TISSUE CULTURE AND CELLULAR BEHAVIOUR STUDIES OF RICE (*Oryza sativa* L. CV. MRQ 74)

AZANI SALEH

FACULTY OF SCIENCE
UNIVERSITY OF MALAYA
KUALA LUMPUR

2017

TISSUE CULTURE AND CELLULAR BEHAVIOUR STUDIES OF RICE (*Oryza sativa* L. CV. MRQ 74)

AZANI SALEH

THESIS SUBMITTED IN FULFILMENT OF THE REQUIREMENT FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

INSTITUTE OF BIOLOGICAL SCIENCES
FACULTY OF SCIENCE
UNIVERSITY OF MALAYA
KUALA LUMPUR

2017

UNIVERSITY OF MALAYA

ORIGINAL LITERARY WORK DECLARATION

Name of Candidate	:	**Azani Binti Saleh**
I.C/Passport No	:	
Registration/Matrix No	:	**SHC090063**
Name of Degree	:	**Doctor of Philosophy (Ph.D.)**

Title of Project Paper/Research Report/Dissertation/Thesis ("this Work"):

TISSUE CULTURE AND CELLULAR BEHAVIOUR STUDIES OF RICE (*Oryza sativa* L. CV. MRQ 74)

Field of Study: (Science) Plant Biotechnology

I do solemnly and sincerely declare that:

(1) I am the sole author/writer of this work;
(2) This work is original;
(3) Any use of any work in which copyright exists was done by way of fair dealing and for permitted purpose and any excerpt or extract from, or reference to or reproduction of any copyright work has been disclosed expressly and sufficiently and the title of the Work and its authorship have been acknowledged in this Work;
(4) I do not have any actual knowledge nor do I ought reasonably to know that the making of this Work constitutes an infringement of any copyright work;
(5) I hereby assign all and every rights in the copyright to this Work to the University of Malaya ("UM"), who henceforth shall be owner of the copyright in this Work and that any reproduction or use in any form or by any means whatsoever is prohibited without the written consent of UM having been first had and obtained;
(6) I am fully aware that if in the course of making this Work I have infringed any copyright whether intentionally or otherwise, I may be subject to legal action or any other action as may be determined by UM.

Candidate's Signature Date **14 August 2017**

Subscribed and solemnly declared before,

Witness's Signature Date **14 August 2017**

Name : **Rosna Mat Taha**
Designation : **Professor**

ABSTRACT

Tissue culture or *in vitro* studies of rice (*Oryza sativa* L. cv. MRQ 74) locally known as "padi Mas Wangi" has been successfully investigated in this project. Callus induction was obtained on MS media supplemented with various concentrations of 2,4-D, applied singly and in combinations with BAP. Stem was identified as the most responsive explant, followed by root, while leaf explants failed to produce any callus. The highest means of callus dry weight of stem (71.60 ± 6.40 mg) and root (66.70 ± 10.90 mg) explants were recorded on MS media supplemented with 0.5 mg/L 2,4-D and 0.5 mg/L 2,4-D in combination with 0.5 mg/L BAP, respectively. Stem explants produced either creamy white, globular and compact or creamy white, globular and friable callus. On the other hand, creamy white, globular and sticky or mucilageneous callus was observed from root explants. Somatic embryos were induced by transferring the obtained callus from MS media supplemented with 2.0 mg/L BAP in combination with 1.0 mg/L 2,4-D onto MS media containing various concentrations of ABA, kinetin and L-Proline. MS media supplemented with 1.0 mg/L ABA in combination with 1.0 mg/L kinetin showed the highest mean number of somatic embryos (14.33 ± 0.27). The addition of 400 mg/L L-Proline had significantly ($P<0.05$) increased the mean number of somatic embryos (17.37 ± 0.66). Stem was found to be the only responsive explant for *in vitro* regeneration of this species. The best hormone for shoot induction was BAP at the concentration of 1.5 mg/L with mean number of shoots per explant of 4.03 ± 0.31. The highest mean number of roots produced (25.33 ± 1.89) was achieved when stem explants were cultured on MS media supplemented with 0.1 mg/L BAP in combination with 0.1 mg/L NAA. The addition of TDZ at the concentration of 0.1 mg/L had significantly increased the mean number of shoots per explant (8.23 ± 1.09). Synthetic seeds were created from microshoots of stem explants that were cultured on MS media containing 1.5 mg/L BAP. The best encapsulation matrix was Ca-free MS supplemented

with 30 g/L sucrose with survival rate of 100 %, after 30 days of culture. The survival rate of plantlets (100 %) were best achieved on MS basal and MS media supplemented with 0.1 mg/L BAP. It was found that the viability of seeds decreased from 93.33 % to 3.33 % after one month of storage at 4 ^{o}C. Regenerated plantlets from stem explants cultured on MS media containing 0.5 mg/L 2,4-D were successfully acclimatized on all types of growing substrates with different survival rates of plantlets. A combination of black soil and red soil at a ratio of 1:1, showed the highest survival rate after 4 and 8 weeks of acclimatization, 90.00 ± 1.53 % and 83.33 ± 1.20 %, respectively. Cytological studies revealed that Mitotic Index (MI) values of root tip meristem cells was significantly lower in MS media supplemented with NAA, kinetin and 2,4-D as compared with hormone-free MS. The obvious effect of 2,4-D was observed on nuclear DNA content, mean cell and nuclear areas.

ABSTRAK

Kajian kultur tisu ke atas pokok padi (*Oryza sativa* L. cv. MRQ 74) atau nama tempatannya "Mas Wangi" telah berjaya dijalankan di dalam projek ini. Induksi kalus telah didapati dalam media MS yang ditambah dengan berbagai kepekatan 2,4-D dan dicampur dengan BAP. Eksplan batang telah dikenalpasti sebagai eksplan yang paling responsif, diikuti oleh akar, manakala eksplan daun gagal menghasilkan kalus. Purata berat kering kalus yang paling tinggi daripada eksplan batang (71.60 ± 6.40 mg) dan (66.70 ± 10.90 mg) dari akar telah direkodkan dalam media MS yang telah dibekalkan dengan 0.5 mg/L 2,4-D dan 0.5 mg/L 2,4-D dengan kombinasi 0.5 mg/L BAP, masing-masing. Eksplan batang menghasilkan kalus samada krim keputihan, globular dan padat atau krim keputihan, globular dan rapuh. Sebaliknya, kalus krim keputihan, globular dan melekit atau berlendir telah diperhatikan daripada eksplan akar. Embrio somatik telah diaruh dengan memindahkan kalus yang terhasil daripada media MS yang ditambah dengan 2.0 mg/L BAP dikombinasikan dengan 1.0 mg/L 2,4-D ke atas media MS yang mengandungi berbagai kepekatan ABA, kinetin dan L-Proline. Media MS yang ditambah dengan 1.0 mg/L ABA dikombinasikan dengan 1.0 mg/L kinetin menunjukkan purata bilangan embrio somatik yang paling tinggi (14.33 ± 0.27). Penambahan L-Proline pada kepekatan 400 mg/L telah meningkatkan purata bilangan embrio somatik dengan bererti (17.37 ± 0.66). Hanya eksplan batang didapati responsif untuk regenerasi lengkap secara *in vitro* bagi spesies ini. Hormon yang paling sesuai untuk induksi pucuk adalah BAP pada kepekatan 0.5 mg/L dengan purata bilangan pucuk per eksplan sebanyak 4.03 ± 0.31. Penghasilan akar yang paling banyak (25.33 ± 1.89) telah dicapai apabila eksplan batang dikultur di atas media MS yang ditambah dengan 0.1 mg/L BAP dengan kombinasi 0.1 mg/L NAA. Penambahan TDZ pada kepekatan 0.1 mg/L telah meningkatkan purata bilangan pucuk per eskplan dengan bererti (8.23 ± 1.09). Biji benih sintetik telah dihasilkan dengan menggunakan pucuk-

pucuk mikro yang diperolehi daripada eksplan batang yang telah dikultur dalam media MS yang dibekalkan dengan 1.5 mg/L BAP. Matrik kapsul yang paling baik adalah MS tanpa kalsium yang mengandungi 30 g/L sukrosa dengan 100 % kadar kelangsungan hidup, selepas 30 hari dikultur. Didapati bahawa daya kehidupan biji benih berkurangan daripada 93.33 % ke 3.33 % selepas satu bulan disimpan pada suhu 4 ^{o}C. Anak pokok yang didapati secara *in vitro* telah berjaya diaklimatisasi ke atas semua jenis substrat pertumbuhan dengan kadar kelangsungan hidup yang berbeza. Kombinasi tanah hitam dan tanah merah pada nisbah 1:1 menunjukkan kadar kelangsungan hidup yang paling tinggi selepas 4 dan 8 minggu diaklimatisasi, 90.00 ± 1.53 % dan 83.33 ± 1.20 %, masing-masing. Kajian sitologi mengesahkan bahawa nilai indek mitotik (MI) sel-sel meristem akar lebih rendah dengan bererti bagi media MS yang telah dibekalkan dengan NAA, kinetin dan 2,4-D berbanding dengan media MS tanpa hormon. Kesan 2,4-D yang ketara telah diperhatikan ke atas kandungan DNA nukleus, luas sel dan nukleus.

ACKNOWLEDGEMENT

Bismilahhirahmanirahim.

Alhamdulillah, all praises to Allah for His blessing and guidance, giving me strength in completing this PhD thesis.

I would like to acknowledge the Ministry of Higher Education (MOHE) and the Universiti Teknologi MARA (UiTM) for the financial support throughout the completion of my PhD studies.

I would also like to express my deepest gratitude to my supervisor, Prof. Dr. Rosna Mat Taha for her excellent supervision. Her invaluable guidance, encouragement and patience throughout the experimental work and writing of this thesis had contributed the success of this research.

Sincere thanks to my fellow labmates (Lab B2.5), Azah, Kinah, Shima, Ina, Ain, Noraini, Sha, Diha, Anis, Azimah and Umi for making my four years in UM very meaningful. The times that we have spent together have been so cheerful and it will always be forever in my memory.Thanks for the help and friendship.

