitol on expression of genes involved in regeneration of upland rice (*Oryza sativa* L.). *Plant Cell, Tissue Organ Cult*. 2011; **106**, 455-63.

[12] HK Khanna and SK Raina. Genotype X culture medium interaction effects on regeneration response of three indica rice cultivars. *Plant Cell, Tissue Organ Cult.* 1998; **52**, 145-53.

[13] Y Hiei and T Komari. Agrobacterium-mediated transformation of rice using immature embryos or calli induced from mature seed. *Nat Protoc*. 2008; **3**, 824-34.

[14] MM Islam, ME Haque, MA Islam, B Sikdar and M Khalekuzzaman. Establishment of an efficient protocol for in vitro callus induction and regeneration system using mature embryo in elite rice (Oryza sativa L.) cultivars. *Res Plant Biol.* 2014; **4**, 09-20.

[15] TTT Xa and NT Lang. Rice breeding for high grain quality through anther culture .*Omonrice*. 2011; **18,** 68-72.

[16] A Karthikeyan, SK Pandian and M Ramesh. Agrobacterium-mediated transformation of leaf base derived callus tissues of popular indica rice (*Oryza sativa* L. sub sp. indica cv. ADT 43). *Plant Sci*. 2011; **181**, 258-68.

[17] M Yatazawa, K Furuhashi and M Shimizu. Growth of callus tissue from rice-root *in vitro*. *Plant Cell Physiol*. 1967; **8,** 363-73.

[18] H Rashid, A Toriyama, K Qurashi, Hinta and KA Malik. An improved method for shoot regeneration from calli of indica rice. *Pak. J. Biol. Sci*. 2000; **3**, 2229-31.

[19] D Verma, R Joshi, A Shukla and P Kumar. Protocol for in vitro somatic embryogenesis and regeneration of rice *(Oryza sativa* L.). *Indian J. Exp. Biol*. 2011; **49**, 958-63.

[20] T Murashige and F Skoog. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant*. 1962; **15**, 473-97.

[21] A Hussain, H Nazir, I Ullah and IA Qarshi. *Plant tissue culture: current status and opportunities*. Intech Open Access Publisher, Croatia, 2012, p. 4-8.

[22] CN Chowdhury, AK Tyag, N Maheshwari and SC Mheshwari. Effect of L-proline and L-trypotophan on somatic embryogenesis and plantlet regeneration of rice (Oryza sativa L.) cv. Pusa 169. *Plant Cell, Tissue Organ Cult.* 1993; **32**, 357-61.

[23] E Shahsavari, AA Maheran, A Siti Nor Akmar and MM Hanafi. The effect of plant growth regulators on optimization of tissue culture system in Malaysian upland rice. *Afr. J. Biotechnol*. 2010; **9**, 2088–94.

[24] DE Wetherell and DK Dougall. Sources of nitrogen supporting growth and embryogenesis in cultured wild carrot tissue. *Physiol. Plant*. 1976; **37**, 97- 103.

[25] M Raval and BB Chatto. Role of media constituents and proline in callus growth, somatic embryogenesis and regeneration of *Oryza sativa* cv Indica. *Indian J. Exp. Biol.* 1993; **31**, 600-03.

[26] Sara Ageel and Khaled Elmeer. Effects of casein hydrolysates and glutamine on callus and somatic embryogenesis of date palm (*Phoenix dactylifera* L.). *New York Science Journal*. 2011; **4**, 121-25.

[27] B Pawar, KALE Prashant, J Bahurupe, A Jadhav, KALE Anil and S Pawar. Proline and glutamine improve in vitro callus induction and subsequent shooting in rice. *Rice Science*. 2015; **22**, 283-9.

[28] F Jaseela, VR Sumitha and GM Nair**.** Somatic embryogenesis and plantlet regeneration in an agronomically important wild rice species *Oryza nivara*. *Asian J. Biotechnol*. 2009; **1**, 74-8.

[29] Z Yinxia and S Te – chato. Callus induction and plantlet regeneration from mature embryos of indica rice (*Oryza sativa* L.) cultivar Hom Kra Dang Ngah. *Int. J. Agric. Technol*. 2012; **8**, 2423-33.

[30] J Summart, S Panichajakul, P Prathepha and P Thanonkeo. Callus induction and influence of culture condition and culture medium on growth of Thai Aromatic rice, Khao Dawk Mali 105, cell culture. *World Appl Sci J*. 2008; **5**, 246-51.

[31] H Rashid, F Mohammad Abbasi and A Quraishi. Plant regeneration from seed derived callus of three varieties of Basmati rice. *Plant Tissue Cult*. 2003; **13**, 75-9.

[32] A Libin, PJH King, KH Ong, JK Chubo and P Sipen. Callus induction and plant regeneration of Sarawak rice (*Oryza sativa* L.) variety Biris. *Afr. J. Agric. Res*. 2012; **7**, 4260-5.

[33] ARJ Mohd Din, FL Ahmad, A Wagiran, AA Samad, Z Rahmat and MR Sarmidi. Improvement of efficient in vitro regeneration potential of mature callus induced from Malaysian upland rice seed (Oryza sativa cv. Panderas). *Saudi J. Biol. Sci*. 2016; **23**, 69-77.

[34] K Matsumoto. *Micro-propagation of bananas. In*: Jain SM and Ishii K (eds.). Micropropagation of Woody Trees and Fruits. Kluwer Academic Publishers, Netherlands, 2003, p. 353 –80.

[35] L Khaleda and M Al-Forkan. Stimulatory effects of casein hydrolysate and proline in in vitro callus induction and plant regeneration from five deepwater rice (*Oryza sativa* L.). *Biotechnology*. 2006; **5**, 379-84.

[36] K Rattana, P Theerakulpisut and S Bunnag. The effect of plant growth regulators and organic supplements on callus induction and plant regeneration in rice (*Oryza sativa* L.). *Asian J. Plant Sci*. 2012; **11**, 182-9.

[37] CMZ Che Radziah, AK Siti Nurkhalida, Z Zamri and I Ismanizan. Effect of illumination, casein hydrolysate and proline on callus induction of (*Oryza sativa* L.) cv. MR219. *Malays. Appl. Biol*. 2012; **41**, 37-41.

[38] AB Siddique, Israt Ara, SM Shahinul Islam and Narendra Tuteja, Effect of Air Desiccation and Salt Stress Factors on In vitro Regeneration of Rice (*Oryza sativa* L.). *Plant Signaling Behav*. 2014, DOI: 10.4161/15592324.2014.977209.

[39] N Matsuta and T Hirabayashi. Embryogenic cell lines from somatic embryos of grape (*Vitis vinifera* L.). *Plant Cell Rep*. 1989; **7**, 684-7.

