CALLUS INDUCTION AND DEVELOPMENT OF SUSPENSION CELL CULTURE OF MALAYSIAN UPLAND RICE CULTIVAR *PANDERAS*

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To my beloved family, friends and lecturers

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ABSTRACT

The aim of the study is to evaluate the effect of amino acid (tryptophan and glutamine) on the callus induction and to develop suspension cell culture protocol for Malaysian upland rice, *Panderas* cultivar. The research revealed that callus induction varied depend on amino acid tested. In callus induction study, dehusked *Panderas* mature seeds were placed on MSB_5 medium supplemented with 3mg/L 2,4-D and 2mg/L NAA with four different concentrations of tryptophan and glutamine separately. After three weeks in culture, inclusion of tryptophan showed positive effect on percentage of callus induction and fresh weight. It was observed that the optimum concentration of tryptophan was 25mg/L with 96.67% of callus induction and 73.33mg of average fresh weight. At week six, the average fresh weight of callus induced on MSB_5 media supplemented with 25mg/L tryptophan was increased to three fold (215mg). Treatment without amino acids (control) showed similar callus percentage (96.67%) and three times increased in fresh weight after six weeks. However addition of 50mg/L glutamine alone showed lower percentage of callus induction and fresh weight compare to tryptophan and control treatment. The callus proliferation resulted in yellowish colour and nodular appearance showing embryogenic potential. The embryogenic callus was then immersed in 1% Evans blue to validate the viability of cells. Five weeks old potential embryogenic callus was then selected to initiate suspension cell culture. The N_6 liquid medium supplemented with 3mg/L 2,4-D, 1mg/L kinetin and 0.005% pectinase resulted in higher fresh weight (1.84g) of suspension cells compared to without pectinase on day ten of incubation. This study concludes that tryptophan and glutamine did not show significant response on percentage of callus induction and fresh weight. Besides that inclusion of pectinase in suspension cell culture may increase fresh weight of suspension cell.

ABSTRAK

Tujuan kajian ini dijalankan adalah untuk mengenal pasti keberkesanan asid amino (triptofan dan glutamin) dalam penginduksian kalus embriogeni dan mengkaji pembangunan kultur sel ampaian bagi padi bukit Malaysia kultivar Panderas. Untuk penginduksian kalus embriogeni, sampel biji benih yang telah dibuang kulitnya diletakkan di atas permukaan media MSB_5 yang mengandungi 3mg/L 2,4-D dan 2mg/L NAA serta ditambah dengan empat kepekatan berbeza triptofan and glutamin secara berasingan. Selepas tiga minggu dikultur, penambahan triptofan menunjukkan kesan positif. Berdasarkan pemerhatian, media penambahan 25mg/L triptofan dapat menginduksikan kalus sehingga 96.67% dan purata berat basah sebanyak 73.33mg. Purata berat basah kalus yang dikultur di atas media ini meningkat sebanyak tiga kali ganda selepas enam minggu. Media tanpa asid amino (kawalan) mengekalkan peratusan penginduksian kalus (96.67%) tetapi berat basah meningkat sebanyak tiga kali ganda pada minggu keenam. Bagaimanapun, penambahan 50mg/L glutamin sahaja menunjukkan peratus pertumbuhan kalus dan berat basah yang rendah berbanding dengan triptofan dan kawalan. Morfologi kalus embriogeni yang diperolehi menunjukkan warna kuning dan berbentuk nodular. Penentuan kebolehidupan sel diperolehi dengan merendamkan kalus ke dalam 1% Evans biru. Seterusnya, kalus embriogeni yang berumur lima minggu dipilih untuk membangunkan kultur sel ampaian. Media cecair N_6 yang ditambah dengan 3mg/L 2,4-D, 1mg/L kinetin dan 0.005% pektinase menghasilkan berat basah sel ampaian yang tinggi (1.84g) pada hari ke-sepuluh pengeraman. Kajian mendapati triptofan dan glutamin tidak menunjukkan perbezaan ketara terhadap peratus pertumbuhan kalus dan berat basah. Selain daripada itu, penambahan pektinase di dalam kultur sel ampaian dapat meningkatkan berat basah sel ampaian.