Special thanks goes to my sisters, brother and nephews for their endless love, prayers, care, support, encouragement and understanding. You were always there for me whenever I needed to hear your voice, which made me feel so calm and at peace. To my beloved husband, thank you very much for your tolerance, love and care.

To those who indirectly contributed in this research, your kindness means a lot to me. Thank you very much.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

ABA	Abscisic acid
ANOVA	Analysis of variance
BAP	6-Benzylaminopurine
2,4-D	2,4 – Dichlorophenoxyacetic acid
DMRT	Duncan Multiple Range Test
g/L	gram per liter
HCl	Hydrochloric acid
IBA	Indolebutyric acid
Kinetin	6-furfurylaminopurine
kPa	Kilo pasca
LSD	Least significant differences
mg/L	Milligram per liter
MI	Mitotic index
MS	Murashige and Skoog
MSO	Murashige and Skoog (without hormone)
NAA	Naphthalene acetic acid
NaOH	Sodium hydroxide
SE	Standard error
SEM	Scanning electron microscope
TDZ	Thiadiazuron
v/v	Volume per volume
w/v	Weight per volume

LIST OF APPENDICES

CHAPTER 1

INTRODUCTION

1.1 RESEARCH BACKGROUND

Tissue culture or *in vitro* studies are very useful tool to many plant species. Almost all plant species can regenerate into complete plants provided the media, hormones and cultural conditions are identified precisely. Generally, dicotyledons generate more easily than monocots. Rice (*Oryza sativa* L.) is the most important staple food for more than half of the world's population and is a model monocot plant due to its relatively small genome size, approximately 430 Mb, which has been completely sequenced (Summart *et al*., 2008). Rice consumers are increasing at the rate of 1.8 % per year (Saharan *et al*., 2004). Therefore, rice production has to be increased to 50 % in the year 2025 (Rashidun, 2012). Most Asian countries are trying to achieve and maintain self-sufficiency in rice production. Malaysia's self-sufficiency level for rice production is about 71.4% and the balance is imported from other countries abroad (Chamhuri *et al*., 2014).

Consumption of aromatic rice has been gaining popularity in Malaysia and around the world. For example, rice imported into the United States is mostly aromatic Thai jasmine and Indian and Pakistan basmati. In Malaysia, Mas Wangi or rice cultivar MRQ 74 is preferred by Malaysian consumers due to less starch content and low glycemic index (GI) and hence it is good for health and suitable for diabetics (Golam *et al*., 2012). Thailand, India, and Pakistan are predominantly leading producers and exporters of high quality aromatic rice. Nevertheless, recent success developments of new aromatic rice emerged from countries outside Asian continent such as the United States. The first adapted aromatic rice released in the United State was Jasmine 85, the cultivar derived from the International Rice Research Institute (IRRI) in 1989. However, it was

not popular among the United States consumers because of its color, creamy grain appearance, weak aroma and flavor. Therefore, the new American aromatic rice variety, Jazzman-2 was released in 2011. Its color and softness are close to Thai Jasmine rice with better aromatic fragrant than Thai Jasmine rice. However, aromatic rice varieties have undesirable agronomic characters such as susceptible to diseases and pests, prone to abiotic stresses and they generate relatively low yield compared to other varieties (Napasintuwong, 2012). These problems could be solved through innovations of science and technology.

Biotechnology is the most important tool for many aspects in rice improvement. However, the success of this technology requires information and knowledge in the field of rice tissue culture. Production of callus and its subsequent regeneration are the prime steps in crop plants to be manipulated by biotechnology means (Saharan *et al*., 2004). Somatic cell culture that has been employed widely in tissue culture system provides a source of variations. In China, anther culture has been used successfully to developed new cultivars of indica rice with good agronomic quality such as high yield, short maturation period and favourable in flood-prone zone (Zheng, 2003).

1.2 PROBLEM STATEMENT

Successful tissue culture of rice has been reported by a number of researchers. Several methods have been used to establish rice tissue culture system such as anther culture, protoplast fusion and culture, leaf culture, root culture, mature seed culture and immature embryo culture. Many types of rice explants have been utilized including immature and mature embryos, anthers, pollen, shoots, root tips and coleoptiles. However, immature zygotic embryos have long been considered as the most suitable explants for many obstinate species including rice. This is due to the fact that younger tissues have greater potential to produce embryos and organs compared with more differentiated mature tissues (Delporte *et al*., 2014). Nowadays, immature and mature zygotic embryos have been the preferred explants for many plant species. According to Lee *et al*., (2002), mature embryos have distinct advantages over immature embryos as starting materials for *in vitro* regeneration in producing transgenic rice.

To date, a highly efficient and reproducible regeneration method from root and stem explants are still lacking in rice. Thus, the question arises whether the root and stem explants could give significant response in tissue culture system. In addition, the success of regeneration is determined by several factors with genotype being the most important factor. In fact, the significant variation response in tissue culture system was observed within indica rice varieties. This indicates that the developed protocol is only destined for a particular variety or cultivar or species. Therefore, tissue culture of Malaysian aromatic rice (*Oryza sativa* L. cv. MRQ 74) from stem explants was investigated in the present work.

1.3 RESEARCH OBJECTIVES

Plant cells are totipotent, i.e., whole plants can be regenerated from single cell by manipulating culture conditions. In the present study, the aromatic variety of *Oryza sativa* L. cv. MRQ 74 with high economic value was subjected to *in vitro* culture system. The general objective of the study was to establish an efficient protocol for callus induction, somatic embryogenesis induction and *in vitro* regeneration of aromatic rice (*Oryza sativa* L. cv. MRQ 74) via tissue culture system using root and stem explants.

The specific objectives were: 1) To find the best combinations and concentrations of plant growth regulators for optimum callus and somatic embryogenesis induction, and *in vitro* regeneration; 2) To determine the best encapsulation matrix and germination substrate for synthetic seeds of rice; 3) To identify the best sowing substrate to increase survival rate of the acclimatized plantlets and 4) To perform cytological investigation to detect somaclonal variation by considering mitotic index, chromosome count, cell and nuclear areas and nuclear DNA content.

1.4 SCOPE OF RESEARCH

The present study was undertaken in order to establish efficient protocols for callus induction, somatic embryogenesis and *in vitro* regeneration of the most popular and economically important Malaysian aromatic rice, *Oryza sativa* L. cv. MRQ 74 (Mas Wangi). The best callus induction media was identified as MS supplemented with 2,4-D and BAP. An experiment on somatic embryogenesis induction was carried out using ABA, kinetin and L-Proline. Different types, concentrations and combinations of plant growth regulators such as BAP, IBA, kinetin, NAA and TDZ were utilized to determine the optimum concentration for *in vitro* regeneration. of *Oryza sativa* L. cv. MRQ 74.

Since rice is consumed by more than one third of the world's population, rice production should be increased to fulfill human need. Furthermore, world population is estimated to increase to 45.7 million in 2030. Therefore, the ability of *in vitro* regenerated plantlets to grow under normal environmental conditions was investigated. The production of synthetic seeds from microshoots and synthetic seeds germination on various sowing substrates was also examined. Microscopic study was carried out using Scanning Electron Microscope (SEM) to compare the ultra structures of leaves from *in vivo*, *in vitro* and synthetic seeds.

The effects of plant growth hormones on cellular activities of root tips meristem cells grown *in vivo* and *in vitro* was also investigated. This is due to the fact that the presence of plant growth hormones in culture media may induce somaclonal variations in generants. Hence, mitotic index, mean cell and nuclear areas, chromosome counts and nuclear DNA content of *in vitro* grown root tip meristem cells were taken into consideration in determining the occurrence of somaclonal variation, and compared it with the root tip meristem cells grown *in vivo* as a reference or background data. The

growth of primary roots was also measured in order to obtain the standard root length of the samples.

CHAPTER 2

LITERATURE REVIEW

2.1 PLANT TISSUE CULTURE

Plant tissue culture is a technique of *in vitro* cultivation of plant cells and organs, in which the cells divide and regenerate into callus or particular plant organs. Tissue culture techniques are well established for dicotyledonous and most monocotyledonous plants. However, there are some restrictions for culturing species belonging to graminae family including rice because of their extremely recalcitrant behaviour for *in vitro* manipulation. Tissue culture of rice was started with the culture of excised roots by Fujiwara and Ojima (1955), followed by Amemiya *et al*., (1956) using immature embryos. Rice was reported as the first cereal to regenerate into whole plant (Vasil, 1983). Morphogenic and regeneration studies of 66 rice varieties was done by Abe and Futsuhara (1986). They observed large differences for the tissue culture ability between japonica and indica rice varieties. The japonica varieties produced high callus yield and regeneration ability than indica. These results indicate that the successful of tissue culture technique relies on factors such as genotype, explant types, aseptic environment and composition of culture medium.

2.1.1 Factors Influencing the Success of Tissue Culture

The composition of culture medium is a major determinant of *in vitro* growth of plants. Sugar is an important component in culture medium and its addition is essential as energy source for *in vitro* growth and development of plants due to unsuitable conditions for photosynthesis in culture containers. Since most plant cultures are unable to photosynthesize effectively due to inadequately developed cellular and tissue development, lack of chlorophyll, limited gas exchange and carbon dioxide in tissue culture vessels, they need external carbon source for energy. The commonly used sugar

is sucrose at a concentration of 2 – 5%. While autoclaving the medium, sucrose is hydrolysed to glucose and fructose which are then used up for growth. The sugar concentration chosen is dependent on the type and age of growth materials. A very young embryo requires a relatively high sugar concentration. Sucrose not only acts as an external energy source but also contribute to the osmotic potential of the medium (Nowak *et al*., 2004) which would permit the absorption of mineral nutrients present in medium. A significant effect of carbon source concentration in culture media on the frequency of callus formation has been observed in many plants including rice (Shahnewaj and Bari, 2004).

