Isolation and Culture of Protoplasts from Embryogenic Cell Suspension of *Rhynchostylis gigantea* var. *rubrum*

Rahmat Dzulfikry

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of

Master of Science in Plant Science

Prince of Songkla University

2009

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Bib Key 312119
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Thesis Title	Isolation and Culture of Protoplasts from Embryogenic Cell Suspension of *Rhynchostylis gigantea* var. *rubrum*.
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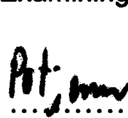
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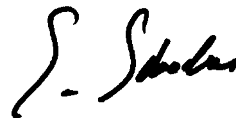
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Major Program	**Plant Science**
Academic Year	**2008**

Abstract

Cell in suspension culture of *Rhyncostylis gigantean var. rubrum* at 9 days after subculture were collected and 1 ml packed cell volume exposed to various concentrations of enzymes. The enzymes were dissolved with mannitol at concentration of 0.75 M in the presence of 100 mM $CaCl_2.2H_2O$, 3 mM MES and adjusted pH to 5.7. The mixtures were incubated on gyratory shaker at 75 rpm for 3 h under dark condition. Isolation of protoplasts was carried out and cultured in three different methods. The result revealed that 0.5 % Cellulase Onozuka R-10 with 0.1 % Macerozyme R- 10 gave the highest yield of protoplasts at 8.9×10^5 /gFW with a high percentage of viability at 94.67%. First division of protoplasts was seen within 24 h in standard plating culture methods. Plating at density of 1.5×10^5 protoplasts / ml using 0.15 % Gellan gum in 0.45 M mannitol provided the highest micro-colony formation at 16 %. However, combination of 1.0 mg/l NAA with 0.1 mg/l BA could induce more microcolony formation in all culturing treatments.

Acknowledgement

I would like to express my gratitude and thankful to Allah S.W.T who gave me the possibility and power to completing this thesis.

I want to thank and expressing my deepest appreciation to my advisor, Associate Professor Dr. Sompong Te-chato for his kindness, helpful suggestions and encouragement throughout the course of this work.

I have furthermore to thank to the chairman of thesis examination : Assistant Professor Dr. Potjaman Suraninpong and Associate Professor Dr. Sayan Sdoodee

I am deeply indebted to Associate Professor Dr. Charassi Nualsri for help, stimulating and encouragement and for her kindness raising inspiration

I deeply wish to thank, to all members at Laboratory of Biotechnology, Faculty of Natural Resources, Prince of Songkhla University for supporting and helping.

Thank to State Educational Department at Indonesia, who being financial supports and gave this program and opportunity to express dedication, inspiration and creation. Appreciated also to Brawijaya University, Malang, Indonesia,

I would like to express my warmest thanks to Prof. Dr. Liliek Sulistyowati as a coordinator of Double Degree Program between Faculty of Agriculture, Brawijaya University, Indonesia and Faculty of Natural Resources, Prince of Songkla University, Thailand, to give me opportunity to follow this program.

For the last part of my expression, I would like to thank to all my lovely family,. Keluarga besar M. Dahlan B.A., my mother, my eldest sister and my youngest sister (love and miss you all).

Rahmat Dzulfikry

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List of Abbreviation and Symbols

BA	: benzyle adenin
CPW	: cell protoplast washing
CW	: coconut water
DNA	: deoxyribonucleic acid
EM	: elution media
g	: gram
gc	: guard cell
MES	: 2-(*N*-morpholino) ethanesulfonic acid
mg/l	: milligram per litre
NAA	: 1-naphtaleneacetic acid
PGRs	: plant Growth Regulators
VW	: vaccin and went
%	: percentage
gFW	: gram fresh weight

Chapter 1
Introduction

Most economically important, orchid cut-flower trade in Asean countries, are thick-leaved. They include *Aranda, Dendrobium, Mokara, Vanda and Oncidium.* Orchid cut-flower production has contributed substantially to the economies of Thailand, Singapore, and Malaysia in particular (Hew, 1994). The orchid cut flower industry in Thailand has been a major foreign exchange earner and 92% of the orchids grown for cut flowers are *Dendrobium* (Kanchanapoom *et al.*, 2001).

Rhynchostylis gigantea var. *rubrum* are on of famous orchids in Thailand. It has a local name, called "Chang Daeng". This orchid has characteristic amethyst-dark purple flowers and is found in Kabinburi, western of Thailand. This species have long period of flowering, between December until February. It's difficult to produce in large scale because time consume for asexual propagation and limited time of flowering. Most orchids have developed highly specialized pollination systems and thus the chances of being pollinated are often scarce (Hew, 1994). This is why orchid flowers usually remain receptive for very long periods and why most orchids deliver pollen in a single mass. Most of the orchids species such as *Rhynchostylis* and especially *Paphiopedilium delenati* found in Asiatic region need to be protected from the danger of extermination through deforestation (Le *et al.*, 1999).

Based on the main problem above therefore a rapid multiplication is needed. It's generally accepted that plant micropropagation is applied for plant breeding in order to overcome some limitations of the conventional breeding. To date protocol for propagation of orchid were successful established such as : high frequency shoot regeneration from *R. gigantea* (Orchidaceae) using thin cell layers (Le *et al.*, 1999), rapid multiplication of *Vanda coerulea* through shoot tip and leaf base culture (Seeni and Latha, 2001), protocorm like body (PLB) formation and plant regeneration from the callus culture of *Dendrobium candidum* (Zhao *et al.*, 2008), and enhancement of growth and regeneration efficiency from embryogenic callus cultures of *Oncidium Gower Ramsey* by adjusting carbohydrate sources (Jheng *et al.*, 2006) Culture of protoplasts

is one kind of propagation techniques which offer many agronomic values. Plant regeneration from protoplasts is possible for large number, and this techniques now seen ideal to be starting point for the genetic manipulation. This phenomenal open a chance for genetic transformation of *R. gigantea* var. *rubrum* using protoplast. Further precise screening techniques provide a great potential for plant improvement through the above -mentioned methods to produce of new variant.

1.1 Objectives of this research

The aim of this research is to isolate high yield and viable protoplasts of *R. gigantea* var. *rubrum* using various concentrations of Cellulase R-10 and Macerozyme R-10 enzymes and set up culturing protocol in order to use protoplasts as a tool for propagation of this species.

1.2 Benefit of this research

Protoplast is an invaluable tool for a variety of studies, including uptake of exogenously supplied materials, such as bacteria, algae, organelles, viruses, and macromolecules like DNA, as well as physiological investigations, transformation assessments, ultra structural studies, and the isolation of sub cellular components, including nuclei, chromosomes, and vacuoles review. The potential of plant regeneration using protoplasts provides the basis for possible cell selection, somatic cell hybridization and genetic manipulation. To pursue this approach, efficient protocols for isolation and fusion as well as plant regeneration are essential.

1.3 Literature Review

1.3.1 Tissue culture

Plant tissue culture (micropropagation) is a technique which can propagate in a short period of time. It is a fascinating and useful tool which allows the rapid production of many genetically identical plants using relatively small amounts of space, supplies and time. Basically the technique consists of taking a piece of a plant (such as a stem tip, node, meristem, embryo, or even a seed) and placing it in a sterile nutrient medium to produce undifferentiated callus tissue, multiply the number of plantlets, grow roots, or multiply embryos for "artificial seed" (Kyte, 2005).

Since the introduction of orchid meristem culture in 1960, a divergence in orchid micropropagation techniques has developed. This divergence manifests itself in the utilization of two distinctly different culture schemes. In one method, the initial explant is induced into an undifferentiated callus or protocorm like body (PLB) state. PLBs are spherical tissue masses that resemble an early stage of orchid embryo development. Proliferation occurs via PLB multiplication, and differentiation into plantlets is permitted only after the desired volume of callus is achieved. The alternate method minimizes the role of callus, and encourages the differentiation of cultures into plantlets early in the procedure. Consequently, proliferation is accomplished by the induction of axillary shoots from plantlets derived from the original explant. These two methods and the attempts to optimize them constitute the bulk of all recent propagative studies (Daniel and Tisserat, 1990). To date the protocols for propagation of orchid were successful established such as : rapid multiplication of *Vanda coerulea* through shoot tip and leaf base culture (Seeni and Latha, 2001), PLB formation and plant regeneration from the callus culture of *D. candidum* (Zhao *et al.*, 2008) and enhancement of growth and regeneration efficiency from embryogenic callus cultures of *O. Gower* var. *Ramsey* by adjusting carbohydrate sources (Jheng *et al.*, 2006).

1.3.2 Callus

Callus is a coherent and amorphous tissue, formed when plant cells multiply in a disorganized way. Callus can be initiated *in vitro* by placing small pieces of the whole plant (explants) onto a growth-supporting medium under sterile conditions. Under the stimulus of endogenous growth regulators or growth regulating chemicals added to the medium, the metabolism of cells, which were in a quiescent state, is changed, and they begin active division. During this process, cell differentiation and specialization, which may have been occurring in the intact plant, are reversed and the explant gives rise to new tissue, which is composed of meristematic and unspecialized cell types. During dedifferentiation, storage products typically found in resting cells tend to disappear. New meristems are formed in the tissue and these give rise to undifferentiated parenchymatous cells without any of the structural order that was characteristic of the organ or tissue from which they were derived. Although callus remains unorganised, as growth proceeds, some kinds of specialised cells may again be formed. Such differentiation can appear to take place at random, but may be associated with centres of morphogenesis, which can give rise to organs such as roots, shoots and embryos. Callus formed on an original explant is called 'primary callus'. Secondary callus cultures are initiated from pieces of tissue dissected from primary callus (George, 2008).

Orchids callus could be induce from many part of orchid explant such as : root, leaf and PLB. Callus formation have been successfully induced from PLB (Khosravi, *et al.*, 2008 and Zhao, *et al.*, 2008). From this observation indicated that the PLB explants contained sufficient endogenous auxin for the induction of calli. To date protocols for callus induction of orchid were successful established such as : callus formation and plant regeneration from callus through somatic embryo structures in *Cymbidium* orchid (Le *et al.*, 2004), callus induction from protocorm-like body segments and plant regeneration in *Cymbidium* (*Orchidaceae*) (Le and Tanaka, 2004), callus induction and somatic embryogenesis of *Phalaenopsis* (Ishii *et al.*, 1998) and induction of callus and plant regeneration from shoot – tip explant from *D. fimbriatum* Lindl. Var. *oculatum* HK. f. (Jonojit and Nirmalya, 2002). For callus of *R. gigantea* var. *rubrum* were successfully

induce with Vaccin and Went (VW) solid media supplemented with 15% Coconut Water (CW), 2% sucrose (Te-chato, Unpublished data).

1.3.3 Cell suspension

Unorganized plant cells can be grown as callus in aggregated tissue masses, or they can be freely dispersed in agitated liquid media. Techniques are similar to those used for the large-scale culture of bacteria. Cell or suspension cultures, as they are called, are usually started by placing an inoculum of friable callus in a liquid medium. Under agitation, single cells break off and, by division, form cell chains and clumps which fracture again to give individual cells and other small cell groups. The walls of plant cells have a natural tendency to adhere, it is not possible to obtain suspensions that consist only of dispersed single cells. Some progress has been made in selecting cell lines with increased cell separation, but cultures of completely isolated cells have yet to be obtained. The proportion and size of small cell aggregates varies according to plant variety and the medium in which the culture is grown. As cells tend to divide more frequently in aggregates than in isolation, the size of cell clusters increase during the phase of rapid cell division. Because agitation causes single cells, and small groups of cells, to be detached, the size of cell clusters decreases in batch cultures as they approach a stationary growth phase (George, 2008).