[40] C Liu, L Kwanhoon, HM Honda and T Kobayashi. Enhanced regeneration of rice (*Oryza sativa* L.) embryogenic callus by light irradiation in growth phase. *J. Biosci. Bioeng*. 2001; **91**, 319 –21.

[41] JP Nitsch and C Nitsch. Haploid plants from pollen grains. *Science.* 1969; **163**, 85-7.

[42] AS Afolabi, O Oyebanji, O Odusanya, ME Abo, M Misra and GH Ogbadu. Regeneration of plants from rice caryopsis derived callus culture of Nigerian local cv. Suakoko 8 and a Nerica cv. Faro 55. *Afr. J. Plant Sci.* 2008; **2**, 109-12.

[43] M Inoue and E Maeda. *Control of Organ Formation in Rice Callus Using Two- Step Culture Methods. In:* Fujiwara A (ed.). Plant Tissue Culture. Maruzen, Tokyo, 1982, p. 183-6.

[44] P Bhausaheb,_K Prashant,_B Jyoti, J Ashok,_K Anil_and P Sharad._Proline and glutamine improve in vitro callus induction and subsequent shooting in rice. *Rice Science*. 2015; **22**, 283-9.

[45] RM Subhadra, G Divya and RR Gyana. In vitro somatic embryogenesis of high yielding varieties of rice (*Oryza sativa* L.). *Afr. J. Biotechnol*. 2013; **12**, 6113-8.

[46] BE Moghaddam, M Mesbah and N Yavari. The effect of in planta TIBA and proline treatment on somatic embryogenesis of sugar beet (*Beta vulgaris* L.). *Euphytica*. 2000; **112**, 151-6.

[47] SK Ghanti, KG Sujata, S Rao, M Udayakumar and PB Kavi Kishor. Role of enzymes and identification of stage-specific proteins in developing somatic embryos of chickpea (*Cicer arietinum* L*.). In Vitro Cell. Dev. Biol.* 2009; **45**, 667–72.

[48] S Rueb, M Leneman, RA Schilperoort and LAM Hensgens. Efficient plant regeneration through somatic embryogenesis from callus induced on mature rice embryos (*Oryza sativa* L.). *Plant Cell, Tissue Organ Cult*. 1994; **36**, 259-64.

[49] FA Joyia and MS Khan. Scutellum-derived callus-based efficient and reproducible regeneration system for elite varieties of indica rice of Pakistan. *Int. J. Agric. Biol*. 2013; **15**, 27-33.

[50] C Coenen and TL Lomax. Auxin-cytokinin interactions in higher plants: old problems and new tools. *Trends Plant Sci.*1997; **2**, 351-6.

[51] A Meneses, D Flores, M Muñoz, G Arrieta and AM Espinoza. Effect of 2,4-D, hydric stress and light on indica rice (*Oryza sativa*) somatic embryogenesis. *Rev. Biol. Trop.* 2005; **53**, 361-8.

[52] MW Nabors, CS Kroskey and DM Mchugh. Green spots are predictors of high callus growth rates and shoot formation in normal and in salt stressed tissue cultures of oat (*Avena sativa* L.). *Z. Pflanzenphysiol*. 1982; **105**, 341-9.

[53] Z Yinxia and S Te-chato. Improved plantlet regeneration systems in indica rice (*Oryza sativa* L.) landrace Hom Kra Dang Ngah. *Int. J. Agric. Technol*. 2013; **9,** 1641-54.

[54] TA Jubair, U Salma, N Haque, F Aktar, IJ Mukti, AKMF Haque and MR Ali. Callus induction and regeneration of local rice (Oryza sativa L.) variety topa. *Asian J. Plant Sci*. 2008; **7**, 514-7.

[55] AJ Faiz and MS Khan. Reproducible and expedient rice regeneration system using *in vitro* grown plants. *African Journal of Biotechnology*. 2011; **11**, 138-44.

[56] EH Hoque and JW Mansfield. Effect of genotype and explant age on callus induction and subsequent plant regeneration from root – derived callus of Indica rice genotypes. *Plant Cell, Tissue Organ Cult*. 2004; **78**, 217-23.

[57] T Werner, V Motyka, V Laucou, R Smets, H Van Onckelen and T Schmülling. Cytokinin-deficient transgenic *Arabidopsis* plants show multiple developmental alterations indicating opposite functions of cytokinins in the regulation of shoot and root meristem activity. *Plant Cell. 2003;* **15**, 2532–50.

[58] F Skoog and CO Miller. Chemical regulation of growth and organ formation in plant tissue cultures in vitro. *Symp. Soc. Exp. Biol*. 1957; **11,** 118–31.

[59] MBH Sikder, PK Sen, M Abdullah-Al Mamun, MR Ali and SM Rahman. In vitro regeneration of aromatic rice (*Oryza sativa* L.). *Int. J. Agri. Biol*. 2006; **8**, 759-62.

[60] JS Sandhu, SS Gosal and MS Gill. Micropropagation of indica rice through proliferation of axillary shoots. *Euphytica*. 1995; **81**, 139-42.

[61] S Lavanya, N Rosimah and QZ Faridah. Effects of plant growth regulators on in vitro regeneration of Malaysian Indica rice (*Oryza sativa* L.) cv. MR219 by shoot apical meristem. *Asian J. Agric. Res.* 2012; **6**, 180-7.

Paper 2

Effect of Culture Media and Plant Growth Regulator on Callus Induction and Regeneration of *Indica* Rice (*Oryza sativa* L. cv Sangyod)

Effect of Culture Media and Plant Growth Regulator on Callus Induction and Regeneration of *Indica* Rice (*Oryza sativa* L. cv Sangyod)

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1.Introduction

Rice (*Oryza sativa* L.) is one of the most strategical important cereal crop in Asia and a fundamental food source for over half of the world's population. Aromatic rice is very popular in southeast Asia and known as fragrance rice or scented rice. It has built up larger approval in the Western and Middle East nations recently (Sarhadi *et al*., 2008; Myint *et al*., 2009; Hashemi *et al*., 2015). Rice grain of Sangyod is regarded as one of the economically important traits for rice grain quality with dark-red color dehusk seed, soft and aromatic of cooked, grown in Patthalung province, Thailand for hundred years. Sangyod grain contains more minerals (iron), vitamins, bioactive compounds (anthocyanin, flavonoid, phenolic compounds). In addition, to meet its demand for healthier rice products are being increasing globally. Emphasizing a need for the research and development of efficient mass propagation tools for Sangyod rice variety in the future is required.