The type and concentration of sugar used in media influences somatic embryogenesis. Sucrose has been most frequently employed to induce somatic embryogenesis (Malgorzata, 2004). Besides sucrose, glucose and fructose are also known to support good growth of some tissues (Bhojwani and Razdan, 2004). It was reported that glucose was the optimal type of sugar for somatic embryo development in culture of *Panax ginseng* callus (Tang, 2000). On the other hand, fructose promoted somatic embryogenesis of *Linum usitatissinum* (Cunha and Fernandes-Ferreira,1999). Besides sugar, plant growth regulators play an important role in plant tissue culture system. The effects of plant growth regulators on *in vitro* regeneration have been reported for many plant species (Mroginski *et al*., 2004). The type of morphogenesis that occurs in plant tissue culture system largely depends upon the ratio and concentrations of auxins and cytokinins present in the culture medium. Callus formation is controlled by growth regulating substances present in the medium containing auxin and cytokinin (Shah *et al*., 2003). Vietiz and San Jose (1996) reported that the exogenous supply of growth regulators is frequently necessary in callogenesis. The specific concentration of plant growth regulators needed to induce callus varies from species to species and depends on the type of explants (Charriere *et al*., 1999). It

has been demonstrated that in many cases, 2,4-D is usually the choice of auxin for callus induction and subculture of grasses (Baskaran and Smith, 1990). The addition of a low concentration of cytokinin in callus induction medium often enhances callus regeneration (Bradley *et al*., 2001).

Cytokinins have important roles in plant growth and development by promoting cytokinesis, regulating mitosis cell division (Carle *et al*.,1998) and increasing mitotic activity. The most commonly used cytokinins are 6-benzyladenine purine (BAP), kinetin and zeatin. BAP either applied singly or in combination with auxin is one of the most efficient cytokinins to break bud dormancy and subsequent regeneration of multiple shoots (Barik *et al*., 2007). Pandeya *et al*., (2010) reported that BAP played an important role in multiple shoots induction. Ismail *et al*., (2011) found that the highest rate of shoot multiplication was obtained on MS medium supplemented with 1.0 mg/L BAP. In synthetic seeds production of aromatic rice (*Oryza sativa* L. cv. MRQ 74), an addition of 0.1 mg/L BAP into sowing medium gave the highest germination rate (100 %) and plantlets survival rate (100 %) (Taha *et al*., 2012).

Besides BAP, thidiazuron (TDZ) is also widely applied in culture media which influences shoots production. Numerous plant species were induced to feasible regeneration via TDZ application (Malik and Saxena, 1992; Cocu *et al*., 2004; Faisal *et al*., 2005). According to Murthy *et al*., (1998), TDZ is a powerful hormone for *in vitro* plant regeneration and subsequent growth in many plant species. It was also reported that TDZ induced better response than BAP in shoot regeneration of peanut (Victor *et al*., 1999; Gairi and Rashid, 2004).

Auxins induce cell division, cell elongation, apical dominance, adventitious root formation and somatic embryogenesis. Auxins also play an important role in the mobilization of carbohydrates in leaves and upper stem as well as in the increase of their transport to the rooting zone (Husen and Pal, 2007). When used in low

concentration, auxins induce root initiation and in high, callus formation occurs. The commonly used synthetic auxins are 1-naphthaleneacetic acid (NAA), 2,4 dichlorophenoxyacetic acid (2,4-D), indole-3 acetic acid (IAA) and indolebutyric acid (IBA). IBA is a synthetic auxin that is used commercially worldwide to initiate root growth in many species (Ludwig-Müller *et al*., 2005).

Abscissic acid (ABA) is involved in many plant development processes. One of the crucial functions of this hormone is to inhibit seeds germination. In plant tissue culure system, ABA is among the most frequently applied of plant growth hormone in the process of somatic embryos maturation induction. A low concentration of ABA stimulates the elongation of embryos at globular stage, while at a high concentration resulted in growth inhibition of cactus plant (Lema-Ruminska *et al*., 2013).

Besides sugar and plant growth regulators, other nutrients can also be added in culture media. Addition of amino acids to media is important for stimulating cell growth in protoplast cultures and also in inducing and maintaining somatic embryogenesis. This organic nitrogen is more readily taken up by plants than the inorganic nitrogen. L-glutamine, L-asparagine, L-cystein, L-glycine and L-Proline are commonly used amino acids which are added to the culture medium in form of mixtures as individually they inhibit cell growth.

Previous studies recommended that culture media fortified with amino acids, expanded the shoot organogenesis or embryogenesis. Pinto *et al*., (2002) reported that glutamine and casein have been employed in order to improve embryogenesis in eucalyptus, while L-Proline was used to boost up early stages of somatic embryogenesis in *Miscanthus* and Cherries cultures (Holme *et al*., 1997; Cheong and Pooler, 2004). The accumulation of amino acids during somatic embryogenesis was also reported in alfalfa (Andarwulan and Shetty, 1999), cowpea (Ramakrishnan *et al*., 2005) and for the shoot regeneration of strawberry (Qin *et al*., 2005).

Complex organics such as casein hydrolysate, coconut milk, yeast extract, orange juice, tomato juice are often used when no other combination of known defined components produce the desired growth. Casein hydrolysate has given significant success in tissue culture and potato extract also has been found useful for anther culture. Activated charcoal is reported to stimulate growth and differentiation in orchids, carrot, ivy and tomato whereas inhibits tobacco and soybean. It absorbs brown-black pigments and oxidized phenolics produced during culture and thus reduce toxicity. It also absorbs other organic compounds like plant growth regulators and vitamins which may cause the inhibition of growth. Another feature of activated charcoal is that it causes darkening of medium and so helps root formation and growth.

The effect of explant types on successful tissue culture of various crops has been reported by many researchers (Gubis *et al*., 2003; Blinstrubiene *et al*., 2004; Tsay *et al*., 2006). The use of the suitable explant type is important for the success of regeneration and controls the type of morphogenic reaction. Nodal segments have been widely used for *in-vitro* shoot proliferation of woody plants such as *Citrus limon* (Rathore *et al*., 2004), rough lemon (Ali and Mizra, 2006). According to Siwach *et al*., (2011), nodal segments of *Ficus religiosa* L. was found to be the best explant for callus proliferation and induction.

2.1.2 Micropropagation

Micropropagation or clonal propagation is referred to *in vitro* propagation of plants vegetatively by tissue culture to produce genetically identical copies or true to type of a cultivar, variety or species. Micropropagation is important for propagation of sexually sterile species like triploids, aneuploids which cannot be perpetuated by seeds, seedless plants, cross bred perennials where heterozygosity is to be maintained, mutant lines like auxotrophs which cannot be propagated *in vivo* and disease free planting material of fruit trees and ornamentals. Efficient plant regeneration through *in vitro*

micropropagation is very essential for the successful utilization of biotechnology in rice crop improvement.

Several studies have been carried out to develop *in vitro* micropropagation protocols of rice through callus culture from seed explants (Abolade *et al*., 2008; Zhang and Te-chato, 2013). According to Puhan and Siddiq (2013), dehusked rice seed is the most preferred explant compared to other types of explant for rice tissue culture due to its distinct advantages such as easily accessible materials. On the other hand, very limited number of the developed protocols using other explants such as stem was reported for this species (Faiz and Mohammad, 2012). Therefore, in this study, leaf, root and stem explants from aseptic seedling were used for micropropagation, callus and somatic embryogenesis induction.

2.1.3 Callus Induction

Callus is undifferentiated mass of cells that can be induced from various parts of plant such as stem, leaf, petiole, root and ecetera via tissue culture system. In rice, an efficient callus induction protocols have been reported by many researchers using mature seeds (Abolade *et al*., 2008; Golam *et al*., 2012; Zahida *et al*., 2014). According to Rashid *et al*., (2000), rice seeds have higher potential in callus formation as compared to node and root tips. The morphology of the produced callus is highly influenced by rice genotype and culture conditions (Zuraida *et al*., 2012). Studies on a few selected rice varieties showed that, the indica varieties produced either light yellow, compact, smooth-surfaced or yellowish with hair-like projections and tiny green spots. In contrast, the calli of the japonica and javanica varieties were yellowish, compact and had smooth surface (Josefina and Kazumi, 2010).

Characterization of callus either embryogenic or non embryogenic is based on their ability to regenerate whole plants. This is due to the fact that plant cells have totipotency, which means whole plant can be regenerated from single cells by

manipulating culture conditions. Zhang and Te-chato (2013) reported the morphological characters of embryogenic calli of indica rice (Hom Kra Dang Ngah) were creamy white, some compact, friable and globular, while non embryogenic calli were completely yellow or bright brown with soft and compact texture. The presence of 2,4-D in the culture medium was crucial for the induction of embryogenic calli in many plants species (Daniela *et al*., 2013). However, long periods of exposure to this hormone may cause genetic alterations due to the occurrence of anomalous embryos (Pescador *et al*., 2008). Therefore, identification of optimum concentration of plant growth hormones and suitable culture period are important for each variety and cultivar before carrying out any transformation experiments.

2.1.4 Somatic Embryogenesis

Somatic embryogenesis is a regeneration process of somatic cells that develop by division to form complete embryos. As the embryos develop, they progress into the distinct structural stages of the globular, heart, torpedo and coteledonary in dicots. However, monocots have a more complex embryo structure in the mature seed as compared to dicots. Studies have shown that in monocots somatic embryos pass through globular, scutellar, and coleoptilar stages. There are two types of somatic embryogenesis, direct somatic embryogenesis and indirect somatic embryogenesis. Direct somatic embryogenesis is characterized by the induction of somatic embryos directly from pro-embryogenic cells from leaves, stems, microspores or protoplasts without the proliferation of calli, whereas indirect somatic embryogenesis, somatic embryos are developed from friable embryogenic calli (Jiménez, 2001; Molina *et al*., 2002; Quiroz-Figueroa *et al*., 2002; Quiroz-Figueroa *et al*., 2006). Somatic embryogenesis is a unique process in plants and it is of remarkable interest for biotechnological applications such as clonal propagation, artificial seeds and genetic engineering (Quiroz-Figueroa *et al*., 2006; Namasivayam, 2007). Furthermore, when it

is integrated with conventional breeding programs, molecular and cell biological techniques, it provides a valuable tool to enhance genetic improvement of important crops (Quiroz-Figueroa *et al*., 2006). In rice, somatic embryogenesis is the most common regeneration pathway and has been obtained from various plant organs.