The degree of cell dispersion in suspension cultures is particularly influenced by the concentration of growth regulators in the culture medium. auxinic growth regulators increase the specific activity of enzymes, which bring about the dissolution of the middle lamella of plant cell walls. Thus by using a relatively high concentration of an auxin and a low concentration of a cytokinin growth regulator in the culture medium, it is usually possible to increase cell dispersion. However, the use of high auxin levels to obtain maximum cell dispersion will ensure that the cultured cells remain undifferentiated. This may be a disadvantage if a suspension is being used to produce secondary metabolites. Well-dispersed suspension cultures consist of thin-walled undifferentiated cells, but these are never uniform in size and shape. Cells with more differentiated

structure, possessing, for example, thicker walls and even tracheid-like elements, usually only occur in large cell aggregates. Many different methods of suspension culture have been developed such as : induction of embryogenic callus and cell suspension culture from shoot tips excised from flower stalk buds of *Phalaenopsis* (Orchidaceae) (Tokuhara and Masahiro, 2001). Generally cell suspension could induce liquid VW media supplemented with 2% sucrose and 15% CW like previously reported by Te-chato *et al* (unpublish data).

1.3.4 Embryogenic cell suspension

Suspension cultures can sometimes be initiated from friable callus (FC) or friable embryogenic tissue (FET) in liquid culture medium under shaking conditions and controlled environments. The cells in suspension still retain the capacity to regenerate somatic embryos freely. Obtaining such cultures is not always a simple matter, for the auxin levels that are often used to promote cell dispersion may result in the loss of morphogenic capability. Embryoids were induced to develop into somatic seedlings when plated onto an agar medium without growth regulators, or with lower concentration of auxin than used at the previous stage. Embryogenesis can be induced in cell suspensions of some plants when the cultures are produced from non-morphogenic callus and have been maintained without morphogenesis for one or more transfers. Induction occurs most readily in recently isolated suspensions and usually becomes much less probable with increasing culture age. Loss of regenerative ability is often associated with the appearance of some cells with abnormal chromosome numbers, but it can also be due to culture on an inappropriate medium. Embryogenesis in suspension cultures seems to require media at Stage I and Stage II with similar compositions to those necessary for somatic embryo formation in callus cultures. Somatic embryos can be formed in suspension cultures in very large numbers. Embryogenesis ceased on a medium containing low concentration of nitrogen, but it was re-induced for several transfers after the culture was returned to a high concentration of nitrogen containing

medium. In a few kinds of plants it is possible to induce embryogenesis in previously unorganised suspension cultures. Success is, so far, recorded only in members of the families Apiaceae (Umbelliferae), Cruciferae and Scrophulariaceae. This is not therefore a method of propagation which can be readily utilised. There is a greater chance of obtaining an embryogenic suspension culture from embryogenic callus. In both callus and cell suspension cultures, cells and embryoids are seldom synchronous, so that there are embryoids at different stages of development. This presents a major drawback for plant propagation, especially from suspensions. A proportion of the seedlings developing from somatic embryos can also be atypical: abnormalities include the possession of multiple or malformed cotyledons, more than one shoot or root axis, and the presence of secondary adventive embryos. Embryos with three cotyledons have been observed to give rise to well-formed somatic embryos do however produce secondary embryos, which are usually of normal morphology (George, 2008).

1.3.4.a Cell growth measurement of *R. gigantea* var. *rubrum*

The importance of plant cell suspension cultures as a research tool in various aspects of plant biology is well established. A basic requirement of work with plant cell suspension cultures is the ability to monitor growth on the basis of various parameters. Reliable and reproducible measurement of growth is essential in order to asses the performance of the culture in general and pinpoint the occurrence of certain metabolic events at given growth stages (Stepan *et al.*,1990).

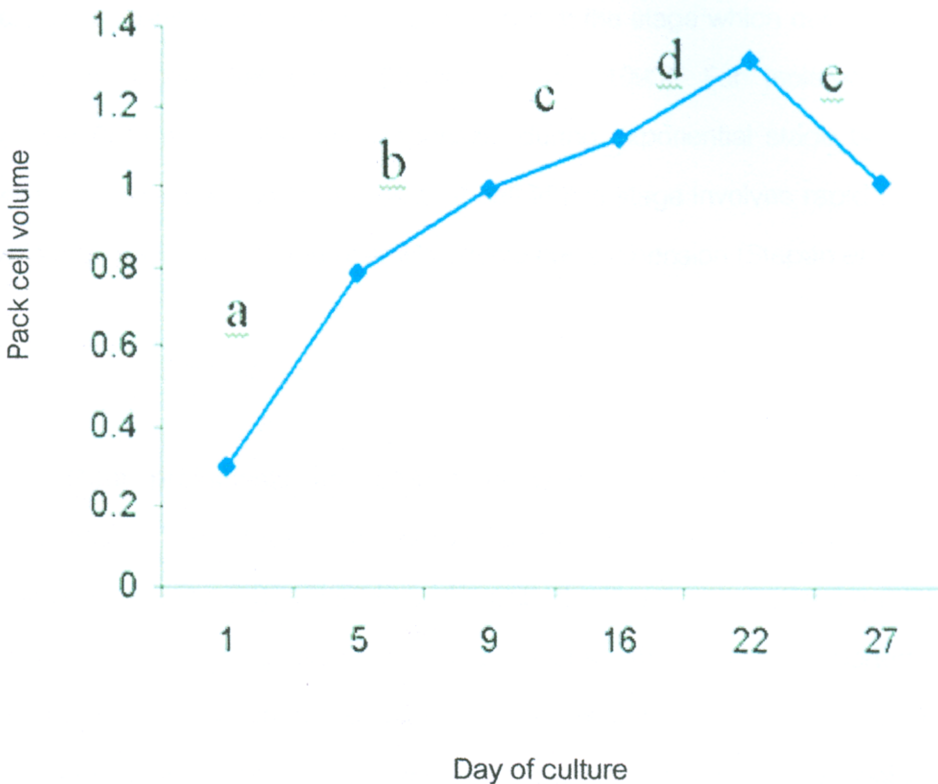

Figure 1 Model curve relating culture growth to time in cell suspension of *Rhyncostylis gigantea* var. *rubrum*.

The various stages of growth are: (a) lag phase, (b) exponential phase, (c) progressive deceleration phase, (d) stationary phase and (e) declaration phase.

Sources : Te-chato (Unpublished data)

Figure 1 showed about growth of cell in suspension culture of *R. gigantea* var. *rubrum*. The data showed lag phase start at first day until 6 days after subculture, then the cells turned to exponential phase and divided rapidly during 9 days until 12 days. After that they divided slowly and produced some secondary metabolites which cause a decrease in growth rate. This state so called stationary phase. After the cells reach to this phase it is necessary to subculture otherwise they are gradually die. So, cell

suspension culture of *R. gigantea* var. *rubrum* were routinely subculture every two week intervals. In general, subculture is carried out at the stage which cell growth rapidly and usually in exponential stage (Stepan *et al.,* 1990). For Isolated protoplasts it's appropriate to isolate the cell suspension during exponential stage between 8-9 days after subculture, because the growth of cell at this stage involves rapid cell division and give a high pack cell volume of embryogenic cell suspension (Stepan *et al.,* 1990).

1.3.4.b Protoplast

Plant protoplasts are cells from which the cell wall has been removed enzymatically. Thus, they retain all the normal cell organelles plus the nucleus which capable of expressing totipotency through the conversion of the protoplast to the regenerated plant using tissue culture technology. Many authors reported that plantlet regeneration from culturing protoplasts is possible from a large number of species. From this success, its open a wide view in improving protoplasts technology. Moreover protoplasts are special tool for genetic engineering technology designed to bring many of prime agronomic values, means that the protoplast is now seen as an ideal starting point for the gamut of genetic engineering technology designed to bring about plant improvement. The protoplast can be manipulated in other area of studies including uptake of exogenously supplied materials, such as bacteria, algae, organelles, viruses, and macromolecules DNA, as well as physiological investigations, transformation assessments, ultrastructural studies, and the isolation of subcellular components, including nuclei, chromosomes and vacuoles (Power and Davey, 1990).

1.3.4.c Isolation of protoplast

At present, isolated protoplasts are used chiefly in research into plant virus infections, and modifying the genetic information of the cell by inserting selected DNA fragments. Protoplasts may also be fused together to create plant cell hybrids. Genetically modified cells will be only of general practical value if whole plants having

the new genetic constitution can be regenerated. The ability to recover plants from protoplast cultures is therefore of vital importance to the success of such genetic engineering projects in plant science. (George, 2008). There are several different methods by which protoplasts may be isolated : 1). by mechanically cutting or breaking open the cell wall; 2). by digesting away the cell wall with enzyme; 3). by a combination of mechanical and enzymatic separation (George, 2008).

Enzymatic method offers the advantage over the first method in terms of viability and quantity. Mechanical and enzymatic methods can also be combined which the cells are firstly separated by mechanical method followed by enzymatically transformed into protoplasts. To date, protoplasts have been isolated from almost every part of the plant (Bajaj, 1976). For successful isolation, it has been found essential to cause the protoplast to contract away from the cell wall, to which, when the cell is turgid and tightly adpressed. Contraction is achieved by plasmolysing cells with solutions of salts such as potassium chloride and magnesium sulphate, or with sugars or sugar alcohols (particularly mannitol) (George, 2008).

To date, many protocols has been reported how to isolated protoplast of orchids, Isolation and fusion of protoplasts from mesophyll cells of *Dendrobium* has been reported by Kanchanapoom *et al.* (2001), they used combination of cellulase Onozuka R-10 (Yakult Honsha Co., Ltd. Lot # 201059) and macerozyme R-10 (Yakult Honsha Co., Ltd. Lot # 201059). Combination of both enzymes was found to be effective in releasing protoplasts from the leaves. Other studies recommended that the mixed of cellulase and macerozyme could be applied effectively in an economic sense for *Dendrobium* Prathum Red. (Kunasakdakul and Smitamana, 2002). Beside the combination of both enzymes, we must consider other chemical that influence high yield and viability of protoplasts. The concentration of mannitol used was found to be effective since lysis of protoplasts was not evident. (Kanchanapoom *et al.*, 2001). These osmotica must be of sufficient concentration to prevent brusting of the protoplasm, but of insufficient strength to cause cellular damage.