Rice improvement via tissue culture technique base on effective protocols through callus formation and suitable plantlet regeneration under *in vitro* conditions (Vega *et al*., 2009; Khatun *et al*., 2012; Siddique *et al*., 2014). *In vitro* rice plantlet regeneration can be established via organogenesis (shoot, leaf, root) and somatic embryogenesis (somatic embryos). However, somatic embryogenesis is one of the most auspicious methods for expeditious propagation due to induction of large- scale of plantlets and for the utilization of genetic transformation to resist to biotic and abiotic stresses (Zuraida *et al*., 2011). Successful achievement of somatic embryogenesis and plantlet regeneration of rice are highly depended on genotype, kinds of culture media, plant growth regulators (PGRs), gelling agent and carbon sources (Deo *et al*., 2009).

Many basal culture media such as MS, NN, N_6 and ARDA usually used for in vitro propagation of rice. Auxin (2,4-D or NAA) plays critical role for cell growth and development (Su *et al*., 2009; Rademacher *et al.*, 2012). In rice, it prompts somatic embryogenesis and plantlet regeneration on cultured callus (Vega *et al*., 2009). Auxin was recognized as a key component of somatic embryogenesis that impacts carbohydrate metabolism on shoot regeneration and osmotic requirement (Lee and

Huang, 2014). Huang *et al*. (2012) reported that endogenous auxin stimulate shoot regeneration in rice callus. PGRs is key factor controlling many growth processes including organ regeneration from varied tissues (Rueb *et al*, 1994). Osmotic water promising influences by kinds of media, concentrations of gelling agent and carbon sources (Hadeler *et al*., 1995; Klimaszewska *et al*., 1997; Triqui *et al*., 2008). Hence, choice of suitable culture medium in combination with PGRs are vital for optimum callus induction and subsequent plantlet regeneration in order to apply genetic transformation of rice. The additions of casein hydrolysate and proline have been reported to enhance callusing reaction (Lin and Zhang, 2005). The optimum conditions for efficient callus induction and regeneration from mature seeds of *indica* rice cultivars is one of prerequisites for the successful application of genetic transformation in crops.

The objectives of this study were to find out the most efficient culture medium and concentrations of PGRs (NAA, BAP, TDZ, Kn) in order to optimize productive protocol for callus induction and plantlet regeneration in Thai rice cultivar Sangyod.

2. Materials and methods

Plant material and sterilization

Mature seeds of *indica* rice cultivar Sangyod were used as explant source. They were obtained from Department Plant Science, Faculty Natural Resources, Prince of Songkla University, Thailand. The seeds were dehusked, washed with running tap water for 20 minutes, then surface sterilized with 70% (v/v) ethanol for 2 minutes, followed by immersion in 20% (v/v) Clorox (commercial bleach containing 0.05-0.1 ml of a wetting agent"Tween-20" per 100 ml Clorox solution) on orbital shaker at 100 rpm for 6 minutes. Finally, the seeds were rinsed five times with sterile distilled water in laminar air flow hood before blotted dry on autoclaved tissue paper to remove excess water. Sterile seeds were then cultured on callus induction medium (CIM).

Callus induction and proliferation

The experiments were carried out to optimize culture medium (MS, NN and ARDA) by culturing disinfedted seeds on three types of CIM, MS (Murashige and Skoog, 1962), NN (Nitsch and Nitsch, 1969) and ARDA (Yinxia and Te-chato, 2013). All culture media were supplemented with 2 mg/ L 2,4-D, 750 mg/ L of casein hydrolysate (CH) and 200 mg/ L L-Proline. Each medium was added with 3% (w/v) sucrose, adjusted to pH 5.7, solidified with 0.75% (w/v) agar, and autoclaved at 121^0 C, 1.07 kg/ cm^2 for 20 min. All cultures were maintained at 26 ± 2^0 C in the culture room under 14 hours photoperiod with irradiance of 25 μmol/m^2/s provided by cool white fluorescent tubes. After four weeks of culture frequency of callus induction,

number of days to callus initiation and mean callus fresh weight were recorded and statistical compared.

Effect of subculture on callus growth

Four-week-old calluses obtained from the most suitable medium of previous experiment at 0.1 gram fresh weight were excised and subcultured to the same medium component at weekly intervals for 8 weeks to proliferate and growth index (GI) and fresh biomass (FW) measured. The preparation of culture media and culture conditions were the same as previous experiments.

The average of GI, FW, browning callus frequency and morphological characteristics were evaluated every two-week of culture until the fourth times of subculture (8 weeks). GI was calculated as described by Chan *et al.* (2008) according to the following formula;

$$GI = (\text{Final FW} - \text{Initial FW}) / \text{Initial FW}$$

Effect of PGRs on plantlet regeneration

The calluses from the best subculture stage were transferred to regeneration medium (RM) which was MS supplemented with various concentrations of PGRs (NAA, TDZ, BAP and Kn) as shown in Table 4. The medium was added with 3% (w/v) sucrose and adjusted to pH 5.7 prior to addition with 0.75% (w/v) agar. The cultures were maintained at the same conditions as mentioned in previous experiments. At three weeks of culture the percentage of green spot (GS) formation, percentage of plantlet regeneration and mean number of plantlets per callus were recorded and statistically compared after six weeks of culture.

Statistical analysis

All the tissue culture experiments of callus induction, callus growth, and plantlet regeneration frequency were arranged in completely randomized design (CRD) with 6 replicates per treatment. Data were tested by using one–way analysis of variance (ANOVA) and the significant differences among means were separated by Duncan's multiple range test (DMRT) ($p = 0.01$) using program R statistical package version 2.14.

3. Results and discussions

3.1 Influence of different culture medium on callus induction

Our results showed that different culture media were significant influence on time required for callus initiation, frequency of callus induction and mean FW (Table 1 & 2). After 17 days of culture on ARDA medium, callus was initiated from the scutellum region under 16 hour photoperiod. The highest frequency of callus induction (75.63 ± 5.28 %) and FW (68.05 ± 20.04 mg) were observed on ARDA medium supplemented with 2 mg/ L 2,4 – D, 750 mg/ L CH and 200 mg/ L L-proline after 4 weeks of culture whereas there was not significantly different ($p < 0.01$) with MS medium (Figure 1). Various morphological calluses were found in different culture media. However, embryogenic callus with good characteristics such as yellow or white color with globular structure was observed on MS and ARDA medium.