Since most somatic cells are not naturally embryogenic, an induction phase is required for the cells to acquire embryogenic competence (Namasivayam, 2007). It has been suggested that embryogenic cells are present in direct somatic embryogenesis. Therefore, it requires simple favourable conditions for embryo development as compared with indirect somatic embryogenesis (Quiroz-Figueroa *et al*., 2002). Embryogenic cells are those cells that have completed their transition from a somatic state to one in which no further application of exogenous stimuli are necessary to produce somatic embryo. On the other hand, the term competent cell is restricted to that cells that have reached the transitional state and have started to become embryogenic but still require exogenous stimuli application (Jiménez, 2001).

The use of appropriate medium composition, mainly the type and concentration of plant growth regulators will determine the morphogenetic pathways either shoot organogenesis or somatic embryogenesis. However, some authors reported that organogenesis and embryogenesis, occurring simultaneously, as the regeneration pathway (Boissot *et al*. 1990; Gairi and Rashid, 2004). Somatic embryogenesis is influenced by the presence of auxin in the culture medium. Among different auxins, 2,4-D was the most commonly applied for somatic embryogenesis induction (Malgorzata, 2004). The hormone induces dedifferentiation of explant cells to form embryogenic clumps. When auxin is removed or its concentration is reduced, embryogenic clumps is converted to somatic embryos. Maturation of somatic embryos is achieved by culturing on high sucrose medium. ABA is added as it gives hardening due to water loss which is important for embryo maturation. Nitrogen in form of

ammonium ion is essential for induction of somatic embryogenesis while nitrate ion form is required during maturation phase. Other factors such as explant types and genotype have influenced on somatic embryogenesis. In cereals, the use of maltose as carbohydrate source promotes both somatic embryo induction and maturation.

Nutritional supplements such as casein hydrolysate, proline and glutamine have been reported to enhance callusing response (Lin and Zhang, 2005). The promotive effect of proline on the frequency of callusing and regeneration has been reported by Chowdry *et al*., (1993). Moghaddam *et al*., (2000) also stated that the presence of proline in the culture medium seems to produce a required stress condition, decreasing water potential, increasing the accumulation of nutritional elements in cells and finally enhance embryogenesis. So as to enhance green-plant regeneration, supplements such as proline have been used because the use of proline in the medium has been reported to be effective for the initiation and maintenance of embryogenic calluses (Datta *et al.,*1992).

2.1.5 Synthetic Seed Technology

Synthetic seeds are defined as artificially encapsulated somatic embryos, shoot buds, cell aggregates, or any other tissue that can be used for sowing as a seed and that possess the ability to convert into a plant under *in vitro* or *ex vitro* conditions (Capuano *et al*., 1998). Initially, synthetic seeds were referred only to the somatic embryos produced from tissue cultured. Nowadays, the development of synthetic seed technology has expanded to the artificial encapsulation of various types of micropropagules. Although various micropropagules have been considered for synthetic seed production, the somatic embryos have been largely favoured. This is due to somatic embryos possess the radical and plumule that are able to develop into root and shoot without any specific treatment.

The encapsulation technology has been applied to produce synthetic seeds for a number of plant species belonging to angiosperms and gymnosperms. The essential

prerequisite for the practical application of the synthetic seed technology is the large scale production of high quality of micropropagules, which is a major limiting factor at present (Ara *et al*., 2000). For example, embryogenesis in androgenic calli of indica rice has been comparatively low than japonica and tropical japonica varieties (Roy and Mandal, 2008). Therefore, it is important to establish an efficient protocol for obtaining maximum number of micropropagules for each species, varieties or cultivar.

2.1.6 Acclimatization

Even though *in vitro* micropropagation techniques have been widely used in many plants species, however it is restricted by the high percentage of plants damage during acclimatization process due to extremely different conditions between *in vitro* and *ex vitro*. Under *in vitro* culture conditions, plants grow under low irradiance levels, aseptic conditions, on a medium containing sufficient sugar and nutrients to allow for heterotrophic growth and in an atmosphere with a high level of humidity. These conditions lead to the formation of plantlets that differ in terms of morphology, anatomy and physiology from naturally growing plants, resulting in poor survival under natural environmental conditions when they are directly transferred to *ex vitro* (Pospisilova *et al*. 2007).

Acclimatization of micropropagated plantlets to the natural environment requires several morphological, anatomical and physiological changes (Hazarika, 2006). ABA acts as an anti-transpirant during the acclimatization of tissue culture-raised plantlets and reduces the relative water loss of the leaves of micropropagated plantlets during transplantation even when non-functional stomata are present (Pospisilova *et al*., 2007). Pospisilova *et al.,* (2009) reported that the addition of ABA to the last subculture improved the survival rate of tobacco plantlets transferred to the natural environmental conditions. Acclimatization can also be improved by the positive effect of ABA on Chlorophyll a content and other photosynthetic parameters as well as on plant growth

(Pospisilova *et a*l., 2007). A number of other reports also documented the significant role of ABA in the acclimatization of tissue culture-raised plants (Hronkova *et al*., 2003).

2.1.7 Somaclonal Variation

Somaclonal variation is defined as genetic variation that occurs in plants that have been regenerated through plant tissue culture technique. It is a commonly observed phenomenon in cell and tissue cultures of different species regardless of the regeneration system used (Li *et al.,* 2010). This variation involves changes in both nuclear and cytoplasmic genomes and it can be genotypic or phenotypic, which in later case can be either genetic or epigenetic in origin (Henry, 1998).

Genetic variability is caused by mutations or other changes in DNA (deoxyribonucleic acid). There are two types of mutation namely chromosome mutations and gene mutations. Chromosome mutations caused by inversion, deletion, translocation and duplication of a section of a chromosome. This can lead to genes lost, alteration in gene order, duplication of genes and segment of chromosome moving to new location on different chromosome. Whilst, gene mutations are changes of DNA base nucleotide on DNA strands. These changes occur through addition or insertion, deletion and substitution of DNA base nucleotide. Typical genetic alteration in plant tissue culture are: (1) Changes in chromosome numbers (polyploidy and aneuploidy), (2) Changes in chromosome structure (Chromosome mutations), (3) Changes in DNA sequence (gene mutations). Changes in ploidy originate from abnormalities that occur during mitosis such as, extra chromosomal duplication during interphase, spindle fusion or lack of spindle formation and cytoplasmic division. Another causes are the composition of the growth medium and nutrient limitation. For example, the present of kinetin and 2,4-D in culture medium can trigger changes in ploidy. Thus, the longer the cell remains in culture medium, the greater is its chromosomal instability.

Somaclonal variation can be reduced by selecting a suitable explants and appropriate culture medium. Osuga *et al*., (1999) believed that plants regenerated from somatic embryos carry less *in vitro* induced variation. Prior to this, Deverno (1995) claimed that conifers regenerated through somatic embryos displayed low level of variation and high genetic uniformity. In contrast, regenerants of some *Picea* species derived via somatic embryos exhibited morphology and chromosome number variation (Trembley *et al*., 1999). They believed that this variation was strongly influenced by genotype and the time of the culture. The risk of somaclonal variation is particularly high with increasing culture duration, especially in long term cultivated embryogenic callus (Konstantin *et al*., 2014). Therefore, direct development of somatic embyos from cultured explants and the use of young explants tissue in combination with short-term culture usually limit *in vitro* induced variation (Malgorzata, 2004). Somaclonal variation in regenerants can be detected by morphological characteristics such as plant height, leaf morphology and abnormal pigmentation (Israeli *et al*., 1991). For clarification of these phenomena, cytological studies need to be carried out. Through cytogenetic analysis, chromosomal alteration and ploidy changes could be detected.

2.2 INTRODUCTION TO RICE (*Oryza sativa* L.)

Rice (*Oryza sativa* L.) is one of the most important food crops in Asia and the rest of the world. Rice is normally grown as an annual plant, although in tropical areas it can survive as a perennial and can produce a ratoon crop for up to 30 years. The rice plant can grow to 1-1.8 m tall, occasionally more depending on the variety and soil fertility. The plant has long slender leaves, 50-100 cm long and 2.0-2.5 cm broad. The small wind pollinated flowers are produced in a branched arching to pendulous inflorescence 30 -50 cm long. The edible seed is a grain (caryopsis), 5-12 mm long and 2-3 mm thick.

Oryza sativa L. is an annual monocotyledonous grass, belonging to the family of Gramineae. The genus *Oryza* consists of two cultivated species which are *Oryza sativa* (Asian rice) and *Oryza glaberrima* (African rice), with twenty-one wild species (Dogara and Jumare, 2014). It is classified into two major ecotypes (sub species) namely indica and japonica based on their geographical conditions. Indica refers to the tropical and subtropical diversity grown throughout South and Southeast Asia and Southern China. Meanwhile, japonica varieties are grown in Japan, China, Korea and northern California due to their tolerance to low night temperatures. The scientific classification of the plant is shown below;

Kingdom : Plantae

Division : Magnoliophyta

Class : Liliopsida

Order : Poales

Family : Poaceae

Genus : *Oryza*

Species : *sativa*

2.2.1 World Paddy Production, Import and Export

World paddy production has risen steadily from about 200 million tonnes in 1960 to over 600 million tonnes in 2004. The production increases to more than 700 million tonnes in 2010 (Table 2.1). The top producers were China, India, Indonesia, Bangladesh, Vietnam, Thailand and Myanmar (FAO, 2015). Malaysia had only contributed about 2.6 million tonnes in 2013 and 2014, which was far less as compared with other Asian countries such as Philippines (18.8 to 18.9 million tonnes). Based on FAO forecast, the world paddy production in 2015 will increase to 749.1 million tonnes as compared to 741.8 million tonnes in 2014. The increase would mainly stem from growth in Asia, where paddy production may approach 678 million tonnes, 1.1 percent more than in 2014.