1.3.4.d Osmoticum

Sugar alcohols were thought not usually to be metabolised by plant tissues and therefore unavailable as carbon sources. For this reason, mannitol and sorbitol have been frequently employed as osmotica to modify the water potential of a culture medium. In these circumstances, sufficient sucrose must also be present to supply the energy requirement of the tissues. Adding either mannitol or sorbitol to the medium may make boron unavailable. Mannitol was found to be metabolised by *Fraxinus* tissues (Wolter and Skoog, 1966). Later, studies with carrot and tobacco suspensions and cotyledon cultures of radiata pine showed that although mannitol was taken up very slowly, it was readily metabolized (Thompson *et al.,* 1986). Thus sugar alcohol is only of value as a short-term osmotic agent. In contrast, sorbitol is readily taken up and metabolized in some species. Before attempting to isolate protoplasts for the first time from particular tissue it is advisable to determine the correct level of osmoticum. It is often helpful to isolate the protoplasts with osmotic stabilizer at a number of different concentration. In this way the concentration of osmotic stabilizer required to give the maximum yield of viable protoplasts can be established. As to be expected, the actual concentration of osmotic stabilizer required for successful protoplast isolation varied depending upon the plant tissue and the conditions under which the material grown. The level of osmotic potential during protoplast isolation can have a more subtle influence than simply whether or not viable protoplasts are obtained. A number of different sugars and sugar alcohols have been used as osmotic stabilizers, but mannitol is the most frequent choice, no doubt because it only penetrates very slowly into plant cells (Evan and Cocking, 1977).

1.3.4.e Culture of protoplast

To promote growth of protoplasts, it may also be beneficial to add to the medium supplementary chemicals and growth factors required for the culture of intact cells (George, 2008). Protoplasts of orchids have been cultured in modified PS medium

(Kunasakdakul and Smitamana, 2002) supplemented with *1-Naphaleneacetic acid* (NAA), *Benzyl Amino Purine* (BAP), Zeatin and sucrose in various combination. First cell divisions protoplasts in media were detected within 7 – 10 days. This result appropriate with in *Phalaenopsis* (Shrestha *et al.*, 2007), while secondary cell division was difficult to observe. After being cultured for two months, cell cluster were formed in mannitol free liquid modified PS medium and would be transferred to promote the embryogenic callus induction. Shrestha *et al.* (2007) reported that protoplasts culture of *Phalaenopsis* were proliferated into 1.2–1.5 mm size of calli and turned green within 2 months. However, culture medium must be suitable for growth of the protoplasts. Perhaps the best method for the culture of protoplasts is by plating them in agar solidified medium (Bajaj, 1976). By this method a large number of cells or protoplasts can be handled conveniently.

1.3.4.f Application of protoplast technology

Traditional breeding of most woody plants is more or less confronted with certain barriers. Protoplast isolation is pave way for genetic improvement of woody plants. Since the first try on protoplast isolation was conducted much progress has been made in woody plants, covering most of the important species including fruit trees, such as citrus, apple, pear, mango, kiwifruit, litchi and forestry trees, such as sandalwood, poplar, eucalyptus, mulberry, conifer, etc. The present paper reviews the general protocols concerning protoplast isolation, summarizes the factors affecting protoplast isolation and culture, introduce the variations, inclusive of chromosome number, morphology and resistance, occurring in protoplast-derived plants. In addition, application of protoplast to genetic improvement and basic study of woody plants are discussed herein, such as cryopreservation, somatic hybridization, genetic transformation and other basic researches (Cocking, 1972).

1.3.4.g Preparing media and plant growth regulator for protoplast culture

Plant protoplast is very fragile and particularly liable to either physical or chemical damage. Thus if they are suspended in a liquid medium, it must not be agitated, and an equal concentration of osmotic potential of both enzyme solution and culture medium should be temporarily maintained. As growth depends on adequate aeration, protoplasts are usually cultured in very shallow containers of liquid or solid media; fairly high plating density of $5x10^4$ to $1x10^5$ protoplasts/ml may be necessary (George, 2008). The medium proposed by Vacin and Went (VW) supplemented with 2% sucrose and 15% Coconut Water (CW) is recommended. VW media is widely used for germination of hybrid seeds. In the Singapore Botanic Gardens, four past decades, only the VW has been used for culture protoplast of orchids and suitable for the growth of orchid seedlings have been proposed and utilized (Rao, 1976).

1.3.4.h Preparation media for protoplast isolation

There is two media for isolation protoplast can be used:

1. EM- media (elution media) : This media already mixed with cellulase enzyme and macerozyme R-10 with the allowed concentration to isolate the protoplasts prosess from embryogenic cell suspension of *R. gigantea* var. rubrum.
2. CPW-media (cell protoplast washing) : This media use for washing solution and purification protoplast and ready to culture in the next step.
3. Chemical compounds used are driselase, macerozyme R-10, cellulase Onozuka R-10 at various concentrations. These enzymes were dissolved in 0.75 mannitol.

1.3.4.i Isolation protoplast from embryogenic cell suspension

Actively growing young embryogenic cell suspensions are ideal material for the isolation of protoplasts in large quantities. Protoplasts can be readily obtained from such culture by treating the filtered embryogenic cell suspension with combination of cellulose Onozuka and macerozyme R-10 dissolved in 0.75 M mannitol, for 3 h at 28 $\pm$ 0.5°C by a gently shaking on reciprocal shaker. With slight modifications, depending on the age and nature of the material, protoplasts have been isolated from tissue culture of *R. gigantea* var. *rubrum*. Older cell cultures have a tendency to form giant cells with thick walls which are difficult and sometimes impossible to degrade by the enzymes. So it is highly desirable that the cell suspensions should be periodically subcultured. Addition of very low concentration (0.1%) of celluase to cell suspension cultures two days before use, discourages the formation of thick walls. Certain chemicals like colchicines and chelating agents, when added to suspension cultures, also tend to prevent the formation of aggregates and consequently yield is better cell suspensions.

Chapter 2
Materials and methods

2.1 Materials

2.1.1 Plant material

Embryogenic cell suspension of *R. gigantea* var. *rubrum* were kindly provided by Department of plant sciences lab, Natural resources Faculty, Prince of Songkla University, Hat Yai, Thailand and were maintenance in liquid VW media supplemented with 15% CW, 2% sucrose like previous protocol described by Te-chato *et al.* (unpublished data). The suspension was kept on a rotary shaker at 75 rpm under 14 h photoperiod of light provided by cool white fluorescent lamps (35 μmol^{-2} s^{-1}) at 26 $\pm$ 4^0C.

2.2 Methodology

2.2.1 Protoplast isolation and purification

Embryogenic cell suspension at 9 days after transfer to fresh medium was used for protoplast isolation. This present research has 8 combinations of treatment as shown in Table 1. First of all, an optimum concentration of mannitol as osmoticum and incubation time should be examined. In these two experiments 1% cellulase Onozuka R10, 0.5% macerozyme R10 and 0.1% driselase. Those enzymes were dissolved in two different concentrations of mannitol, 0.45 and 0.75 M and used for incubation cell for 3 h. The better result in released protoplasts from mannitol was selected for further study of incubation time, 3, 4 and 5 h. After obtaining an optimum concentration of the above osmoticum (concentration of mannitol) and incubation period kinds and concentrations of enzymes were investigated to find out the best conditions for isolation protoplasts of this orchid. Completely randomized design (CRD) was used to compare statistically difference among treatment and Duncant multiple range test (DMRT) used to separated mean of treatment.

Table 1. Various concentrations of enzymes using for protoplast isolation.

Treatments	Cellulase R-10(C) (%)	Macerozyme R-10(M) (%)	Pectinase (P) (%)
1.	0.5	0.5	0
2.	0.5	0.5	0.5
3.	0.5	0.1	0
4.	0.1	0.5	0
5.	0.1	0.5	0.5
6.	0.1	0.1	0.5
7.	0.1	2.0	0
8.	0.1	2.0	0.5

All combinations of those enzymes were dissolved in an osmoticum shown in table 2.

Table 2. Composition of osmoticum used for dissolving of enzyme and washing the protoplast during protoplasts isolation.

Chemical Compounds	Concentration
$CaCl_2.2H_2O$	100 mM
MES	0.3 mM
Mannitol	0.75 M
pH	5.7

MES : 2-(*N*-morpholino)ethanesulfonic acid

Methods for protoplasts Isolation were carried out according to the protocol that had been described by Te-chato *at al.* (unpublished data). Embryogenic cells suspension of *Rhynchostylis* were collected on centrifuge tube in which adjust pack cell volume until one milliliter (equal to 1 gram/fresh weight) and were centrifuge at 800 rpm for 5 minutes. After centrifugation liquid media in which contain in centrifuge tube were

replaced with 10 ml of enzyme solution as shown in Table 1. The composition of osmoticum need for dissolving the enzyme was shown in Table 2. Sterilization of the enzyme solution was carried out passing trough sterilized Millipore filter membrane with 0.45 µm pore size (Millipore SLHA 033, Carrigtwohill, Ireland). The mixture was incubated in small Petri dishes (35mm x 10mm) and placed on a rotary shaker (75 rpm) for 3 hours under dark condition at 28 °C to liberate the protoplast. The digested cells were filtered through a 77 µm nylon mesh under aseptic conditions and the filtrate was centrifuged at 800 rpm for 5 min. The pellet of protoplasts was washed thrice with washing medium solution (Table 2) to remove all traces of the enzymatic solution. Before the third washing, the yield of the protoplasts was determined by counting the number of protoplasts using a haemacytometer. The viability of the protoplasts was assessed with fluorescein diacetate (FDA) at a final concentration. Method for protoplasts isolation was described in figure 2 below.

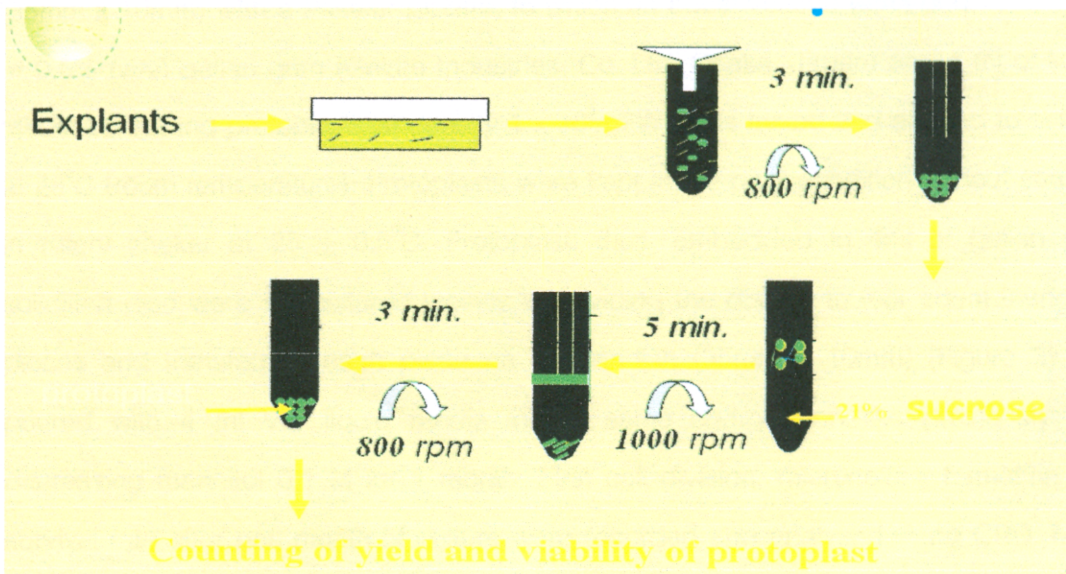

Figure 2 Protocol used for protoplast isolation.

Sources : Te-chato (Unpublished data)

2.2.2 Protoplast culture

We obtained three different methods for culturing protoplasts according to previously reported by Shrestha *et al.* (2007). The best samples out of eight enzyme combination in high yield and viability are taken and cultured in a VW culture medium supplemented with 2% sucrose, 0.45 mannitol and 15 % CW.