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LIST OF SYMBOLS

%	-	percentage
>	-	greater than
°C	-	degree Celsius
g	-	gram
g/L	-	gram/litre
L	-	litre
mg	-	miligram
mg/L	-	milligram/ litre
ml	-	mililitre
mm	-	milimeter
mmol/L	-	milimol/ Litre
M	-	molar
psi	-	pounds force per square
p< 0.05	-	probability at 95% < 0.05
rpm	-	revolutions per minute
v/v	-	volume/ volume
w/v	-	weight/ volume

LIST OF ABBREVATIONS

2,4-D	-	2,4-Dicholorophenoxy acetic acid
AA	-	amino acid
AA_2	-	amino acid with 2mg/L 2,4-D
E	-	embryogenic
EB	-	Evans Blue
HCl	-	hydrochloric acid
IAA	-	indoleacetic acid
MS	-	Murashige and Skoog
MSB_5	-	Murashige and Skoog with B_5 vitamin
N_6	-	Chu N_6 medium
NAA	-	napthyl acetic acid
NaOH	-	sodium hydroxide
NB	-	nutrient broth
NE	-	non embryogenic
pH	-	potential of hydrogen

CHAPTER 1

INTRODUCTION

1.1 Introduction

Rice or *Oryza sativa* L. was classified under the tribe Oryzeae, subfamily Oryzoideae, of the grass family Poaceae (Gramineae) (Lu, 1999). Among 25 species of genus *Oryza* cultivated throughout the world including tropical and subtropical regions of Asia, America, Africa and Australia, the *Oryza sativa* L and *Oryza glaberrima* Steud are the two species cultivated widely. *Oryza sativa* is known as Asian cultivated rice but in fact it is cultivated worldwide while *Oryza glaberrima* cultivation is confined to a few countries in West and Central Africa only. More than half of the world's populations depend on rice as it is one of the most essential cereals and staple food source that provide several important nutrients such as carbohydrate, protein, lipids and minerals. In Asian countries such as Myanmar, Vietnam, Cambodia and Malaysia, rice is a primary diet for more than three billion people that contributed to 50-80% of their daily calorie intake (Khush, 2005). According to FAO (2003), 154 million hectares of land planted with rice harvested 603 million tons of rice worldwide. Asia is the largest rice production countries worldwide coming from China, India and Vietnam with highest rice production in 2012 (FAOSTAT, 2012; Datta, 2004).

It is understood that rice consumers will probably increase two fold by the year 2020 (Khush and Toenissen, 1991) as the population of rice consumers is rising at the rate of 1.8% per annum. Thus production of rice has to be increased by 50% by 2025 to rally the ever growing world population (Khush and Virk, 2000).

Among cultivated rice either as upland or wetland rice, *indica* and *japonica* are the two major subspecies where long grained *indica* contribute 80% of the cultivated rice worldwide (Ramesh *et al*., 2009). Unfortunately the growth in rice yield had decreased to somewhat more than 1% per year (FAO, 2002a) due to the traditional breeding methods that supposed to increase rice yield had reached a plateau. This is due to several impacts imposed by drought, floods, salinity, soil degradation, pests and diseases. However among them, drought stress is considered as serious problem for crop production around the world (Pandey *et al*., 2007). To overcome this crisis, development of the rice varieties with advanced yields, first-rate grain quality, and resistance to various biotic and abiotic stresses must be taken into consideration. Addressing these problems requires the application of tissue culture and genetic manipulation technologies which will contribute further to crop improvement (Khush, 1997). This biotechnological tool depend on genotype of the donor plant, type and physiological status of the explants and plant growth regulators used in the culture medium that manipulate the capability for callus induction and later regeneration in rice tissue culture. Related to that, optimizations for locally favoured rice genotypes were reported in order to utilize upland rice in the gene transformation (Shahsavari *et al*., 2010).