Even though world rice production has increased significantly, only about 5 to 6% of rice produced is traded internationally. In 2014, China, Nigeria and Philippines were among the top rice importers countries (Table 2.2). Although China and India are the two largest producers of rice in the world, these countries consume the majority of the rice produced, leaving a little to be traded internationally. The largest three exporting countries are India, Thailand and Viet Nam (Table 2.3). Malaysia requires about 1.15 million tonnes of rice from abroad to meet the consumption needs of 30.4 million people.

Table 2.1: World paddy production from 2010 to 2015.

	2010-2012 Average	2013	2014	2015
		Million tonnes		
WORLD	723.0	744.9	741.8	749.1
Developing countries	697.1	719.5	715.5	723.5
Developed countries	25.9	25.4	26.3	25.6
ASIA	655.1	676.0	670.7	677.7
Bangladesh	50.6	51.5G	52.4	52.0
Cambodia	8.8	9.4G	9.3G	9.4
China	201.9	205.2G	208.2G	209.5
China (mainland)	200.3	203.6G	206.5G	208.0G
India	153.3	160.0G	153.8G	155.2
Indonesia	67.1	71.3G	70.8G	75.6G
Iran	2.7	2.5G	2.6	2.7
Japan	10.6	10.8G	10.5G	10.5
Korea	5.6	5.6G	5.6G	5.5
Lao PDR	3.2	3.4G	3.3	3.4
Malaysia	2.5	2.6G	2.6G	2.7
Myanmar	29.8	28.3G	28.9	29.2
Nepal	4.7	5.0G	4.8G	4.6
Pakistan	8.3	10.2G	10.5G	10.3
Philippines	17.3	18.8G	18.9G	18.4
Sri Lanka	4.0	4.6G	3.4G	4.1
Thailand	37.4	36.8G	34.3	34.7
Viet Nam	42.0	44.0G	45.0G	44.7
AFRICA	26.4	27.5	28.5	28.7
North Africa	5.4	6.1	6.0	6.0
Egypt	5.3	6.1	6.0	5.9
Western Africa	12.6	13.8	14.0	14.2
Cote d'lvoire	0.7	0.8G	0.8	0.8
Guinea	1.8	2.1G	2.0G	2.0
Mali	2.0	2.2G	2.2G	2.3
Nigeria	4.5	4.7	4.9	4.8
Sierra Leone	1.1	1.3G	1.2	1.2
Central Africa	0.5	0.5	0.6	0.5
Eastern Africa	2.8	2.8	3.2	3.2
Tanzania	2.2	2.2G	2.6G	2.6

'**Table 2.1**, continued'

	2010-2012 Average	2013	2014	2015
		Million tonnes		
Southern Africa	5.0	4.2	4.6	4.7
Madagascar	4.5	3.6G	4.0G	4.1
Mozambique	0.3	0.3G	0.4G	0.4
CENTRAL AMERICA & CAR	3.0	3.2	3.0	3.0
Cuba	0.6	0.7G	0.6G	0.5
Dominican Rep.	0.9	0.9G	0.9G	0.9
SOUTH AMERICA	24.0	24.3	24.8	25.4
Argentina	1.5	1.6G	1.6G	1.6G
Brazil	12.3	11.8	12.1G	12.5G
Colombia	2.0	2.0G	1.8	2.0
Ecuador	1.3	1.2G	1.2	1.2
Peru	2.8	3.0G	2.9G	3.0
Uruguay	1.4	1.4G	1.3G	1.4
NORTH AMERICA	9.5	8.6	10.0	9.4
United States	9.5	8.6G	10.0G	9.4
EUROPE	4.4	4.1	4.0	4.1
EU 1/	3.2	2.9G	2.9G	2.9
Russian Federation	1.1	0.9G	1.0G	1.1
OCEANIA	0.6	1.2	0.8	0.7
Australia	0.6	1.2G	0.8G	0.7G

Source: FAO (2015), I/ : Excluding intra-trade, G: Official figure

Table 2.2: World rice imports from 2010 to 2015.

	2010-2012 Average	2013	2014	2015
		Million tonnes		
WORLD	35.5	37.2	42.8	42.0
Developing countries	30.8	31.8	37.3	36.4
Developed countries	4.7	5.3	5.4	5.6
ASIA	17.0	16.5	20.8	20.1
Bangladesh	0.7	0.2G	1.3	1.0
China	1.6	2.7	3.0	3.2
Of which China (mainland)	1.1	2.2G	2.5G	2.7
Indonesia	1.9	0.5	1.0	0.9
Iran	1.2	1.9G	1.4G	1.4
Iraq	1.3	1.4	1.4	1.5
Japan	0.7	0.7G	0.7G	0.7
Malaysia	1.0	0.9G	1.1	1.2
Philippines	1.6	0.7	1.9	2.0
Saudi Arabia	1.2	1.3G	1.4	1.5
United Arab Emirates	0.6	0.7	0.8	0.8
AFRICA	12.0	14.0	14.4	14.4
Cote d'lvoire	1.1	1.3	1.2	1.2
Nigeria	2.5	2.4	3.0	2.8
Senegal	1.0	1.1	1.3	1.2
South Africa	1.0	1.3G	0.9G	1.1
CENTRAL AMERICA & CAR	2.1	2.0	2.1	2.2
Cuba	0.4	0.3G	0.4	0.4
Mexico	0.6	0.7G	0.7G	0.6
SOUTH AMERICA	1.3	1.5	1.4	1.6
Brazil	0.7	0.7G	0.6G	0.6
NORTH AMERICA	1.0	1.1	1.1	1.1
United States	0.6	0.7G	0.8G	0.7G
EUROPE	1.6	1.7	2.0	2.1
EU 1/	1.2	1.2G	1.4G	1.5G
Russian Federation	0.2	0.2G	0.3G	0.3
OCEANA	0.5	0.5	0.5	0.5

Source: FAO (2015), I/ : Excluding intra-trade, G: Official figure

Table 2.3: World rice exports from 2010 to 2015.

	2010-2012 Average	2013	2014	2015
		Million tonnes		
WORLD	35.4	37.2	42.8	42.0
Developing countries	31.0	33.1	38.8	37.6
Developed countries	4.4	4.2	4.0	4.5
ASIA	27.6	29.3	35.0	33.7
Cambodia	1.0	1.2	1.1	1.2
China	0.5	0.5	0.4	0.5
China (mainland)	0.5	0.5G	0.4G	0.5
India	5.8	10.5G	11.5G	10.0
Myanmar	0.6	0.7	0.7	0.8
Pakistan	3.1	3.1G	3.7G	3.8
Thailand	8.8	6.6G	11.0G	10.9
Vietnam	7.3	6.6G	6.5G	6.3
AFRICA	0.5	0.6	0.6	0.6
Egypt	0.3	0.4	0.4	0.5
SOUTH AMERICA	3.0	3.1	3.2	3.3
Argentina	0.6	0.5G	0.5G	0.5
Brazil	0.9	0.8G	0.8G	0.8
Guyana	0.3	0.4G	0.5G	0.6
Uruguay	0.9	0.9G	0.9G	0.9
NORTH AMERICA	3.5	3.3	3.0	3.5
United States	3.5	3.3G	3.0G	3.5G
EUROPE	0.4	0.4	0.5	0.5
EU I/	0.2	0.2G	0.3G	0.3G
Russian Federation	0.2	0.1G	0.2G	0.2
OCEANA	0.3	0.5	0.4	0.4
Australia	0.3	0.5G	0.4G	0.4

Source: FAO (2015), I/ : Excluding intra-trade, G: Official figure

2.2.2 Rice Cultivation and Production in Malaysia

The rice industry is the oldest organized sub-sector in Malaysia agriculture and underpins the country traditional and cultural heritage. It is grown under different environments which can be classified into three ecosystems viz. irrigated, rainfed lowland and upland ecosystems (Abdullah *et al*., 2003).

The upland rice is grown aerobically in upland environments such as in Sabah and Sarawak (Paul Vincent, 2010). The upland rice can give stable but low yields in adverse environments where rainfall is low, irrigation is absent, soil texture is poor or toxic, weed infestation is high, farmers are too poor to supply high inputs and rice is grown as a subsistence crop (Chan *et al*, 2012). Furthermore, upland rice are not responsive to external inputs such as fertilizer and water. On the other hand, lowland rice was relatively sensitive to water stress in generative stage after panicle initiation as yield dropped up to 47% when drought stress occurred during that period. Aerobic rice is a combination of both characteristics of the upland and the high yielding lowland rice varieties (Tuong and Bouman, 2003). According to Bouman *et al*., (2006), yield indicated little differences in sensitivity for water stress before panicle and after panicle initiation development stages in aerobic rice.

In Malaysia, most of the rice is grown in irrigated areas especially in Kedah. Even though rice production increased from 1,351,461 metric tonnes in 2001 as compared to 1,685,236 metric tonnes in 2013, Malaysia still only produces 8% of what it needs to support itself (Table 2.4). Therefore, to fulfil the demand of this crop, Malaysia has imported about 0.9 to 1.1 million tonnes of rice per year from other countries (Table 2.2). The shortage in the locally produced rice had caused domestics rice prices rose almost double during food global crisis in 2008. Therefore, Malaysia nowadays, is looking to be self-sufficient. According to Ahmad and Mohd. Razi (2009), Malaysia rice production is estimated to increase to 4.4 and 9.1 million t/ha in 2020 and 2030,

respectively with the predicted population of 36.7 million (2020) and 45.7 million (2030).

Table 2.4: Planted Area, Yield, Production of Paddy and Rice in Malaysia, 2001 – 2013.