2.2.2.a Culturing methods investigation

The cultures were carried out by three different methods; (1) standard plating (2) liquid thin layer and (3) beads method. All cultures were kept in the dark at 25 $\pm$ 0.5°C.

In the standard plating method, purified protoplasts are re-suspended at 1.5 x 10^5/gFW/ml in culture medium containing VW liquid medium, 0.45 mannitol, 2% sucrose, 15% coconut water and adjust pH to 5.0. The mixture was dispensed into a Petridish (35mm x 10mm) with a Pasteur pipette, to which an equal volume (4ml) of 0.15%, 0.3% or 0.5% (w/v) gellan gum (Gelrite Industries, Co. Ltd, Osaka, Japan) and 4 ml of liquid media containing protoplasts at density 1 x 10^5/gFW /ml is mixed and allowed to solidify at 28°C (room temperature). Protoplasts were kept in the dark condition without shaking in rotary shaker at 25 $\pm$ 0.5°C. Protoplast, thus, embedded in 4ml of gellan gum solidified disc were subcultured weekly by dividing the disc in to four equal triangular pieces and transferring each piece on to Petridish (35mm x 10mm) (Figure 3) and poured with 4 ml VW liquid media in the same composition like previously only decreasing mannitol 0.1 M for 1 month. First cell division, microcolony formation and survival rate after one month of culture were recorded and analysed using CRD. Mean among treatments was separated by DMRT.

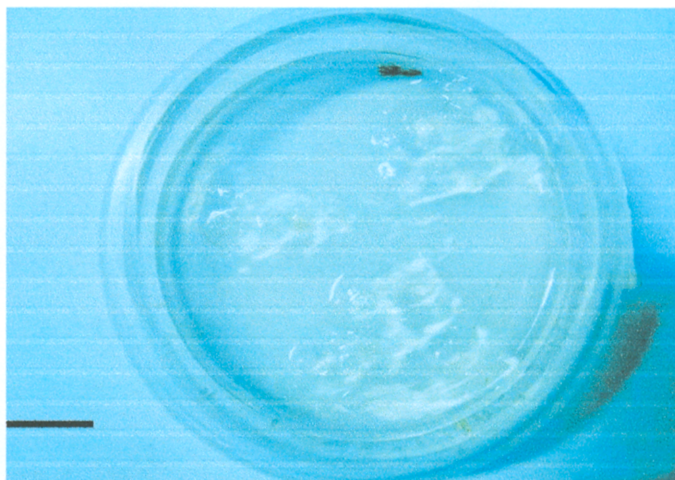

Figure 3 Standard plating method in different concentrations of gellan gum (Gelrite) Industries, Co. Ltd, Osaka, Japan) (bar 35mm).

In beads method, purified protoplasts were mixed and adjusted the density to 1 x 10^5 cells/ml with different concentrations of Na-alginate at 1%, 1.5% or 1.8% (w/v). One ml of the suspension in Na-alginate was mixed with 1 ml of liquid VW medium containing 2 % sucrose, 15 % CW and 0.45 M mannitol and dropped into 100 mM $CaCl_2H_2O$ solution and gave approximately 30 beads from a Pasteur pipette in to each Petri dish (35mm x 10mm) (Figure 4). Protoplasts were kept in the dark condition without shaking in rotary shaker at 25 $\pm$ 0.5°C. The solution of 100mM $CaCl_2H_2O$ was replaced by 4ml of VW liquid culture medium after 1 h. The protoplasts in the beads method were subcultured every week by replacing the surrounding culture medium with the fresh liquid VW medium containing a 0.1M decrease in mannitol concentration. First cell division, microcolony formation and survival rate after one month culture were recorded and analysed by CRD.

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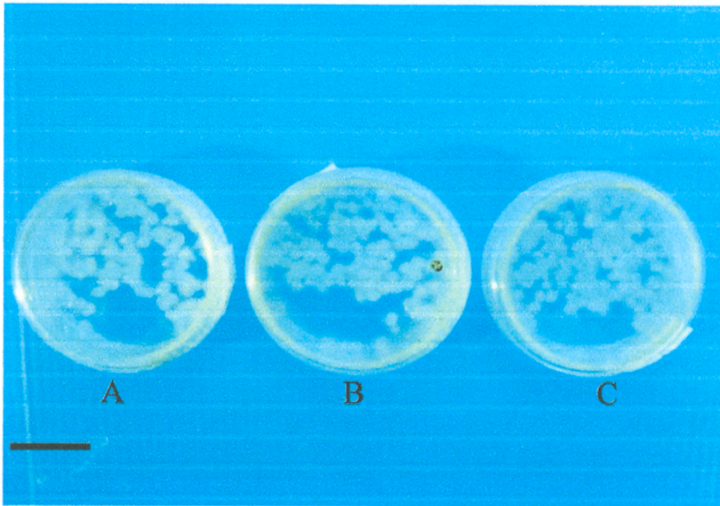

Figure 4 Bead method in different concentrations of Na–alginate as the gelling agents. Each replicate contain 30 beads (bar 35mm).

A: 1% Na-alginate B: 1.5% Na-alginate C: 1.8% Na-alginate

For liquid thin layer method, purified protoplasts were cultured in different densities of protoplasts at 1 x 10^5, 1.5 x 10^5 and 2x10^5 cell/ml. Protoplasts at the last step of washing were centrifuged at 800 rpm for 5 min. Liquid media as washing solution in the last step were replaced with new liquid VW media containing 2 % sucrose, 15 % coconut water and 0.45 M mannitol at 2 ml of each petridish. After gently pipetting one time, protoplasts were plating on small petridish (35mm x 10mm) (Figure 5). Protoplasts were floating on top of liquid media and lead to divide at first day and forming microcolony formation after one month of culture. First cell division, microcolony formation and survival rate after one month culture were recorded and analysis by CRD.

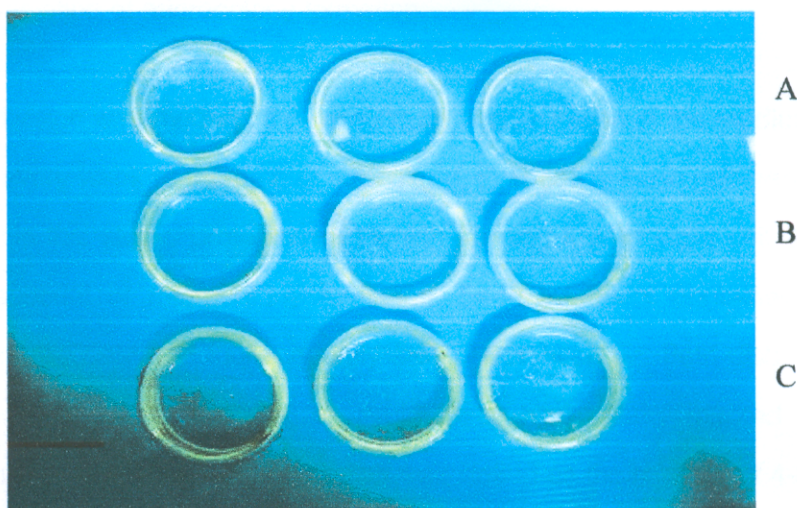

Figure 5 Method in liquid thin layer with different densities of protoplasts. Each treatment contains three replicates (bar 35 mm).

A: $1x10^5$ protoplast/ml B: $1.5x10^5$ protoplast/ml C: $2x10^5$ protoplast/ml

2.2.2.b Plant growth regulator (PGR) investigation

Protoplasts in which gave highest result of division from previously experiment were cultured in VW medium containing 2 % sucrose, 15 % CW, 0.45 M mannitol and adjusted pH to 5.0. The culture medium was supplemented with different concentration of plant growth regulator (PGR), 1.0 mg/l NAA in combination with 0.1 mg/l BA, 0.1 mg/l NAA in combination with 0.5 mg/l BA and 0.1 mg/l NAA in combination with 0.1 mg/l BA. First cell division, microcolony formation and survival rate after one month culture were recorded and analysis by CRD. Mean among treatments was separated by DMRT.

2.2.2.c Observation of microcolony formation

When cell division was evident, the osmoticum concentration of the medium was gradually reduced every week at the time of subculture. First cell division occured in the first day of culture. In this study, protoplasts in which forming four to eight cell division was taken in to account of microcolony formation. Each treatment contained three replicates, and each replicate had four randomly area observation per Petri-dish. In one area of observation at least 25 cells or protoplasts have been marked under microscope. Percentage of microcolony formation was defied as the number of 4-8 cells (obtaining from dividing protoplast) divided by total number of protoplasts in that area (>25 cell) multiply by 100. After culture for 4 weeks first cell division, microcolony formation and survival rate after one month culture were recorded and analysis by CRD

Chapter 3
Results

In order to optimize protoplast isolation, the following parameters were considered : 1) osmolarity, 2) incubation time, 3) Types and concentrations of enzymes, and 4) protoplasts culture

3.1 Osmolarity

The osmolarity of the enzyme solution had a substantial effect on yield and viability of the protoplasts. In this study, two concentrations of mannitol were tested in the enzyme incubation medium and washing medium. From the two concentrations of mannitol used (0.45 M and 0.75 M), the highest number of protoplasts was obtained from 0.75 M mannitol (Table 3) at 8.9 x 10^5 cells/gFW and viability at 94.667%. For 0.45 M mannitol maximum result of yield reached at 6.6x10^5cells/gFW and viability at 83.667% were obtained. There were significant different observed from the both yield treatments. From the observation, it shown that protoplasts derived from embryogenic cell suspension of *R. gigantea* var. *rubrum* in which isolated by using 0.75 M mannitol gave intact and stable protoplasts shape. Mannitol at concentration 0.75 M seem to be more appropriate and significantly different than 0.45 M mannitol. This suggested that the distinct optimum concentration of osmoticum for protoplast releasing was 0.75 M.

Table 3. Effect of different concentrations of Mannitol for protoplasts yield and viability

Mannitol concentration	Yield (X 10^5 protoplasts / gFW)	Viability (%)
0.45 M	6.6	83.76
0.75 M	8.9	94.76
LSD 0.5	2.76	18.76
C.V. (%)	20	5.9

3.2 Incubation time

Incubation time played significant role in yield of protoplast. The longer time of incubation resulted in lower yield and viability. The highest result was found at 3 h after incubation (8.9 x 10^5 cells/gFW), followed by 5 h (6.7 x 10^5/gFW) and 7 h (6.3 x 10^5 cells /gFW) (Table 4). For the viability of protoplasts, the significant difference was observed. The highest protoplast viability was found at 3 h of incubation time (94.7%) followed by 5 h (79.7%) and 7 h (76.3%). It was shown that incubation time longer than 3 h gave lower yield and viability. it is indicated that longer time incubation promoted bursting of the protoplasts. Three hours of incubation time seem to be an appropriate for releasing of protoplasts from embryogenic cell suspension of *R. gigantea* var. *rubrum*.

Table 4. Effect of different incubation times on protoplast yield and viability.

Incubation time (h)	Yield (X 10^5 protoplasts/gFW)	Viability (%)
3	8.9^a	94.67^a
5	6.7^a	79.67^b
7	6.3^a	76.63^b
F- test	*	*
C.V. (%)	14.9	6.3

ns : non significant

* : significant different at $p<0.05$

Mean sharing the same letter in common does not differ significantly by DMRT.