Mature seeds cultured on NN medium in combination with 2 mg/ L 2,4 – D, 750 mg/ L CH and 200 mg/ L L-proline gave the longest time for callus initiation after 20 days of inoculation, lowest frequency of callus induction at 50.22 ± 6.51 % and callus FW at 44.39 ± 25.36 mg. The earliest period time for callus appearance was obtained on MS medium after 10 days. Some researchers reported that the positive influence of NN medium on rice callus induction in some varieties such as Khao Dawk Mali 105, Chai Nat 1, Supanburi 1 (Rattana *et al*., 2012). Furthermore, the highest percentage of callus induction (100%) and mean FW of callus (341.2 mg) was obtained on NN medium suplemented with 1 mg/ L 2,4 –D in Nam Roo rice (Anurug *et al*., 2016). Recently, Yinxia and Te-chato (2013) reported that ARDA medium gave the best result for the percentage of green spot callus formation (61.3%) in rice cv. Hom Kra Dang Ngah. However, the effect of ARDA medium on callus induction has not been reported.

Nitrogen could apply to culture media as organic or inorganic sources. Nitrogen in form of organic source may be apply as amino acid and vitamins. The inorganic nitrogen is commonly provided through the structure of ammonium or nitrate ions (Kaushal *et al*., 2014). The ratio of NO_3^-/NH_4^+ is known to be the main key for the successful efficient of callus induction in rice (Grimes and Hodges, 1990). MS and ARDA medium contain higher level of NH_4NO_3 than that of NN medium. Beside, ARDA medium also contains K_2SO_4 while the others two culture media (MS or NN medium) do not consist this component. It may be one of reason to improve embryogenic callus incuction. Minyaka *et al.* (2008) reported the positive effect of K_2SO_4 on induction a high frequency of somatic embryo formation in cacao genotypes IMC67 and Sca6 on DKW medium (Driver and Kuniyuki, 1984). Zinc and Copper are two crucial micronutrients for plant cells which effect on embryogenesis

(Silva, 2012). Copper plays important roles in respiration process which is the functional components of oxidative enzymes in plant tissue (Sumner and Somers, 1953). Similarly, it has also reported that the copper enhanced the *in vitro* response in *japonica* rice cultivars (Yang *et al*., 1999). Furthermore, zinc is essential co-factors for the process of Krebs' and glycolysis cycle (Macelroy and Nason, 1954).

Casein hydrolysate was known as organic nitrogen which provides calcium sources, vitamins, several micronutrient and amino acids supplements on culture media which have been useful for callus induction. The addition of L-proline in the medium acts as stress condition due to the reduction of water potential, thus, enhancing the development of embryogenic callus through the accumulation of nutritional items in cells (Mallick *et al.*, 2013). Base on our results, ARDA medium was the most suitable for callus induction and proliferation in Sangyod rice and this culture medium was used for next experiments. The composition of basal salts, strength of micronutrients and amino acids supplements in culture medium are the main factors influencing callus induction frequency and fresh weight of callus. In rice, callus formation depend on the kinds of culture medium used (Zhao, 1999). Therefore, it is necessary to optimize culture medium for each genotype before establishment of genetic transformation experiment.

Table 1 Compositions of culture medium used for callus induction of Sangyod rice

Components	MS	ARDA (mg/L)	NN
NH_4NO_3	1650	1025	720
KNO_3	1900	950	950
KH_2PO_4	170	170	68
$K_2 SO_4$	-	495	-
$MgSO_4.7H_2O$	370	185	185
$CaCl_2.2 H_2O$	440	268	219
H_3BO_3	6.2	6.2	10
$MnSO_4.4 H_2O$	16.90	16.9	25
$ZnSO_4.7 H_2O$	10.60	9.6	10
$Na_2MoO_4.2 H_2O$	0.25	0.25	0.25
KI	0.83	0.415	-
$CuSO_4.5 H_2O$	0.025	3.138	0.025
$CoCl_2.6 H_2O$	0.025	0.013	-
$FeSO_4.7 H_2O$	27.8	27.8	27.80
Na_2EDTA	37.3	37.3	37.30
Thiamine-HCl	0.1	0.55	0.50
Nicotinic acid	0.5	0.5	0.50
Pyridoxine-HCl	0.5	0.5	0.50
Glycine	2.00	2.00	5.00
Myo -inositol	100	100	2.00
Biotin	-	-	0.05
Folic acid	-	-	0.50
Sucrose	30,000	30,000	30,000

Table 2 Influence of different culture media supplemented with 2 mg/ L 2,4-D, 750 mg/ L CH and 200 mg/ L L-proline on Sangyod callus induction after 4 weeks of culture.

Culture medium	Days to callus initiation	Frequency of callus induction (%)	Mean callus FW (mg)
MS	10	72.75 ± 4.67^{a}	66.51 ± 14.70^{a}
ARDA	17	75.63 ± 5.28^{a}	68.05 ± 20.04^{a}
NN	20	50.22 ± 6.51^{b}	44.39 ± 25.36^{b}
F- Test	-	**	**
C.V. (%)	-	8.79	36.23

** Significant difference at $p \leq 0.01$ level
Values are means of 6 replicates ± SD. Means followed by different letters within column are significantly different by DMRT

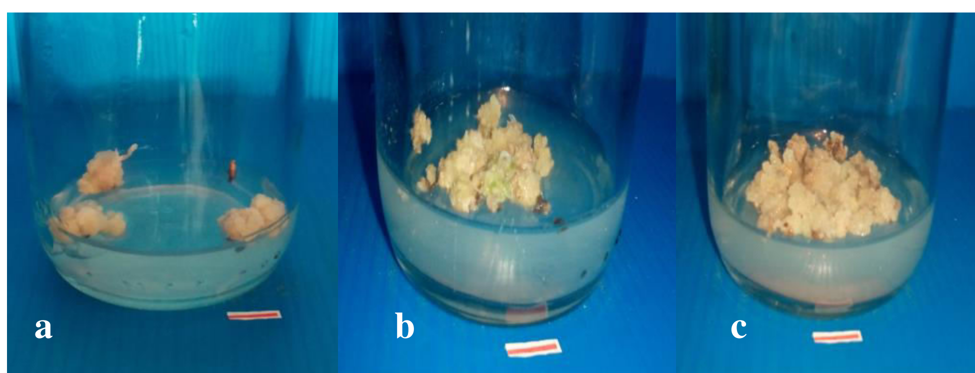

Figure 1 Characteristics of callus induction and callus proliferation in different subculture stages of Sangyod rice (bar = 1 cm)
(a): Callus induction on ARDA medium supplemented with 2 mg/ L 2,4-D, 750 mg/ L CH and 200 mg/ L L-proline after 4 weeks of culture
(b): Callus proliferation after 35 days of subculture
(c): Callus proliferation after 56 days of subculture

3.2 Influence of repeated subculturing on callus growth

Based on the results in Figure 2 & 3, GI and mean FW of callus increased exponentially from day 7 to day 35 after subculture. After this period growth of callus was slow and enter stationary stage. Model of the callus growth curve is one of main parameters that determine the period for maximum callus induction and growth rate