Year	Planted Area (hectare)	Average Yield (Kg/ha)	Paddy Production (Metric Tonnes)	Rice Production (Metric Tonnes)
2001	673,634	3,110	2,094,995	1,351,461
2002	678,544	3,238	2,197,351	1,415,117
2003	671,820	3,360	2,257,037	1,453,137
2004	667,310	3,434	2,291,352	1,467,052
2005	666,823	3,471	2,314,378	1,490,015
2006	676,034	3,236	2,187,519	1,407,221
2007	676,111	3,514	2,375,604	1,530,971
2008	656,602	3,584	2,353,036	1,516,474
2009	674,928	3,720	2,511,043	1,620,256
2010	677,884	3,636	2,464,831	1,588,456
2011	687,940	3,748	2,578,519	1,661,260
2012	684,545	3,797	2,599,382	1,674,981
2013	674,332	3,879	2,615,845	1,685,236

Source: Paddy Statistic in Malaysia 2011, 2013

2.2.3 Glycemic Index (GI) and Amylose Content in Rice

Glycemic index (GI) is a measurement carried out on carbohydrates-containing foods and their impact on human blood sugar levels. In order to classify foods as low, medium or high GI, glucose is used as standard reference with GI value is 100. Foods can be classified as low GI (55 or less), medium GI (56-69), or high GI (70 and above). Low-GI foods are digested and absorbed by the body more slowly and produce gradual increases in blood sugar and insulin levels after meals. High-GI foods are associated with dramatic increases and drops in blood sugar levels believed to be damaging to

arteries and various blood vessels while triggering far too much production of insulin. These are linked to higher risks of developing type-2 diabetes.

According to Fitzgerald *et al*., (2011), there was large variability in GI between the different varieties of rice, ranging from a low of 48 to a high of 92, with an average medium GI of 64. They also found that amylose content of rice affects the GI of rice. As the amylose content increased, the GI decreased, and vice versa. Therefore, rice varieties with high amylose content such as Swarna, India's most widely grown rice which has high amylose content, has a low GI. In contrast, sticky or glutinous rice, which has low amylose content, has a higher GI. The popular Basmati rice falls in the middle with a medium amylose content and medium GI. The finding that different varieties of rice have different GI values allows consumers with health problems to choose the right rice that can be safely incorporated into their regular diet.

2.2.4 Rice Nutrients Content

Rice is the main source of energy as it is rich in carbohydrates, plenty of B vitamins, low in fat and contains some proteins (Resurreccion *et al*., 1979). Rice is also a good source of insoluble fibre and gluten free. Hence, it is suitable for celiac and a wonderful food for the very young and elderly people. The quality of rice is influenced by a few factors such as the genetic makeup of a particular variety or cultivar, environmental conditions and processing techniques (Jennings *et al*., 1979). The nutrients content of white rice is shown in Table 2.5. Besides carbohydrates, it is also high in calcium, iron, magnesium, phosphorus and potassium.

Most of the high quality preferred varieties in the major rice growing countries are aromatic such as the Basmati rice of India and Pakistan, Dulhabhog of Bangladesh, Khao Dawk Mali and Leuang Hawn of Thailand, Azucena and Milfor of the Philippines, Rojolele of Indonesia, Sadri varities of Iran, Barah of Afghanistan and Della of the United States (Rosniyana *et al*., 2010). Table 2.6 shows the nutritional

composition of aromatic rice (Mas Wangi) of Malaysia. As compared to white rice, Mas Wangi contains higher values of all minerals with high amylose content (24.4) (Rosniyana *et al*., 2010). This indicates that the cultivar is suitable for diabetic.

Table 2.5: Rice nutrients content (white rice, medium-grain, cooked).

Content/Properties per 100 g	Unit
Energy	544 kJ (130 kcal)
Carbohydrates	28.59 g
Dietary fiber	0.3 g
Fat	0.21 g
Saturated	0.057 g
Monounsaturated	0.065 g
Polyunsaturated	0.056 g
Protein	2.38 g
Water	68.61 g
Vitamin A equiv.	0 µg
Thiamine (vit. B_1)	0.167 mg
Riboflavin (vit. B_2)	0.016 mg
Niacin (vit. B_3)	1.835 mg
Vitamin B_6	0.05 mg
Folate (vit. B_9)	2 µg
Vitamin B_{12}	0 µg
Vitamin C	0 mg
Calcium	3 mg
Iron	1.49 mg
Magnesium	13 mg
Phosphorus	37 mg
Potassium	29 mg
Sodium	0 mg
Zinc	0.42 mg

Source: USDA Nutrient Database.

Table 2.6. Nutritional composition of brown rice and milled rice of Mas Wangi.

Properties (mg/100 g sample)	Organic rice Milled rice	Brown rice	Inorganic rice Milled rice	Brown rice
Calcium	5.0 ± 0.5	48.0 ± 0.3	45.0 ± 0.3	54.0 ± 0.3
Potassium	69.0 ± 0.1	69.0 ± 0.1	49.0 ± 0.3	67.0 ± 0.5
Sodium	21.0 ± 0.1	47.0 ± 0.4	1.7 ± 0.5	45.0 ± 0.3
Magnesium	30.0 ± 0.3	152.0 ± 0.1	59.0 ± 0.1	174.0 ± 0.5
Iron	1.6 ± 0.1	7.5 ± 0.5	1.2 ± 0.5	6.4 ± 0.1
Phosphorous	123.0 ± 0.3	201.0 ± 0.1	89.0 ± 0.1	198.0 ± 0.5
Thiamine	0.23 ± 0.5	0.34 ± 0.3	0.34 ± 0.1	0.47 ± 0.3
Riboflavin	0.03 ± 0.5	0.04 ± 0.4	0.03 ± 0.3	0.03 ± 0.3
Niacin	1.5 ± 0.1	1.60 ± 0.5	1.9 ± 0.1	2.9 ± 0.5
Pyrodoxine	0.7 ± 0.5	0.45 ± 0.5	0.74 ± 0.1	0.60 ± 0.5

Source : Rosniyana *et al*., (2010)

2.2.5 Malaysian Aromatic Rice Cultivar MRQ 74 (Mas Wangi)

Aromatic rice has a stronger aroma than ordinary rice. It constitutes a small but a special group of rice, which is considered best in quality. This rice has long been popular in the orient, very popular in South-East Asia and now becoming popular in the middle East, Europe and United States. Most of the trade in aromatic rice is from India, Pakistan and Thailand.

Malaysian Aromatic Rice Cultivar, MRQ 74 also known as 'Mas Wangi' has been introduced officially to Malaysian farmers in 2005. The cultivar has developed from crosses between aromatic rice from India, Q 34 and Khaw Dok Mali from Thailand in 1991. It was then crossed again with Kasturi from India in 1994 to produce high quality of aromatic rice. The rice has remarkable characteristics such as 80% similar attributes to Basmati type, long and slender grain shape, non-sticky and aromatic. After 13 years of investigation on aromatic rice cultivar MRQ 74, Malaysian Agricultural Research and Development Institute (MARDI) has revealed that the cultivar can also be grown in Malaysia. Therefore, the cultivar is now widely grown in Penisular Malaysia especially in Kedah and Kelantan as pioneer project. This cultivar is currently planted both in

inorganic and organic practices (Rosniyana *et al*., 2010). It was also proposed to be grown as aerobic rice due to its high potential to be commercialised.

The plant's height is about 60 to 70 cm with 125 days maturity period and produces about 65 to 85 seeds per stalk. It is estimated to produce 4 to 5 tonnes of paddy seeds per hectare. The cultivar is also resistant to a few diseases such as 'karah', 'hawar bakteria' and 'benah'.

CHAPTER 3

DETERMINATION OF STANDARD PRIMARY ROOT GROWTH OF *Oryza sativa* L. cv. MRQ 74

3. 1 EXPERIMENTAL AIMS

Plant growth occurs by cell division and elongation. Meristematic cells in the root tips are capable of undergoing cell division through mitosis. Under favourable conditions such as enough nutrients, water and oxygen, the cells increase in size, vacuolated, stretched, elongated and soon become differentiated into one or another different types to serve different functions. These processes will lead to root growth and development. Root growth can be measured as average values of the root length and standard root age from a population of roots that are as homogenous as possible (Pilet, 1991).

The root tip has been widely used to study plant cell proliferation and cell growth due to it relatively simple structure and distinct region of meristem. In this chapter, the aim of the experiment was to determine the standard growth of primary root of *in vivo* grown *Oryza sativa* L. cv. MRQ 74. The mean root length was obtained from the samples and was used in the subsequent experiments.

3.2 MATERIALS AND METHODS

3.2.1 Seeds Sterilization and Germination

The dehusked seeds (obtained from MARDI, Penang, Malaysia) were surface sterilized by soaking and shaking them in 70 % (v/v) Clorox with two drops of 1 ml/L tween 20, followed by 50%, 40%, 30%, 20% and 10% (v/v) Clorox. Subsequently, the seeds were rinsed three times in sterilised distilled water. Finally, the seeds were rinsed in 70 % (v/v) alcohol for one minute followed by three times in sterilised distilled water for complete removal of Clorox and ethanol. Approximately, 100 of the sterilised seeds were then germinated on sterilized moist cotton wools in petri dishes and were maintained for two weeks at 25 ± 1 ^{o}C under 16 hours light and 8 hours dark with 1000 lux of light intensity. This experiment was carried out for determination of standard growth of primary root from the population grown *in vivo*.

As for comparison with standard growth of *in vitro* primary root length, the sterilised seeds were cultured in sterile tubes containing MS basal media. The cultures were maintained at 25 ± 1 ^{o}C, 16 hours light and 8 hours dark in the culture room. The length of primary roots of seedlings were measured every day until secondary roots appeared in most of the primary roots. The mean root length of the sample was recorded and was plotted into a graph of the average length of the primary roots (Y-axis) against time (X-axis). A linear regression analysis was then conducted to find the relationship between both factors. Based on these results, standard root length was selected for subsequent experiments.