3.3 Type and levels of enzymes

Type of enzymes and their concentrations affected the number of viable protoplasts. To determine the optimum time for cell wall digesting enzyme action which obtaining high yield and viability of protoplasts, eight enzyme mixtures were used. It was found that 8.9 x 10^5 protoplasts /gFW of embryogenic cell suspension and for the

viability reached 94.667% (Table 5). Combination between 0.5 % Cellulase plus 0.1 % Macerozyme seem to be effective for releasing protoplasts of *R. gigantea* in the 0.75 M mannitol, and 3 h incubation time. Freshly isolated protoplasts were spherical in shape at the size of 30 µm (Figure 6A). Viability of protoplasts was higher than 90% after assessment by FDA staining (Figure 6B).

Table 5. Effect of types and concentrations of enyzmes on protoplast yield and viability.

Enzyme Concentration	Yield ($X 10^5$ protoplasts/gFW)	Viability (%)
0.5 % Cell + 0.5 % Mac	7.30[b]	87.67[ab]
0.5 % Cell + 0.5% Mac + 0.1 % Pec	7.26[b]	86.23[bc]
0.5 % Cell + 0.1 % Mac	8.90[a]	94.67[a]
0.1% Cell + 0.5% Mac	2.06[d]	78.67[cd]
0.1% Cell + 0.5% Mac + 0.1% Pec	3.70[c]	76.00[d]
0.1% Cell + 0.1% Mac + 0.1% Pec	3.63[c]	83.67[bcd]
0.1% Cell + 2.0% Mac	2.20[d]	76.33[d]
0.1% Cell + 2.0% Mac + 0.1% Pec	1.96[d]	79.67[bcd]
F- test	*	*
C.V. (%)	13.65	5.42

ns : non significant

* : significant different at $p<0.05$

Mean sharing the same letter in common does not differ significantly by DMRT.

Cell: Cellulase Onozuka R10

Mac: Macerozyme R10

Pec: Pectolyase Y23

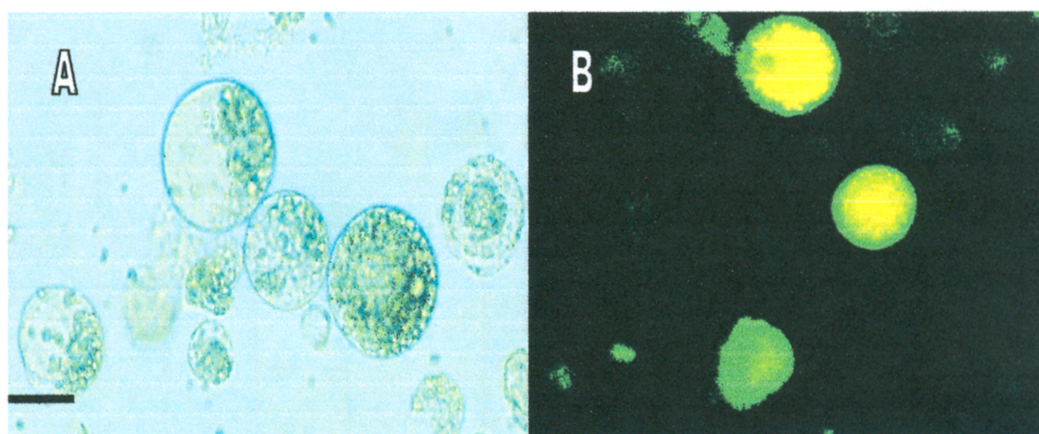

Figure 6 Freshly protoplasts after isolatation by using 0.5% Cellulase and 0.1% Macerozyme **(A)** (bar 20mm) and their viability assessed by FDA staining **(B)** (bar 10 mm).

3.4 Protoplast culture

3.4.1 Effect of gellan gum

3.4.1. a Effect different concentration of gellan gum on protoplast division

The highest yield and viability of protoplasts from samples out of eight enzyme combinations were cultured in VW medium supplemented with 2% sucrose, 0.45 mannitol and 15% CW. First of all, it was necessary to decide the suitable gelling agent. The result from this experiment showed that cell division differed depending on the gelling agents used. The highest microcolony formation after one month of culture was obtained when protoplasts resuspended in 0.15 % gellan gum (16 %), 0.3 % gellan gum (14 %) and 0.5 % gellan gum (12 %), respectively (Figure 7).

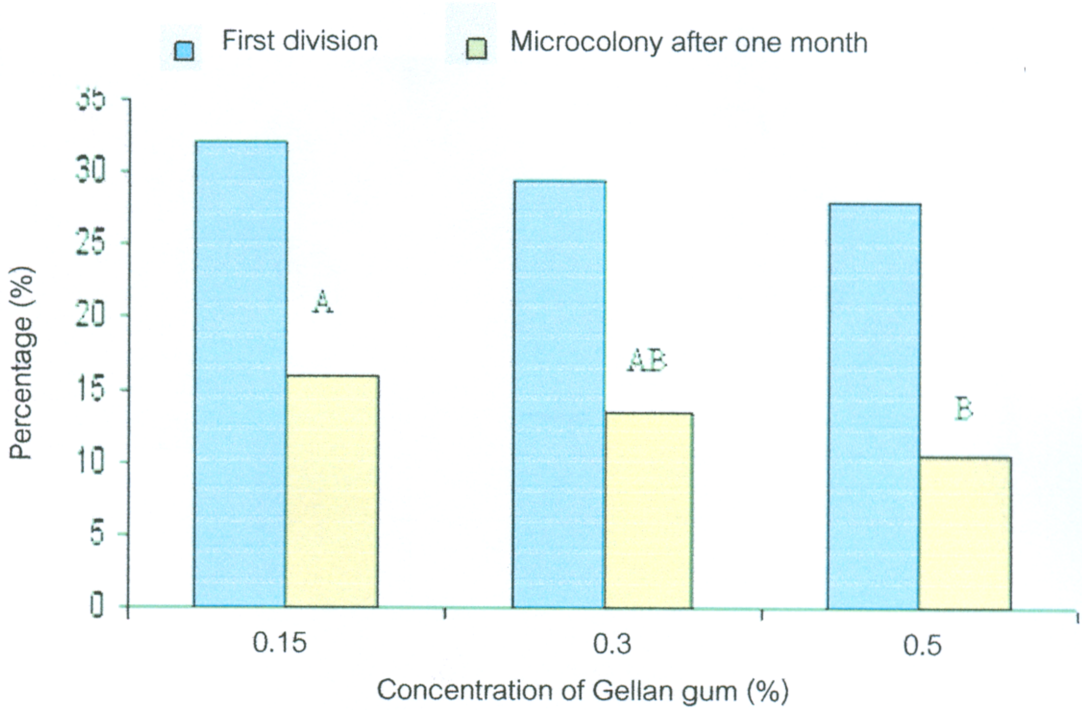

Figure 7 Percentage of first division and microcolony formation after one month of cultured in different concentration of gellan gum.

Different letters on the bars represent significant difference at $p<0.05$.

Among the three different concentrations of gellan gum, significant difference in microcolony formation was observed. The highest percentage of first division was found at 0.15% gellan gum (32.00 %), followed by 0.3% (29.33%) and 0.5% (28.00%). The first division occurred within 24 h of culture and second division was evident after 2– 3 days after culture (Figure 8).

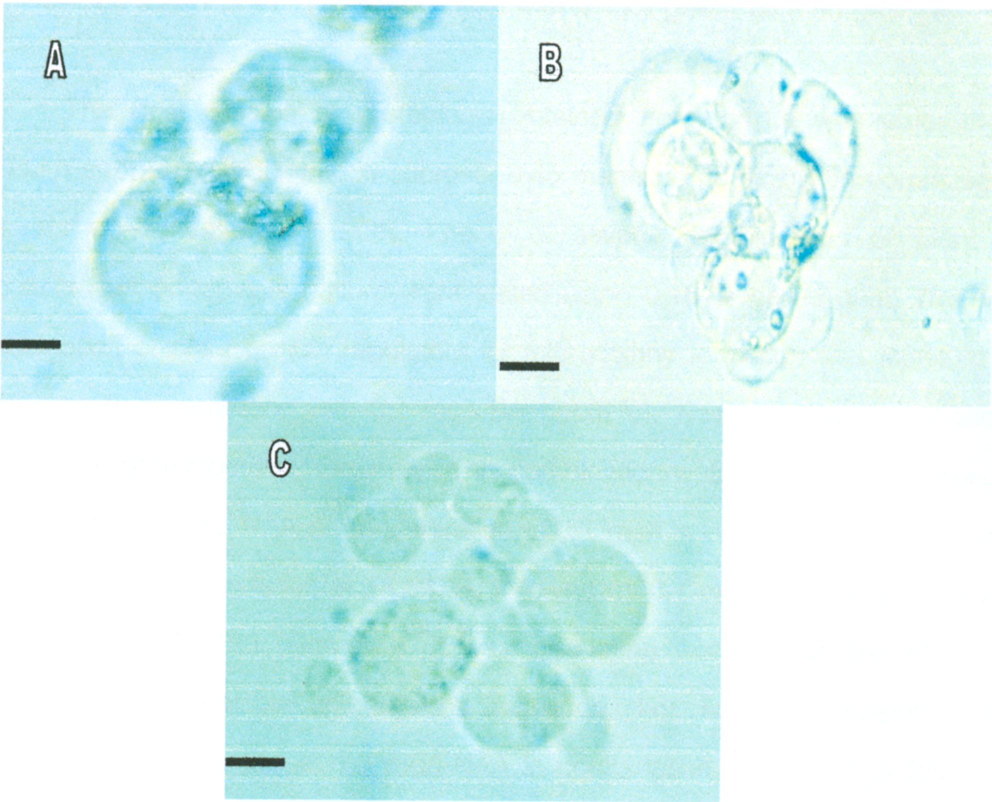

Figure 8 Growth and development of protoplasts after culturing in VW medium supplemented with 2% sucrose, 0.45 M mannitol, 15% CW and various concentration of gellan gum for one month.

A: first division obtained in all concentration of gellan gum (bar 40mm).

B: second division after 2 -3 days of culture (bar 20 mm).

C: microcolony formation after two weeks of culture in 0.15% gellan gum containing VW medium (bar 20 mm).

3.4.1.b Effect of PGR in combination with 0.15% gellan gum on protoplast division

The best division of protoplasts obtained from 3.4.1.a was cultured in VW medium supplemented with 2% sucrose, 0.45 mannitol, 15 % CW in combination with different concentrations of PGR. Effects of several combinations of plant growth regulators on protoplasts culture were tested using 0.15 % gellan gum. The optimum combination of plant growth regulators for microcolony formation was found to be 1.0 mg/l NAA in combination with 0.1 mg/l BA (22.67%). This combination enhanced the percentage of microcolony formation. This combination was significantly difference from the other treatments. NAA at concentration of 0.1 mg/l in combination with 0.5 mg/l BA gave microcolony formation at 18.667%, followed by 0.1 mg/l NAA in combination with 0.1 mg/l BA (17.33%) (Figure 9). It was interesting that combination of auxin with cytokinin could promote microcolony formation. For first cell division significant difference was observed at 1.0 mg/l NAA in combination with 0.1 mg/l BA (36.00%), followed by 0.1 mg/l NAA in combination with 0.5 mg/l BA (33.33%) and 0.1 mg/l NAA in combination with 0.1 mg/l BA gave 30.67% of survival rate. However, microcolony formation up to ten cells were formed after one month of culture (Figure 10).