(Hussein *et al*., 2016). Four–week-old or 28-day-old callus were suitable period for routine subculture to ARDA medium supplemented with 2 mg/ L 2,4–D, 750 mg/ L CH and 200 mg/ L L-proline. This peroiod showed significant response on mean fresh weight of callus and GI for 56 consecutive days of culture (Figure 2). The callus growth curves of Sangyod rice revealed according to a sigmoidal shape that the GI and FW were initially slow during first 7 days of culture (lag phase). After this period growth of callus increased dramatically different ($p<0.001$) until 35 days (log phase). The maximum mean FW at 1272.83 ± 48.63 mg was obtained at day 35 of culture. GI was also the highest at 11.73 folds of the initial FW (100 mg) at this date of culture (Figure 2). In case of browning of callus, increase in times of culture promoted the increment of callus browning. After the 5th time of culture browning of callus was severely increased, significantly different ($p< 0.01$) with the 1st to 4th time of culture (Figure 3). The highest browning callus at 69.17 ± 10.56 % was observed by day 56 after culture whereas the greatest mean FW of callus (1423 ± 37.59 mg) and GI (13.23) were obtained. Therefore, the good quality and high GI of Sangyod callus were at 35 days after subculture which was in log phase and should be the most suitable for producing plant regeneration and genetic transformation experiment.

Calluses are known as unorganized cell masses that could be produced from one differentiatiated cell. The quality of the callus in rice is one of the key factors to define the growth rate of regeneration (Amarasinghe, 2010). Callus growth, alike bacterial growth, is connected procedure that exposes unique phases with the specific GI. There were three main stages for callus growth (Figure 2). Commonly, the cycle of callus growth of rice in first stage could be maintained until the callus attain stationary phase.

It has been reported that the second subculture (42 successive days of culture) gave the best subculture period for the highest proliferation rate of callus and good quality such as nodular, compact, yellow or white color in ten rice varieties, especially, the maximum GI of callus-in varieties Pachcha Perumal (4.993) and Taipei 309 (6.187) (Amarasinghe, 2010). Additionally, the callus growth rate of seven rice genotypes (IR24, Zhenshan 97, IRBB13, IRBB 4, IRBB 10, Minghui 63 and 93-11) on S medium were evalutated by Ge *et al.* (2006). After 20 days of subculturing, the GI of callus increased, particularly, Minghui 63 genotype showed the highest GI (8.13) among the other 7 genotypes.

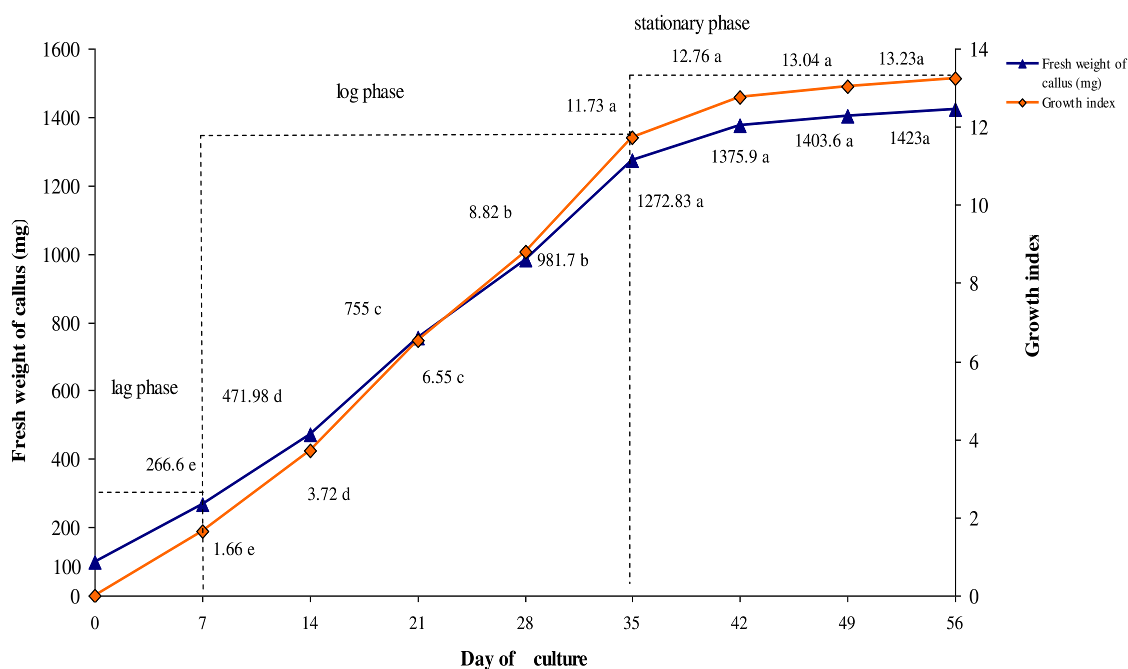

Figure 2 The effect of successive subcultures on mean fresh weight of callus and growth index of Sangyod rice on ARDA medium supplemented with 2 mg/ L 2,4 – D, 750 mg/ L CH and 200 mg/ L L-proline. The cultures were routinely subcultured 56 days intervals.