3.3 RESULTS

3.3.1 Standard Growth of Primary Roots of *Oryza sativa* L. cv. MRQ 74

In the present study, rice seeds germination occurred within two days. The growth of primary roots took place very fast, where the roots started to grow on day 2 with the mean root length was 1.38 ± 0.21 mm. The growth of primary roots increased more than two-fold on day 3 and day 4 before they started to slow down on the following days. However, the maximum growth rate was recorded on day 5 with the mean root length of 8.09 ± 0.79 mm, a day before the emergence of secondary roots. Since most of the secondary roots appeared on the sixth day, primary roots of 5-day-old seedlings were chosen to be used as the standard root length in subsequent experiments (Table 3.1). In order to identify the relationship between the mean root length and time, the data was plotted in a graph. Figure 3.1 shows a linear regression between mean root length (mm) and time (day). The coefficient of determination (R^2), is a number that indicates how well data fit a statistical model. Values of R^2 range from 0 to 1, where a value close to zero means no correlation, close to 0.5 as weak correlation, between 0.5 to 0.7 as moderate correlation and higher than 0.7 as high correlation. An R^2 of 1 indicates that the regression line perfectly fits the data. In this study, the R^2 value was very high (0.944) indicated a highly strong relationship between mean root length and time required for the roots to grow. Based on the equation, the mean of primary root growth rate was 1.68 mm per day.

Table 3.1: The mean of primary roots length of *Oryza sativa* L. cv. MRQ 74 obtained from 100 seedlings.

Day	Mean Root Length (mm ± SE)
1	0.00 ± 0.00
2	1.38 ± 0.21
3	3.29 ± 0.51
4	6.48 ± 0.83
5	8.09 ± 0.79
6	8.85 ± 0.88
7	9.10 ± 0.91

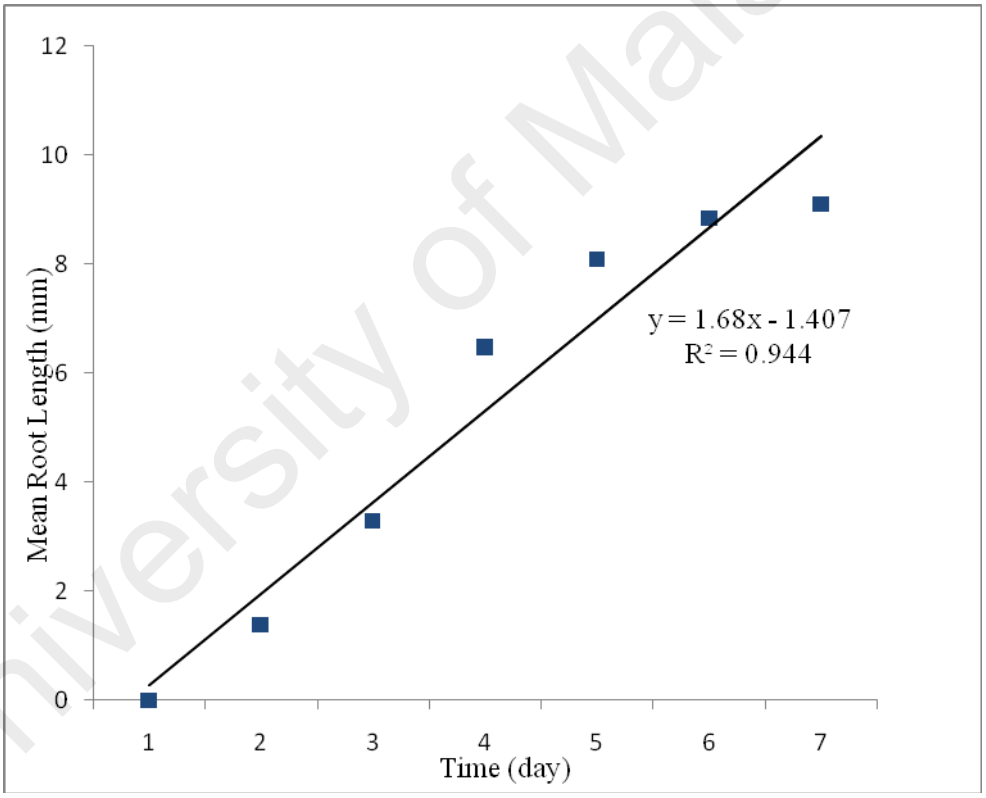


Figure 3.1: The growth of primary roots of *Oryza sativa* L. cv. MRQ 74, germinated on sterilized moist cotton wool.

3.4 SUMMARY OF RESULTS

1. The mean primary root length of *Oryza sativa* L. cv. MRQ 74 was 8.09 ± 0.79 mm, obtained from 5-day-old seedlings.
2. Based on the standard growth of the primary root, the growth rate of the primary roots was 1.68 mm per day.
3. The standard root age was 5-day-old since the secondary roots appeared on the sixth day.
4. Seedlings of 5-day-old with root length of 8.09 ± 0.79 mm were chosen for the subsequent experiments.

CHAPTER 4

CALLUS INDUCTION OF *Oryza sativa* L. cv. MRQ 74

4.1 EXPERIMENTAL AIMS

Callus is a mass of undifferentiated cells, usually cultivated on gel media that developed during tissue culture of plant parts. Callus induction and regeneration potential are influenced by various factors such as genotypes, types of explants, type and concentration of plant growth regulators, carbohydrate sources, culture conditions and others. In particular, the type of explants and plant growth regulators are important factors that determine the success of embryogenic callus induction. It was reported that supplementation of synthetic auxins such as 2,4-D at adequate levels into a basal medium resulted in prolific callus formation from various types of rice explants (Morita *et al*., 1999**)**. Lee *et al*., (2002) stated that the presence of 2,4-D in culture medium is crucial for rice callus induction. In fact, no callus formation was observed in the absence of 2,4-D in N6 medium. Zuraida *et al*., (2010) reported that the highest percentage of callus induction of rice variety MR232 was recorded on the media containing 1 mg/L 2,4-D in combination with 10 mg/L NAA.

The number, colour, size and shape of embryogenic calli are also influenced by these factors. According to Lee *et al*., (2002), rice calli display two major distinct morphological phenotypes. Embryogenic calli are compact, dry, light yellowish, nodular with numerous globular structures. On the other hand, non-embryogenic calli are friable, translucent, rough and rhizogenic. The embryogenic calli of rice can be induced from tissues of various organs such as immature seeds (Masuda *et al*., 1989), immature embryos (Koetje *et al*., 1989) and roots (Abe and Futsuhara, 1986). However, very little information was available on callus induction of rice from stem explants.

Therefore, this study was undertaken to identify the optimal concentration of growth regulators for an efficient callus induction of aromatic rice (*Oryza sativa* L. cv. MRQ 74). Besides root, stem and leaf explants were also used in the present study to induce callus formation.

4.2 MATERIALS AND METHODS

4.2.1 Sterilization of Seeds

Mature rice seeds (*Oryza sativa* L. cv. MRQ 74) obtained from MARDI, Seberang Prai, Penang, were manually dehusked. The dehusked seeds were surface sterilized by soaking and shaking in 70 % (v/v) Clorox with two drops of 1 ml/L Tween 20 followed by 50 %, 30%, 20% and 10% (v/v) Clorox. Each treatment lasted approximately one minute. The dehusked seeds were rinsed three times in sterilised distilled water. The seeds were then rinsed in 70 % (v/v) alcohol for one minute followed by three times in sterilised distilled water for complete removal of Clorox and ethanol in the laminar air flow cabinet.

The sterilised seeds were then cultured onto MS media containing sucrose (30 g/L) and agar (8 g/L). The cultures were incubated in the culture room and the seeds started to germinate after 2 days of culture. The seedlings were maintained in the culture room for 4 weeks before they were used as source of explants for callus induction.

4.2.2 Culture Media and Conditions

Root, stem, and leaf explants were used in this study in order to identify the responsive explants for *in vitro* callus induction. All types of explants were taken from 4-week-old aseptic seedling as described in section 4.2.1. The explants were approximately excised into 5.0 – 10.0 mm segments and cultured on MS media supplemented with 30 g/L sucrose and 8 g/L technical agar fortified with various concentrations of 2,4-D (0.0, 0.5, 1.0. 1.5, 2.0 and 2.5 mg/L) either applied singly or in combinations with BAP (0.0, 0.5, 1.0, 1.5, 2.0 and 2.5 mg/L). The pH of the callus induction medium employed in this experiment was adjusted to 5.8 using 1.0 M HCl and 1.0 M NaOH prior to autoclaving process at 121 ºC, 105 kPa for 21 minutes. The cultures were maintained in the culture room at 25 ± 1 ºC under 16 hours light and 8 hours dark with 1000 lux of light intensity for eight weeks.

Below is the list of culture media with different types and concentrations of plant growth regulators that were employed in this study.