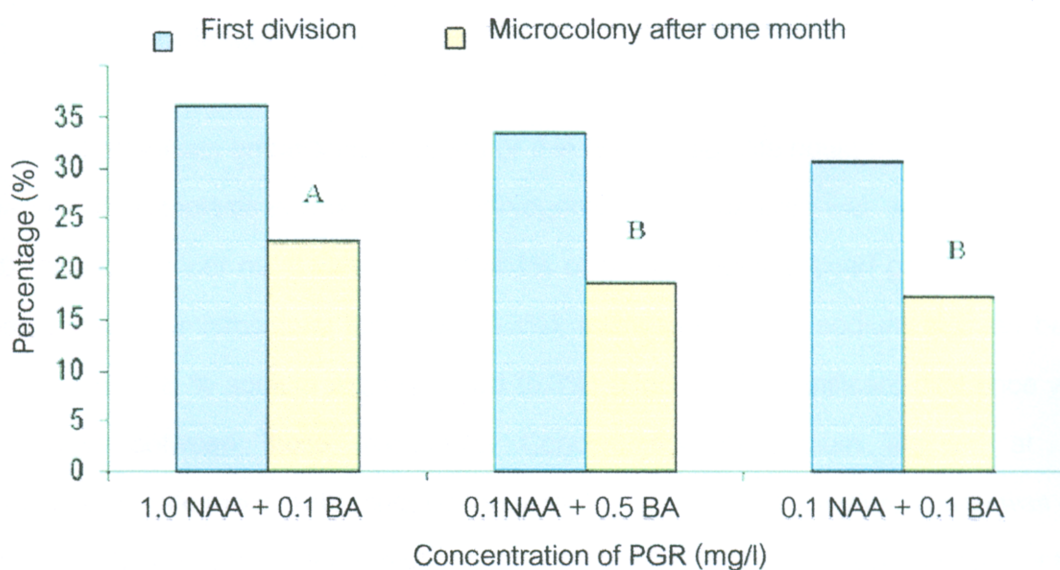

Figure 9 Percentage of first division and microcolony formation in 0.15 % of gellan gum containing VW medium with different concentrations of PGRs.
Different letters on the bars represent significant difference at $p<0.05$.

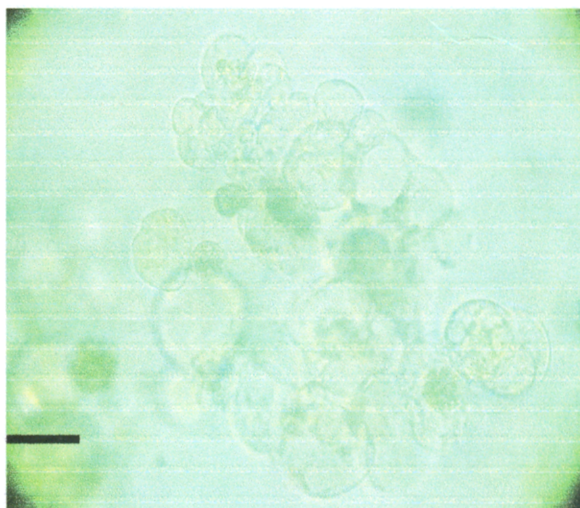

Figure 10 Microcolony formation (up to ten cells) after one month of culture in 0.15 % of gellan gum with 1.0 mg/l NAA in combination with 0.1 mg/l BA (bar 40 mm).

3.4.2 Effect of sodium alginate bead

3.4.2.a Effect of different concentrations of sodium alginate on protoplast division

Protoplasts embedded in beads or thin films of alginate could plate densely. The density of protoplasts isolated from embryogenic cell suspension was adjusted to 1.5 x 10^5 protoplasts per ml. It was found that 1% of sodium alginate bead gave the highest percentage of microcolony formation (9.3%), followed by 1.5% sodium alginate bead (6.6%) and 1.8 % sodium alginate bead (5.3%) (Figure 11). Significant difference was observed between those treatments. Concentration of sodium alginate at 1% significantly difference with another treatment. For first cell division, there was no significantly difference among treatment. It was found that the highest percentage of first cell division was obtained in 1% sodium alginate (24.00%), followed by 1.5% sodium alginate (20.00%) and 1.8% sodium alginate (17.33%). All treatments performed first division at day one and second division at 3 days after culture (Figure 12).

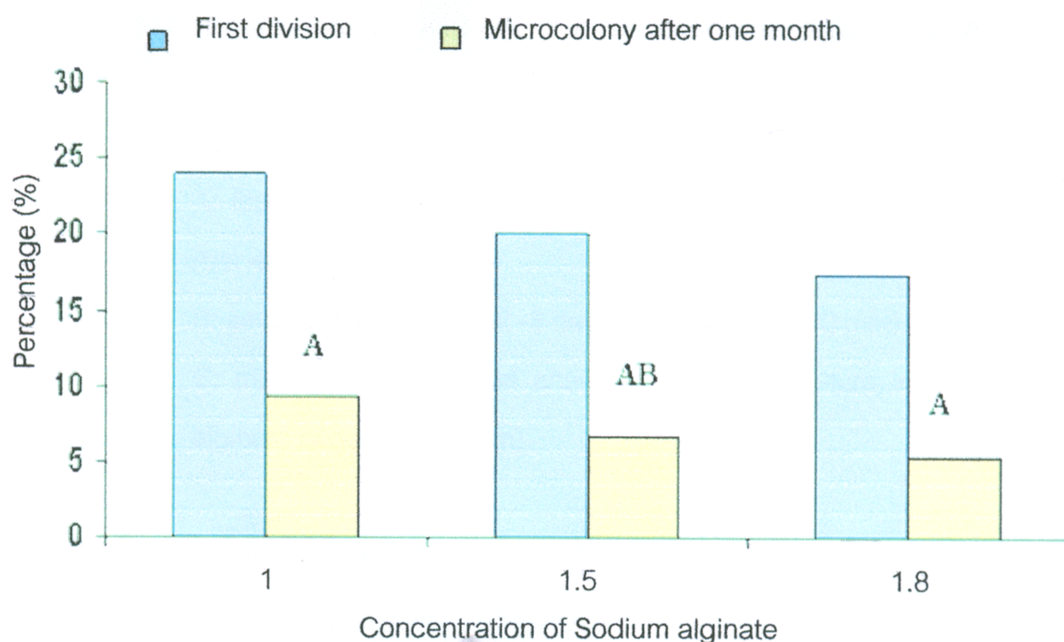

Figure 11 Percentage of first division and microcolony formation in different concentrations of sodium alginate bead.

Different letters on the bars represent significant difference at $p<0.05$.

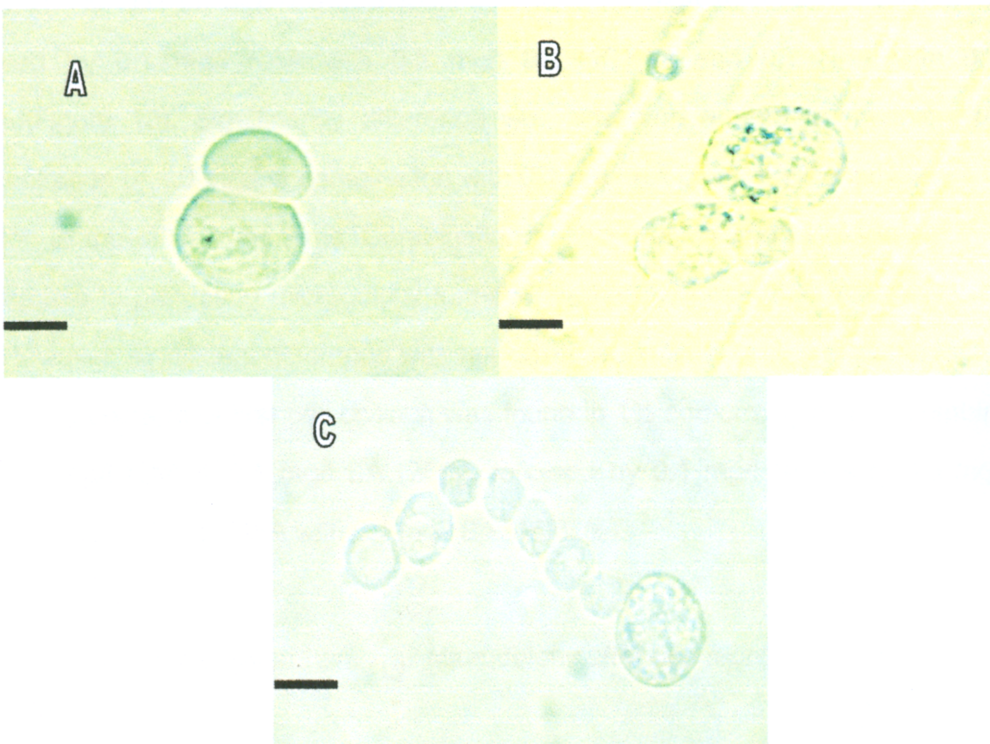

Figure 12 Growth and development of protoplasts after culturing in VW medium supplemented with 2% sucrose, 0.45 M mannitol, 15% CW and various concentration of sodium algenate for one month.

A: first division obtained in all concentrations of sodium alginate bead (bar 40mm).

B: second division after 2 -3 days of culture (bar 20 mm).

C: microcolony formation after two weeks of culture in 0.15% sodium alginate bead (bar 20 mm).

3.4.2.b Effect of PGR with 1% sodium alginate on protoplast division

The best division of protoplasts from previous treatment in an optimum of sodium alginate was used in this treatment. Various concentrations of PGRs were tested together with 1% concentration of sodium alginate containing VW medium supplemented with 2% sucrose, 0.45 M mannitol, 15 % coconut water. Microcolony

formation from isolated protoplasts of embryogenic cell suspension of *R. gigantea* were found in 1% sodium alginate supplemented with 1.0 mg/l NAA and 0.1 BA (13.3%), followed by 0.1 mg/l NAA with 0.5 mg/l BA (10.6%) and 0.1 NAA with 0.1 BA (9.3%)(Figure 13). Significantly difference was observed among treatments. NAA at concentration of 1.0 mg/l in combination with 0.1 mg/l BA gave significantly result in cell division to the other treatments. Combination between auxin and cytokinin seem to be appropriate to promoting microcolony formation rather than the other treatments (Figure 14). However, significant difference was not observed in first division of protoplasts. The highest percentage of first cell division was found in 1% sodium alginate in combination with 1.0 mg/l NAA + 0.1 mg/l BA (26%), followed by 0.1 mg/l NAA with 0.5 mg/l BA (21.33%) and 0.1 mg/l NAA with 0.1 mg/l BA (16.67%).