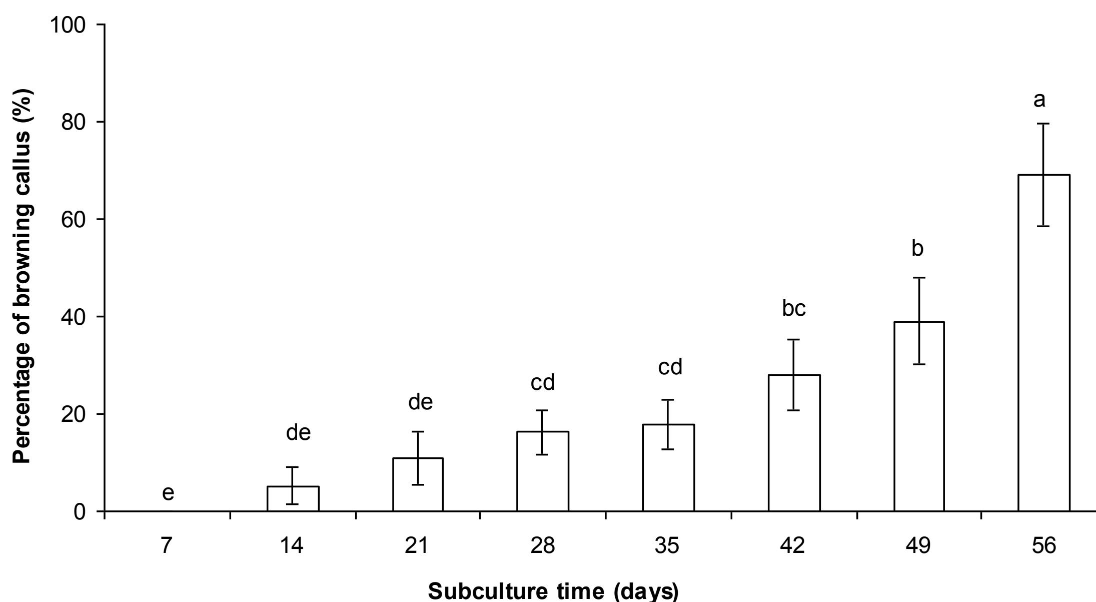

Figure 3 Influence of days after culture on the percentage of browning of Sangyod callus for 56 days. The callus was cultured on ARDA medium supplemented with 2 mg/ L 2,4 – D, 750 mg/ L CH and 200 mg/ L L-proline.

3.3 Influence of various concentrations of PGRs on plantlet regeneration

Different kinds and concentrations of PGRs gave significant influence on green spot callus and plantlet regeneration. The combination of 0.5 mg/ L BAP, 0.5 mg/ L TDZ, 1.0 mg/ L Kn and 0. 5 mg/ L NAA gave the maximum percentage of green spot (GS) formation (72.34 ± 8.75 %), plantlet regeneration frequency (67.25 ± 6.14 %) and mean number of plantlets/ callus (6.63 ± 0.47 plantlets) which was statistically different ($p<0.01$) with the other concentrations of PGRs (Table 4, Figure 4). Plantlet regeneration frequency varied according to the combination of cytokinin and auxin. Kyungsoon *et al.* (2002) reported that the greatest regeneration frequencies were 68, and 77% for cultivars Nak-Dong, Dong-Jin on MS medium containing 2.0 mg/ L NAA and 2.0 and 4.0 mg/ L Kn, respectively. Similarly, Din *et al.*,(2016) revealed that the correct concentration of PGRs together (NAA, BAP, Kn and TDZ) was significantly increased regeneration frequency in Malaysian upland rice cv. Panderas, specifically, the optimum regeneration frequency (100 %) was observed on MS medium containing containing 0.5 mg/ L BAP , 0.5 mg/ L NAA, 1.5 mg/ L Kn, 0.5 mg/ L TDZ. Moreover, the most suitable for plantlet regeneration efficiency of rice cv. BRRI dhan 29 was 80 % on MS medium supplemented with 1.0 mg/ L NAA, 1.5 mg/ L Kn and 2.0 mg/ L BAP (Islam *et al.*, 2014). The action of TDZ could induce more shoot regeneration than BAP in rice cv. Jaumala (Gairi and Rashid, 2004). Raghavendra *et al* (2010) reported that MS medium supplemented with 4 mg/ L TDZ gave the highest plant regeneration frequency (93.33 %) and number of shoots/ explant (10.67 shoots) in rice cv. Rasi. The different responses of variety might be due to recalcitrance and genotype-dependence in *indica* rice. Therefore, it is essential to modify combination of PGRs base on different genotypes to increase regeneration efficiency.

Plant growth regulators play important role in plant tissue culture, where a high the ratio of auxin/cytokinin consistently used for producing embryogenic callus. In contract, a low ratio was used for the plantlets regeneration (Ge *et al.*, 2006**).** In earlier studies, it has been reported that the levels of endogenous auxin in rice callus could promote shoot regeneration (Huang *et al.*, 2012). However, the action of endogenous auxin on the effective of the formation of callus subsequent to shoot induction still unknown. Skoog and Miller (1957) reported that organogenesis in callus and tissue culture occurred by the intake of PGRs (cytokinin, auxin) in medium in the initial phases of culture. At present, the knowledge of mechanisms of auxin and cytokinin in synthesis sites and their molecular level that provides via the plant causes large process of endogenous auxin and cytokinin levels. Auxin may be recognized as a main factor that impacts osmotic prerequisite, phytohormone signal and carbohydrate metabolism on shoot regeneration (Lee and Huang, 2014). The ratio of

plant growth regulators (cytokinin/ auxin) have significant role in growth and differeniation of cells in callus. Cytokinin can promote the growth rate of pro-embryonic masses (Kommamine *et al*., 1992). Some auxins such as 2,4-D or NAA in combination with kinetin at the specified concentrations could improve plantlet regeneration in rice. Using the combination of PGRs promotes more efficient callus induction and plantlet regeneration than using PGR alone (Ge *et al*., 2006).

Table 4 Effects of different kinds and concentrations of PGRs containing ARDA medium on plantlet regeneration from callus of Sangyod rice

Concentrations of PGRs (mg/ L)				GS formation frequency (%)	Plantlet regeneration frequency (%)	Mean number of plantlets/ callus
NAA	TDZ	BAP	Kn			
0.5	1.5	0.0	0.0	36.66 ± 7.04^{de}	31.3 ± 5.15^{c}	2.54 ± 0.21^{cd}
0.5	0.0	1.5	0.0	25.12 ± 10.31^{e}	25.54 ± 4.87^{c}	2.17 ± 0.44^{cd}
0.5	0.0	0.0	1.5	41.45 ± 6.50^{cd}	26.81 ± 5.23^{c}	1.80 ± 0.35^{d}
0.5	0.5	0.5	1.0	72.34 ± 8.75^{a}	67.25 ± 6.14^{a}	6.63 ± 0.47^{a}
1.0	1.5	0.0	0.5	64.50 ± 9.26^{ab}	54.75 ± 5.60^{ab}	4.06 ± 0.55^{b}
1.0	0.0	1.5	0.5	53.84 ± 6.78^{bc}	41.49 ± 7.08^{bc}	4.35 ± 0.28^{b}
1.0	0.5	0.5	1.5	39.26 ± 9.33^{d}	30.17 ± 4.22^{c}	3.33 ± 0.39^{bc}
F - test				**	**	**
C.V.(%)				19.82	13.98	12.39

** Significant difference at $p \leq 0.01$

Values are means of 6 replicates ± SD. Means followed by different letters within column are significantly different by DMRT.