1.2 Background of the Study

Malaysia is one of the countries with increased number of rice consumer that lead the country to import rice to fulfil the demand. Upland rice and wetland rice are the two types of rice grown where upland rice is cultivated in smaller scale by farmers in Malaysia especially in Sabah and Sarawak. The contribution of upland rice was 12% of global rice production but unfortunately it is the lowest-performing ecosystem (Bernier *et al*., 2008) and even in Malaysia, upland rice recorded the lowest rice production (Hanafi *et al*., 2009; Sohrabi *et al.,* 2012). In the present study, tissue culture method was used to carry out study on the effects of inclusion of glutamine and tryptophan in MSB_5 media on frequency of rice callus induction. The fresh weight and embryogenic potential of callus also determined. Later the callus was used to develop suspension cell culture in designated $N_6$3K liquid media (Wagiran *et al*., 2008) and the viability of cell was observed.

1.3 Problem Statement

Rice is a staple food for most developing countries as there is an everyday increasing demand of rice production to fulfil the domestic requirement. In general, wetland and upland rice are two major rice type in Malaysia. The upland rice is largely grown by the rural communities living particularly in Sarawak and Sabah (Paul Vincent, 2010). However, upland rice has been abandoned because of the low and unstable grain yields as the upland rice farmers are poor to contribute the good management of their cultivation, their cultural practices, and the use of the local non-hybrid varieties (Mariam *et al*., 1991). Nevertheless, in recent years, upland rice was taken into attention for more studies because it has competency to survive in drought conditions with high yield (Bernier *et al*., 2008; Geng *et al*., 2008). Therefore, *in vitro* selection and genetic transformation method used to develop natural resistance in plants against all yield-limiting factors. This biotechnology tools provides benefit to

plant breeder as it reduce breeding program times by creating novel rice varieties (Dabul *et al*., 2009).

Nevertheless, finding an effective tissue culture system is important before applying these biotechnology techniques in upland rice because tissue culture system played a major role in production of successful callus induction. The dehusked rice culture approach is one of the many used for callus induction. However, successful callus induction mainly influenced by a number of factors such as plant genotype, explant type, culture medium, plant growth regulator and culture environment (Khanna & Raina, 1998). Furthermore, potential embryogenic calli is mainly required as basic privileges and followed by regeneration system establishment as according to Lee *et al*. (2002), the most crucial step for effective genetic plant transformation is the induction of embryogenic calli in rice. On the other hand, suspension cell culture development with reduced cell aggregation is able to enhance growth rate of cells. Therefore, the aim of this study was to develop efficient protocol for callus induction of *Panderas* rice cultivar since there is very limited study had carried out on the tissue culture system of Malaysian upland rice. In order to do so, the effect of tryptophan and glutamine in various concentrations was investigated and development of efficient suspension cell culture was evaluated in the present study.

1.4 Objectives

The objectives of this research:

i. To study the effect of different concentration of tryptophan and glutamine on embryogenic callus induction
ii. To study the morphology in relation to cell viability of callus
iii. To study the effect of pectinase in suspension cell development

1.5 Scope of Study

Scopes of study for this research is mainly about embryogenic calli formation of *Panderas* cultivar obtained from Kampung Panderas, Jengka, Pahang. Therefore, different concentrations of amino acids (glutamine and tryptophan) were supplemented in tissue culture media to study the callus proliferation. The data obtained from this experiment are based on percentage of callus induction, fresh weight of callus and the embryogenic potential of cells. Later the callus was used to develop suspension cell culture in order to determine the effect of pectinase supplemented in suspension culture media. Data was recorded based on fresh weight and viability of cells.

1.6 Significant of Study

Development of efficient tissue culture system holds the best key in plant biotechnology application and to improve the crop production in upcoming year. Thus this research will be contributing the impact of additive factors such as the role of amino acids as a component of plant tissue culture media in embryogenic calli formation and development based on its morphology observation. Furthermore this study may also be used as reference for further research as limited findings was reported on Malaysian upland rice.

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