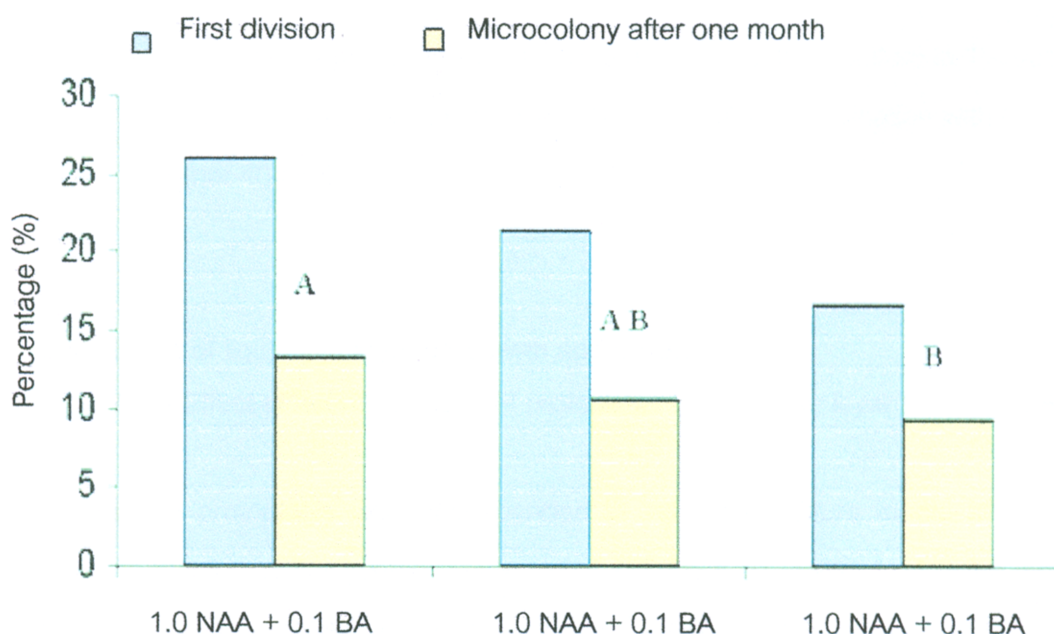

Figure 13 Growth and development of protoplasts in various concentrations of PGRs containing VW medium. The protoplasts were encapsulated with 1% sodium alginate bead.

Different letters on the bars represent significant difference at $p<0.05$.

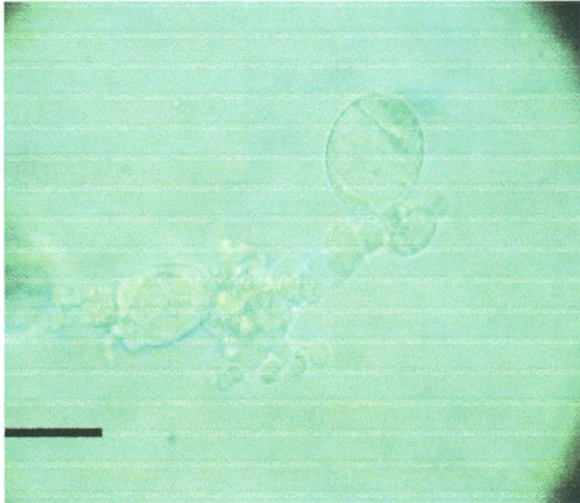

Figure 14 Microcolony formation up to ten cells after one month of culture in 1% sodium alginate bead supplemented with 1.0 mg/l NAA in combination with 0.1 mg/l BA (bar 40 mm).

3.4.3 Effect of liquid thin layer on growth and development

3.4.3.a Effect different densities of protoplasts in liquid thin layer without PGR

Purified protoplasts were resuspended in the liquid culture media at different densities. It was found that protoplasts at density of 1.5×10^5 cells/ml gave the highest microcolony formation (12%), followed by 1×10^5 cells/ml (9.3%) and 2×10^5 cells/ml (8%)(Figure 15). Significant difference was not observed in this treatment. For first cell division it was found that at 1.5×10^5 cells/ml gave the highest percentage of cell division (26.67%) followed by 1×10^5 cells/ml (29.33%) and 2×10^5 cells/ml (25.33%) All treatments provided first division at day one and second division at 3 days after culture (Figure 16).

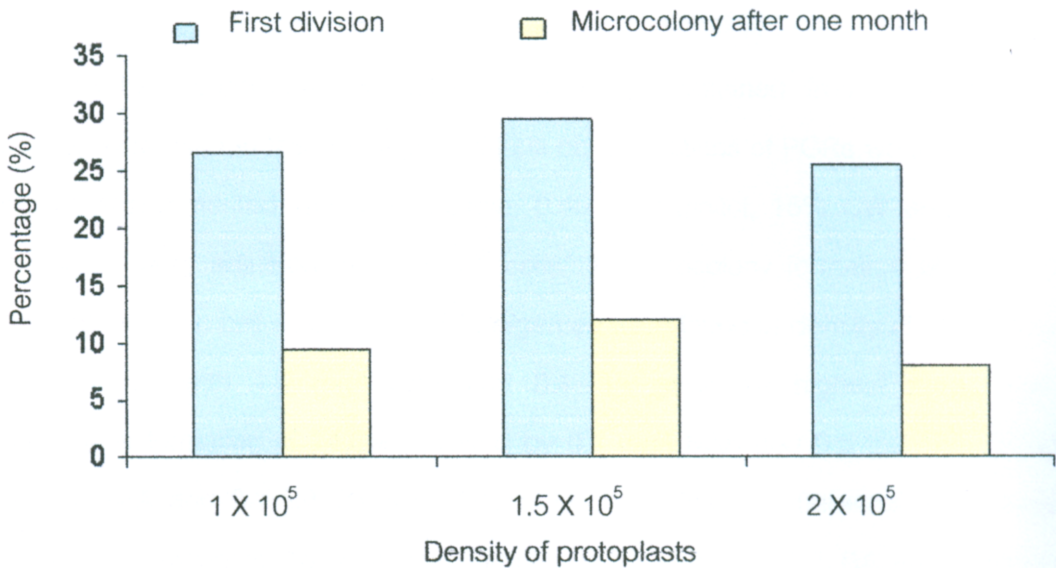

Figure 15 Percentage of first division and microcolony formation of protoplasts after culture as thin layer in liquid VW media with 2% sucrose, 15% CW at different densities.

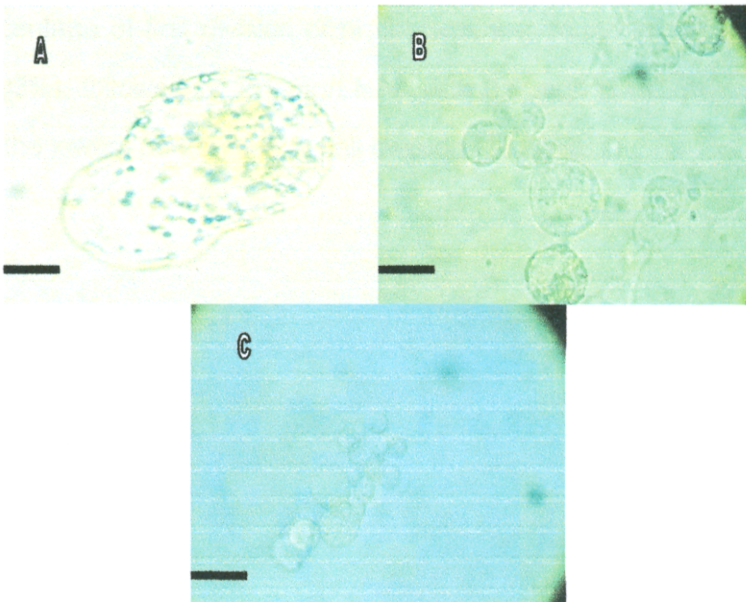

Figure 16 Growth of protoplasts after culture in all liquid thin layer of VW medium with 2% sucrose, 15% CW at density of 1.5×10^5 protoplasts/ml

A: first division after one day of culture (bar 40mm).

B: second division after 2 -3 days of culture (bar 20 mm).

C: microcolony formation after two weeks of culturing (bar 20 mm).

3.4.4.b Effect PGR in liquid thin layer on growth and development of protoplasts

The best conditions for culturing protoplasts obtained from liquid thin layer (3.4.4.a) were used in this treatment. Various concentrations of PGRs were tested in VW medium supplemented with 2% sucrose, 0.45 M mannitol, 15% CW and density of protoplasts were adjusted at 1.5 x 10^5 cells/ml. Microcolony formation of protoplasts from embryogenic cell suspension of *R. gigantea* were found at density of 1x10^5 cells/ml supplemented with 1.0 mg/l NAA and 0.1 BA gave the highest percentage of microcolony formation at 14.6%, followed by 0.1 mg/l NAA and 0.5 mg/l BA (13.33%) and 0.1 NAA and 0.1 BA (10.67%)(Figure 17). Significantly difference was observed among the treatments. NAA at concentration of1.0 mg/l with 0.1 BA gave significant result in growth of protoplasts to the other treatments. Combination between auxin and cytokinin seem to be appropriate for promoting microcolony formation rather than another two treatments (Figure 18). However, Significant difference was not observed in first division. Percentage of first division of protoplasts was found in 1.0 mg/l NAA with 0.1 mg/l BA (29.33%), followed by 0.1 mg/l NAA with 0.5 mg/l BA (28%) and 0.1 NAA with 0.1 BA gave the lowest percentage of cell division (23.33%).

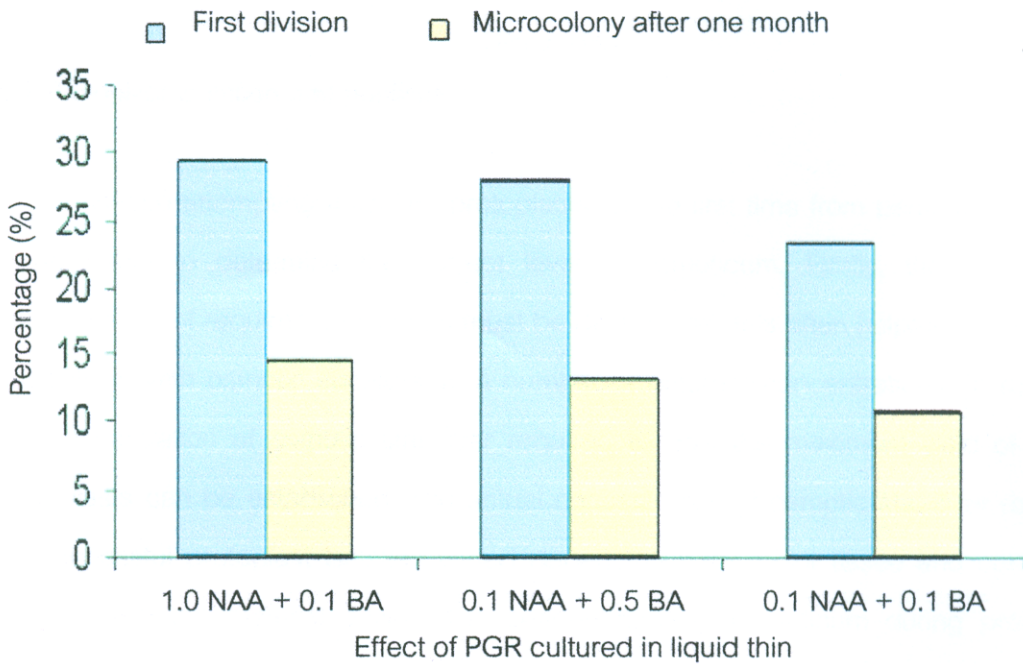

Figure 17 Growth of protoplasts after culture in liquid thin layer VW medium with 2% sucrose, 15% CW and different concentrations of PGRs at density of 1.5 x 10^5 cells/ml.