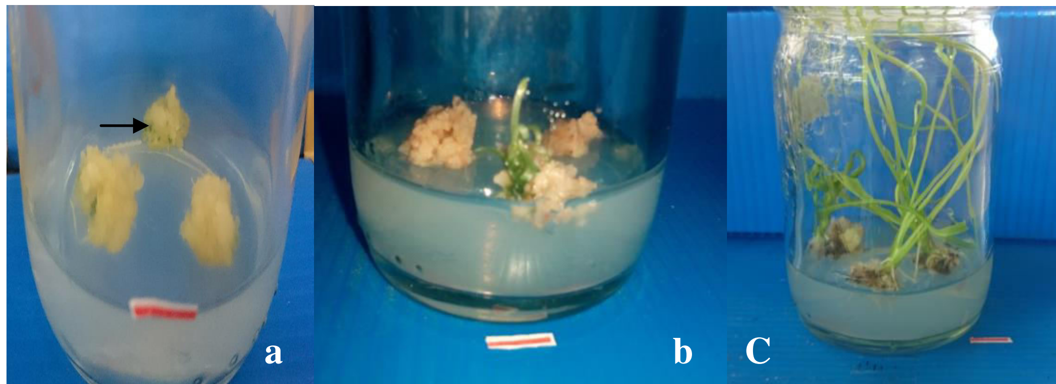

Figure 4 Plantlet regeneration from mature seed-derived callus of Sangyod rice on ARDA medium with 0.5 mg/ L BAP, 0.5 mg/ L TDZ, 1.0 mg/ L Kn and 0. 5 mg/ L NAA (bar = 1 cm).
(a): Green spot formation after 4 weeks of culture
(b): Shoot induction after 6 weeks of culture
(c): Plantlet regeneration after 8 weeks of culture

4. Conclusion

In the present study, culture medium and PGRs play significant role on callus induction, callus growth index and plantlet regeneration protocol for *indica* rice cv. Sangyod from culturing mature seeds. Improvement of callus formation and regeneration efficiency can be used for genetic engineering to create new varieties with desirable traits in the future.

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References

Amarasinghe, A. A. Y. 2010. Effects of copper sulphate and cobalt chloride on in vitro performances of traditional indica rice (*Oryza sativa* L.) varieties in Sri Lanka. Journal of Agricultural Sciences 4: 132– 141.

Chan, L.K., See, K. S., Hoon, L. P., Lim, B. P. 2008. Effect of plant growth regulators and subculture frequency on callus culture and the establishment of

Melastoma malabathricum cell suspension culture for the production of pigments. Biotechnology 7: 678-685.

Deo, P. C., Harding, R. H., Taylor, M., Tyagi, A. P and Becker, D. K. 2009. Somatic embryogenesis, organogenesis and plant regeneration in taro (*Colocasia esculenta* var. esculenta). Plant Cell Tissue Organ Culture 99: 61-71.

Din, A. R. J. M., Ahmad, F. I., Wagiran, A., Samad, A. A., Rahmat, Z., & Sarmidi, M. R. 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: 69- 77.

Driver, J. A. and Kuniyuki, A. H. 1984. In vitro propagation of *Paradox walnut* root stock. Horticultural Science 19:507–509.

Gairi, A. and Rashid, A. 2004. TDZ-induced somatic embryogenesis in non-responsive caryopses of rice using a short treatment with 2, 4- D. Plant Cell, Tissue and Organ Culture 76: 29–33.

Ge, X., Chu, Z., Lin, Y. and Wang, S. 2006. A tissue culture system for different germplasms of indica rice. Plant Cell Reported 25: 392-402. doi: 10.1007/s00299-005-0100-7.

Ge, X., Chu, Z., Lin, Y. and Wang, S. 2006. A tissue culture system for different germplasms of indica rice. Plant Cell Reports 25: 392-402.

Grimes, H. D. and Hodges, T. K. 1990. The inorganic NO_3: NH_4 ratio influence plant regeneration and auxin sensitivity in primary callus derived from immature embryos of indica rice (*Oryza sativa* L.). Journal of Plant Physiology 136: 362-367.

Hadeler, B., Scholtz, S. and Reski, R. 1995. Gelrite and agar differently influence cytokinin-sensitivity of a moss. Journal Plant Physiol 146: 369-371.

Hashemi, F. S. G., Rafi, M. Y., Ismail, M. R., Mohamed, M. T. M., Rahim, H A., Huang, W. L., C. H. Lee and Y. R. Chen. 2012. Levels of endogenous abscisic acid and indole-3-acetic acid influence shoot organogenesis in callus cultures of rice subjected to osmotic stress. Plant Cell Tissuse Organ Culture 108: 257-263.

Hussein, S., Halmi, M. I. E. and Kiong, A. L. P. 2016. Modelling the growth kinetics of callus cultures from the seedling of *Jatropha curcas* L. according to the modified Gompertz model. Journal of Biochemistry, Microbiology and Biotechnology 4: 20-23.

Islam, M. M., Haque, M. E., Islam, M. A., Sikdar, B. and Khalekuzzaman, M. 2014. Establishment of an efficient protocol for in vitro callus induction and regeneration system using mature embryo in elite rice (*Oryza sativa* L.) cultivars. Research in Plant Biology 4: :9 -20.

Kaushal, L., Balachandran, S. M., Ulaganathan, K. and Shenoy, V. 2014a. Effect of media on improving anther culture response of rice (*Oryza sativa* L.). International Journal for Agriculture Innovations and Research 3: 218-224.

Kaushal, L., Sharma, R., Balachandran, S. M., Ulaganathan, K. and Shenoy, V. 2014b. Effect of cold pretreatment on improving anther culture response of rice (*Oryza sativa* L.). Journal of Experimental Biology and Agricultural Sciences 2: 233-242.

Khatun, R., . Islam, S. M. S., Ara, I., Tuteja, N. and Bari, M. A. 2012. Effect of cold pretreatment and different media in improving anther culture response in rice (*Oryza sativa* L.) in Bangladesh. Indian Journal Biotechnology 11: 458-463.

Klimaszewska, K., Devantier, Y., Lachance, D., Lelu, M. A. and Charest, J.P. 1997. *Larix laricina* (tamarack) somatic embryogenesis and genetic transformation. Canadian Journal of Forest Research 27: 538-550.

Komamine, A., Kawahara, R., Matsumoto, M., Sunabori, S., Toya, T., Fujiwara, A., Tsukahara, M., Smith, J., Ito, M., Fukuda, H., Nomura, K. and Fujimura, T. 1992. Mechanisms of somatic embryogenesis in cell cultures: physiology, biochemistry, and molecular biology. In Vitro Cellular and Developmental Biology 28: 11–14.

Kyungsoon, L., Hysung, J. and Kim, M. 2002. Optimazation of a mature embryo-based in vitro culture system for high frequency somatic embryogenic callus induction and plant regeneration from japonica rice cultivars. Plant Cell Tissue and Organ Culture 71: 237-244.