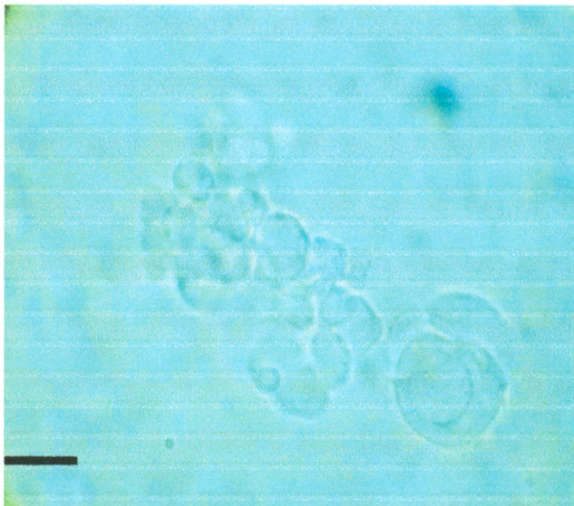

Figure 18 Microcolony formation (up to ten cells) after one month culture at density of 1.5 x 10^5 cells/ml in liquid thin layer VW medium supplemented with 2% sucrose, 15% CW,1.0 mg/l NAA and 0.1 mg/l BA.

Chapter 4

Discussion

4.1 Protoplast isolation and purification

Before attempting to isolate protoplasts for the first time from particular tissue, it is advisable to determine the correct level of osmoticum. Firstly, the appropriate concentration of required osmoticum must be determined. It is often helpful to isolate the protoplasts with osmotic stabilizer at a number of different concentrations. In this way the concentration of osmotic stabilizer required to give the maximum yield of viable protoplasts can be established. The actual concentration of osmotic stabilizer required for successful protoplast isolation varied depending upon plant tissue and conditions under which the material grown. The concentration of osmoticum during protoplast isolation can have a more stable influence than simply whether or not viable protoplasts are obtained. A number of different sugars and sugar alcohols have been used as osmotic stabilizers, but mannitol is the most frequent choice, no doubt because it only penetrates very slowly into plant cells (Evan and Cocking, 1977)..

Mannitol is generally used for osmoticum to keep the cell from bursting like previously reported by Kanchanapoom *et al.* (2001), Kunaksakdakul and Smitamana (2002) who isolated protoplast from mesophyll cells of *D. Pompadaour* and *D. Prathum* red. Successful isolation has been found essential to cause the protoplast to contract away from the cell wall, to which, when the cell is turgid, it is tightly adpressed. Contraction is achieved by plasmolysing cells with solutions of sugars or sugar alcohols (particularly mannitol). These osmotica must be of sufficient concentration to cause shrinkage of the protoplasm, but of insufficient strength to cause cellular damage (Goerge, 2008). From the result revealed that mannitol at concentration 0.75 M gave yield of protoplasts at 8.9×10^5 cells/gFW and viability at 94.667% in compare to concentration of mannitol at 0.45 M which gave yield of protoplasts at 6.6×10^5 cells/gFW. and viability at 83.6%. The higher concentration of osmoticum will obtained high yield and viability of protoplasts too. It is indicating that higher osmoticum concentration could protect protoplasts from bursting. This result contrast to Kaimuk

and Sompong Te-chato (2008) in which using 0.75 M mannitol for isolate leaf of *R. gigantea* var. *rubrum* and gave high yield at 1.28 x 10^6cells/gFW and viability at 92%.

Protoplasts should be incubated in the dark at 25-27°C with shaking under reciprocal shaker at 50 – 75 rpm with different time of incubation depend on the species. Incubation time is needed for enzyme to digesting cellulose and pectin that contain at cell. Data above mentioned at 5 hour for incubation time gave lower yield of protoplasts than 3 hour incubation time, because the activity of enzyme for releasing cellulose and pectin was appropriate at 3 hour of incubation time and many of protoplast were released. At 5 hour incubation period severe damage (bursting) of protoplasts was observed. This evident confirmed by a large number of chloroplasts that burst to outer cell. At 7 hour incubation time gave lowest result, because many cells completely burst. Among these incubation time were tested, it was not significantly difference from protoplasts yield. The optimum incubation period for enzyme activity to release the protoplasts were at 3 hour similar to the result obtained by Kanchanapoom *et al.* (2001).

The use of enzymes for the isolation of protoplasts must take into account to the composition and structure of the primary cell wall. In generally primary cell wall composed of cellulose fibrils coated with a monolayer of hemicellulose, and for dicotyledons this is a xyloglucan. These hemicellulose coated fibrils are linked to each other by pectic polymers which bind covalently to the xyloglucan. Monocotyledons appear to have a cell wall with similar architecture, but hemicellulose is an arabinoxylan rather than a xyloglucan. A further component of the walls of dicotyledons is a glycosylated hydroxyproline rich protein (Evan and Cocking, 1977). In order to degrade such a complex structure as the primary cell wall, a mixture of enzyme would be required with those capable degrading cellulose forming an important component. In general, there are many types of enzymes were used like pectinase, hemicellulase, cellulase and macerozyme (Evan and Cocking, 1977). But now newly come for enzyme preparations and give a more rapid release and often a higher yield of protoplasts and have proved particularly useful in this isolation of protoplasts from cultured cells are

cellulose Onozuka RS or R10 and Macerozyme. These enzymes are now possible to purchase in this slightly more purified and widely used for isolating protoplasts from many plants, especially Orchids spesies (Kaimuk and Te-chato, 2007 ; Kanchanapoom and Tongseedam, 1994 ; Khentry *et al.* 2006 ; Park *et al.*, 1999 ; Pindel, 2007).

The use of 0.5% cellulose Onozuka R-10 with 0.1% Macerozyme R-10 without pectinase gave the highest yield of protoplasts at 8.9 X 10^5 cells/gFW. We know that the main compositions of cell wall of monocotyledon contain cellulose, arabinoxylan and pectin for the middle lamella. High concentration of cellulase was not suggested and from the above result proved that 0.5% of cellulase seem to be appropriate for digesting cell wall. On the other hand, lower concentration of Macerozyme at 0.1% was appropriate to digest middle lamella of cell in which holding cell each other. This result was contrary to Shrestha *et al.* (2007) who isolated protoplasts of *Phalaenopsis* from cell suspension. They reported that 2% Cellulase R-10 and 0.5% Macerozyme R-10 gave the highest result. It seems that in this species *(Phalaenopsis)* higher cellulase (>0.5%) was necessary for releasing of the protoplasts from the cell suspension. But another study reported that concentration of cellulase lower than 0.5% could gave high yield and viability for *Dendrobium* species in which isolated from expanded young leaves of *in vitro* growing plantlets (Kunaksakdakul and Smitammana, 2002).

4.2 Protoplasts culture

One of the important factors for protoplast culture is the kind of gelling agent. As shown in this study, protoplasts isolated from embryogenic cell suspension of – *R.gigantea* divided better in gellan gum than in sodium alginate bead, indicating that gellan gum itself may not be as toxic as the other gelling agents. Protoplasts embedded in bead of alginate or thin layer can plated densely while yet exposed to a large pool of medium that dilutes inhibitors and toxic substances (Goerge, 2008). This result differed to Shrestha *et al.* (2007) who reported that for microcolony formation of *Phalaeonopsis*, Na-alginate used as gelling agent was considered to be better than gellan gum bead culture because it gave compact colonies with small homogenous cells, which readily

turned into green calluses, once transplanted onto regeneration medium. Plant cells are totipotent and have the capacity to unfold their morphogenetic potential to develop into whole plants. By manipulation of the nutritional and physiological conditions this cultured tissue may be induced to regenerate into an intact plant. The highest cell division was seen in 0.15% of gellan gum concentration. It is generally accepted that a gradual reduction of osmotic pressure supports sustained growth of protoplasts derived microcolonies formation. In this study callus formation wasn't observed. Protoplasts in culture started to regenerate new cell wall within few hours, and may take to two several days to complete it. Protoplasts continued to divide in the appropriate conditions, especially nutritional condition. The basic nutrition is needed, however in this study gellan gum was necessary for promoting cell division to form microcolony. Gellan gum at concentration higher than 0.3% affected ability of nutrition uptaking to protoplasts leaded to a low frequency of microcolony formation.

The highest cell division of protoplasts was seen when culturing with standard plating method. However, no callus formation was obtained by using this method. In comparison, standard plating method seem to be appropriate method for protoplast culture over liquid thin layer method and sodium alginate bead method. Sodium alginate bead method gave the lowest percentage of microcolony formation among the other two culturing methods (standard plating and liquid thin layer). These results might imply that bead method gave more damage to the protoplasts. Similar result with Shrestha *et al.* (2007) who reported that in standard method, simply embedding protoplasts in gellan gum gave higher division frequency at the early stage of culture than beads method using gellan gum or alginate as gelling agents. For liquid thin layer method, it seem to give higher result than sodium alginate bead method but still lower result than standard plating method. It is because in liquid thin layer method protoplast wasn't cultured spreadly, protoplasts were collected and floated on the middle of culture Petri-dishes. It makes nutrition cannot transferred to inner cell conveniently. This result was similar to Khentry *et al.* (2007) who reported that percentage of cell division in liquid thin layer method gave lower result than agarose bead culture.

It is generally accepted that plant growth regulator had important role for cell division. Combination between auxin and cytokinin is strongly recommended. It could promoting cell division, transformed mesophyll protoplasts treated with an auxin; NAA together with a cytokinin; BA, strongly enhanced (Goerge, 2008). In all treatments of PGRs, an increase in percentage of cell division was better than without PGRs. High concentration of NAA promoted a high percentage of microcolony formation. Decreasing concentration of NAA caused a low percentage of microcolony formation. Auxin regulation of the cell cycle may involve more diverse mechanisms, including a general stimulation of growth. Combination between auxin and cytokinin could lead microcolony formation.

Chapter 5

Conclusion

From the results of this research work of isolation and culture of protoplasts from embryogenic cell suspension culture of *Rhynchostylis gigantea* var. *rubrum*" the following conclusions could be drawn :

1. The osmolarity of the enzyme solution had a substantial effect on yield and viability of the protoplasts. Mannitol is generally used for osmoticum to keep the cell from bursting. In our study higher mannitol concentration at 0.75 M could gave a high yield and viablity of protoplasts better than mannitol at concentration of 0.45 M.

2. Incubation time is needed for enzyme to digesting cellulose and pectin that contain at cell. Three hour of incubation period gave the best result in releasing protoplasts at 8.9×10^5 cells/gFW with a viability of 94.67%.

3. Cellulase Onozuka R-10 and Macerozyme R-10 were the most widely used enzyme for digesting protoplasts from many species. Cellulase at concentration of 0.5% and 0.1% Macerozyme was chosen because it gave highest yield and viability of the protoplasts.

4. Standard plating methods is the chosen techniques for culturing protoplasts from embryogenic cell suspension of *R. gigantea* var. *rubrum* since it gave a high percentage of microcolony formation than the other techniques.

5. For gelling agent observation, the suitable for culturing protoplasts from embryogenic cell suspension of *Rhnycostylis gigantea* var. *rubrum* was gellan gum in compare to sodium alginate, it is indicating that gellan gum itself maybe not as toxic as other gelling agents.

6. Combination between auxin and cytokinin is strongly recommended. It is indicating that higher concentration of auxin could lead protoplast division in a high percentage of the division.

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