Latif, M. A. and Aslani, M. A. 2015. The genetic and molecular origin of natural variation for the fragrance trait in an elite Malaysian aromatic rice through quantitative trait loci mapping using SSR and gene-based markers. Gene 555: 101-107.

Lee, S. T. and Huang, W. L. 2014. Osmotic stress stimulates shoot organogenesis in callus of rice (*Oryza sativa* L.) via auxin signaling and carbohydrate metabolism regulation. Plant Growth Regulation 73: 193-204.

Lee, S. T.and Huang, W. L. 2014. Osmotic stress stimulates shoot organogenesis in callus of rice (*Oryza sativa* L.) via auxin signaling and carbohydrate metabolism regulation. Plant Growth Regulation 73: 193-204.

Lin, Y. J. and Zhang, Q. 2005. Optimising the tissue culture conditions for high efficiency transformation of indica rice. Plant cell reports 23: 540-547.

Mallick, S. R., Gautam, D.and Rout, G. R. 2013. In vitro somatic embryogenesis of high yielding varieties of rice (*Oryza sativa* L.). African Journal of Biotechnology 12: 6113-6118.

Mcelroy, W. D., and A. Nason. 1954. Mechanism of action of micronutrient elements in enzyme systems. Annual Review of Plant Physiology 5: 1-30.

Minyaka, E., Niemenak, N., Sangare, A. and Omokolo, D. N. 2008. Effect of $MgSO_4$ and K_2SO_4 on somatic embryo differentiation in *Theobroma cacao* L. Plant Cell Tissue and Organ Culture 94: 149 -157.

Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bioassay with tobacco tissue cultures. Physiol Plant 15: 473-497.

Myint, K. K. M., Yasui, H., Takagi, M. and Matsumura. M. 2009. Virulence of long-term laboratory populations of the brown planthopper, Nilaparvata lugens (Stal), and whitebacked planthopper, Sogatella furcifera (Horvath) (Homoptera: Delphacidae), on rice differential varieties. Applied Entomology Zoology 44: 149 -153.

Nitsch, J. P. and Nitsch, C. 1969. Haploid plants from pollen grains. Science 163: 85-87.

Poeaim, A., Poeaim, S., Poraha, R., Pongjaroenkit, S. and Pongthongkam, P. 2016. Optimization for callus induction and plant regeneration from mature seeds of Thai rice variety: Nam Roo (*Oryza sativa L.*). Bioengineering and Bioscience 4: 95-99.

Rademacher, E., Lokerse, A., Schlereth. A., Llavata-Peris, C., Bayer, M., Kientz, M., Freire Rios, A., Borst, J., Lukowitz, W., Jürgens, G. and Weijers. D. 2012. Different auxin response machineries control distinct cell fates in the early plant embryo. Developmental Cell Journal 22: 211-222.

Raghavendra, G., Kumaraswamy, G. K., Ramya, B., Sandesh, H. S., Yogendra, K. N., Deepak, N. and Gowda, P. H. R. 2010. Direct multiple shoot regeneration of indica rice (*Oryza sativa* L.) var.'Rasi'. Asian and Australasian Journal of Plant Science and Biotechnology 4: 71-73.

Rattana, K., Theerakulpisut, P. and Bunnag, S. 2012. The effect of plant growth regulators and organic supplements on callus induction and plant regeneration in rice (*Oryza sativa* L.). Asian Journal of Plant Sciences 11: 182 -189.

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

Sarhadi, W. A., Hien,N. L., Zanjani, M., Yosofzai, W., Yoshihashi, T. and Hirata, Y. 2008. Comparative analysis for aroma and agronomic traits of native rice cultivars from central Asia. Journal Crop Science Biotechnology 11: 17-22.

Siddique, A. B., Ara, I., Islam, S. M. S. and Tuteja, N. 2014. Effect of air desiccation and salt stress factors on in vitro regeneration of rice (*Oryza sativa* L.). Plant Signaling and Behavior 9:12 -19, DOI: 10.4161/15592324.2014.977209.

Silva, T. D. 2012. Microspore embryogenesis. *In:* Ken-Ichi Sato (ed.), Embryogenesis. Intech Open Access Publisher. Available at www.intechopen.com/books/embryogenesis/microspore-embryogenesis. html.

Skoog, F. and Miller, C. O. 1957. Chemical regulation of growth and organ formation in plant tissues cultured. In Vitro Symposia Society Experimental Biology 11: 118-131.

Su, Y. H., Zhao, X. Y., Liu.,Y. B., Zhang, C. L., O'Neill, S. D. and Zhang, X. S. 2009. Auxin-induced WUS expression is essential for embryonic stem cell renewal during somatic embryogenesis in Arabidopsis. Plant Journal 59: 448-460.

Sumner, J.B. and G.F. Somers, 1953. Chemistry and methods of enzymes. Academic Press, New York, USA, pp 166.

Triqui, Z., Guedira, A., Chlyah, A., Souvannavong, V., Haicour , R. and Sihachakr, D. 2008. Effect of genotype, gelling agent, and auxin on the induction of somatic embryogenesis in sweet potato (*Ipomoea batatas* Lam.). Comptes Rendus Biologies 331: 198-205.

Vega, R., N., Espinoza, A. M., Gatica, A. M. and Melara, M. V. 2009. Histology of somatic embryogenesis in rice (*Oryza sativa* cv. 5272). Revíta de Biologia Tropical 57: 141-150.

Yang, Y. S., Jian, Y. Y. and Zheng, Y. D. 1999. Copper enhances plant regeneration in callus culture of rice. Chinese Journal Rice Science 13: 95-97.

Yinxia, Z., Te-chato, S. 2013. Improved plantlet regeneration systems in Indica rice (Oryza sativa L.) landrace Hom Kra Dang Ngah. Journal of Agricultural Technology 9: 1641–1654.

Zhao, J., Zhou, C. and Yang, H. Y. 1999. In vitro development of early proembryos and plant regeneration via microculture in *Oryza sativa*. Plant Cell, Tissue Organ Culture 55: 167–174.

Zuraida, A. R., Naziah, B., Zamri, Z. and Sreeramanan, S. 2011. Efficient plant regeneration of Malaysian indica rice MR 219 and 232 via somatic embryogenesis system. Acta Physiologiae Plantarum 33: 1913-1921.

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Ho, T. L., Te-chato, S. and Yenchon, S. 2017. Effect of culture media and plant growth regulator on callus induction and regeneration of *Indica* rice (*Oryza sativa* L. cv Sangyod). Songklanakarin Journal of Plant Science (in press).

Ho, T.L., Te-chato, S. and Yenchon, S. 2017. Establishment of *Agrobacterium*–mediated transformation Systems in the *Indica* rice cultivar Sangyod (prepare submit).

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