1. MS + 0.0 mg/L BAP + 0.0 mg/L 2,4-D (as control)
2. MS + 0.0 mg/L BAP + 0.5 mg/L 2,4-D
3. MS + 0.0 mg/L BAP + 1.0 mg/L 2,4-D
4. MS + 0.0 mg/L BAP + 1.5 mg/L 2,4-D
5. MS + 0.0 mg/L BAP + 2.0 mg/L 2,4-D
6. MS + 0.0 mg/L BAP + 2.5 mg/L 2,4-D
7. MS + 0.5 mg/L BAP + 0.5 mg/L 2,4-D
8. MS + 0.5 mg/L BAP + 1.0 mg/L 2,4-D
9. MS + 0.5 mg/L BAP + 1.5 mg/L 2,4-D
10. MS + 0.5 mg/L BAP + 2.0 mg/L 2,4-D
11. MS + 0.5 mg/L BAP + 2.5 mg/L 2,4-D
12. MS + 1.0 mg/L BAP + 0.5 mg/L 2,4-D
13. MS + 1.0 mg/L BAP + 1.0 mg/L 2,4-D
14. MS + 1.0 mg/L BAP + 1.5 mg/L 2,4-D
15. MS + 1.0 mg/L BAP + 2.0 mg/L 2,4-D
16. MS + 1.0 mg/L BAP + 2.5 mg/L 2,4-D
17. MS + 1.5 mg/L BAP + 0.5 mg/L 2,4-D
18. MS + 1.5 mg/L BAP + 1.0 mg/L 2,4-D
19. MS + 1.5 mg/L BAP + 1.5 mg/L 2,4-D
20. MS + 1.5 mg/L BAP + 2.0 mg/L 2,4-D
21. MS + 1.5 mg/L BAP + 2.5 mg/L 2,4-D
22. MS + 2.0 mg/L BAP + 0.5 mg/L 2,4-D

23. MS + 2.0 mg/L BAP + 1.0 mg/L 2,4-D

24. MS + 2.0 mg/L BAP + 1.5 mg/L 2,4-D

25. MS + 2.0 mg/L BAP + 2.0 mg/L 2,4-D

26. MS + 2.0 mg/L BAP + 2.5 mg/L 2,4-D

27. MS + 2.5 mg/L BAP + 0.5 mg/L 2,4-D

28. MS + 2.5 mg/L BAP + 1.0 mg/L 2,4-D

29. MS + 2.5 mg/L BAP + 1.5 mg/L 2,4-D

30. MS + 2.5 mg/L BAP + 2.0 mg/L 2,4-D

4.2.3. Measurement of the Callus Formation

The callus produced was carefully separated from culture media using metal spatula. Fresh weight was recorded before it was placed into an oven at 35 ºC. The callus dry weight was only recorded after a constant reading was achieved. Besides that, the morphology of callus was also examined.

4.2.4 Statistical Analysis

Data obtained was analysed using the IBM SPSS statistical package version 20 software (International Business Machines Corp., Armonk, NY). Data was subjected to statistical analysis using Duncan's Multiple Range Test (DMRT) at 5 % significance level. The effect of different treatments was quantified as mean ± SE (standard error).

4.3 RESULTS

4.3.1 Callus Derived from Stem Explants

In the present study, the effect of different concentrations of 2,4-D either applied singly or in combinations with BAP on callus induction from three different explants was investigated. When stem explants were cultured on hormone-free MS media, the explants produced shoots and roots (Table 4.1). On the other hand, on MS media supplemented with 2,4-D and BAP, the stem explants started to become swollen after a week in culture media followed by callus formation at the cut edges after three weeks of culture (Figure 4.1a). The highest mean callus dry weight (71.60 ± 6.40 mg) was obtained when stem explants were cultured on MS media supplemented with 0.5 mg/L BAP. The combination of 0.5 mg/L 2,4-D with 1.5 mg/L BAP showed the second highest of the mean callus produced (62.40 ± 7.40 mg). Meanwhile, MS media supplemented with 2.0 mg/L BAP and 2.5 mg/L BAP in combination with 1.0 mg/L 2,4 -D each produced 50.30 ± 14.70 mg and 50.60 ± 14.20 mg callus dry weight, respectively. The morphology of the callus produced varied depending on the concentrations of hormones used. Creamy white, globular and compact callus was produced at low concentrations of BAP and creamy white, globular and friable at higher concentrations of BAP.

Table 4.1: Mean callus dry weight obtained from stem explants of *Oryza sativa* L. cv. MRQ 74 cultured on MS media supplemented with different concentrations of BAP and 2,4-D.

MS + Hormone (mg/L)		Mean callus dry weight (mg)	Morphology of callus
BAP	2,4-D		
0	0	NR	Explants producing shoots and roots without callus formation
	0.5	$71.60 \pm 6.40_{i}$	Creamy white, globular, compact
	1.0	$16.10 \pm 1.30_{abcd}$	Creamy white, globular, compact
	1.5	$29.70 \pm 4.10_{cde}$	Creamy white, globular, compact
	2.0	$33.30 \pm 3.80_{def}$	Creamy white, globular, compact
	2.5	$13.30 \pm 1.20_{abc}$	Creamy white, globular, compact
0.5	0.5	$12.90 \pm 1.40_{abc}$	Creamy white, globular, compact
	1.0	$20.00 \pm 3.80_{abcd}$	Creamy white, globular, compact
	1.5	$29.50 \pm 5.40_{cde}$	Creamy white, globular, compact
	2.0	$8.50 \pm 1.10_{ab}$	Creamy white, globular, compact
	2.5	$6.30 \pm 0.70_{ab}$	Creamy white, globular, compact
1.0	0.5	$24.00 \pm 5.00_{bcde}$	Creamy white, globular, compact
	1.0	$20.70 \pm 3.90_{abcd}$	Creamy white, globular, compact
	1.5	$8.80 \pm 0.80_{ab}$	Creamy white, globular, compact
	2.0	$7.30 \pm 0.80_{ab}$	Creamy white, globular, compact
	2.5	$6.10 \pm 0.80_{ab}$	Creamy white, globular, compact
1.5	0.5	$62.40 \pm 7.40_{hi}$	Creamy white, globular, compact
	1.0	$10.30 \pm 1.30_{ab}$	Creamy white, globular, compact
	1.5	$10.70 \pm 0.90_{ab}$	Creamy white, globular, compact
	2.0	$17.00 \pm 2.20_{abcd}$	Creamy white, globular, compact
	2.5	$3.50 \pm 0.60_{a}$	Creamy white, globular, compact

'Table 4.1. Continued'

MS + Hormone (mg/L)		Mean callus dry weight (mg)	Morphology of callus
BAP	2,4-D		
2.0	0.5	$46.00 \pm 10.10_{fg}$	Creamy white, globular, friable
	1.0	$50.30 \pm 14.70_{gh}$	Creamy white, globular, friable
	1.5	$22.80 \pm 3.60_{bcde}$	Creamy white, globular, friable
	2.0	$15.50 \pm 1.70_{abc}$	Creamy white, globular, friable
	2.5	$7.30 \pm 0.70_{ab}$	Creamy white, globular, friable
2.5	0.5	$38.10 \pm 5.70_{efg}$	Creamy white, globular, friable
	1.0	$50.60 \pm 14.20_{gh}$	Creamy white, globular, friable
	1.5	$19.70 \pm 2.20_{abcd}$	Creamy white, globular, friable
	2.0	$9.60 \pm 1.20_{ab}$	Creamy white, globular, friable

Mean values with different letters in a column are significantly different at $p<0.05$. (NR: no response)

4.3.2 Callus Derived from Root Explants

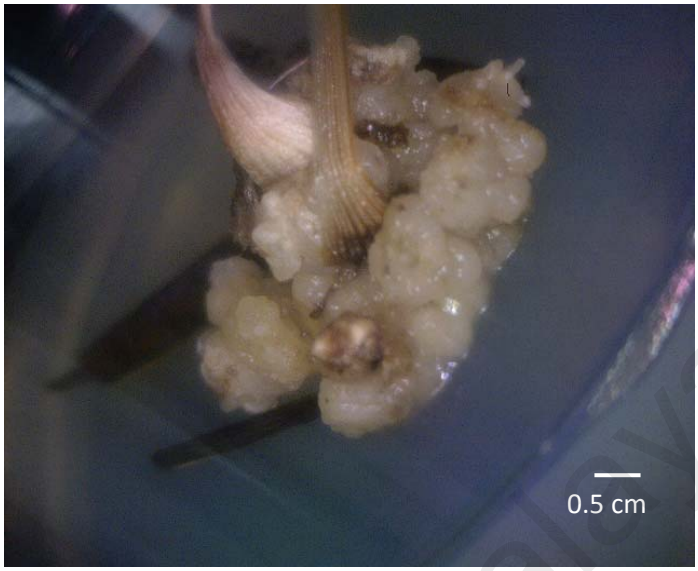
The mean callus dry weight from root explants was significantly higher (66.70 ± 10.90 mg) on MS media supplemented with 0.5 mg/L 2,4-D in combination with 0.5 mg/L BAP (Table 4.2). Overall, the root explants induced significantly lower mean callus dry weight than that of stem explants. It was observed that MS media supplemented with 0.5 mg/L BAP in combination with 2.5 mg/L 2,4-D, 1.0 mg/L BAP in combination with 0.5 mg/L 2,4-D, 1.0 mg/L BAP in combination with 1.0 mg/L 2,4-D and 1.0 mg/L BAP in combination with 1.5 mg/L 2,4-D produced lower mean callus dry weight. The mean callus dry weight of the treatments were 0.60 ± 0.10 mg, 1.20 ± 0.10 mg, 0.20 ± 0.10 mg and 0.50 ± 0.10 mg, respectively. The callus observed was mucilaginous at 1.0 mg/L BAP in combinations with 0.5 to 2.5 mg/L 2,4-D. The morphology of the callus was creamy white, globular and sticky at higher concentrations (1.5 mg/L to 2.5 mg/L) of BAP (Figure 4.1b). There was no green plants regenerated indicating that the callus produced was not embryogenic.

On the other hand, plantlets regenerated were observed from some of the callus induced from stem explants (Figure 4.2). No callus formation was observed from leaf explants cultured on MS media regardless of the types and concentrations of hormones applied. The leaf explants turned brown and died after a few weeks in culture.

Table 4.2: Mean callus dry weight obtained from root explants of *Oryza sativa* L. cv. MRQ 74 cultured on MS media supplemented with different concentrations of BAP and 2,4-D.

MS + Hormone (mg/L)		Mean callus dry weight (mg)	Morphology of callus
BAP	2,4-D		
0	0	NR	No callus formation
0.5	0.5	$66.70 \pm 10.90_{e}$	Creamy white, globular, sticky
	1.0	$9.20 \pm 0.90_{abcd}$	Creamy white, globular, sticky
	1.5	$10.90 \pm 1.20_{abcd}$	Creamy white, globular, sticky
	2.0	$4.10 \pm 1.40_{ab}$	mucilageneous
	2.5	$0.60 \pm 0.10_{a}$	mucilageneous
1.0	0.5	$1.20 \pm 0.10_{a}$	mucilageneous
	1.0	$0.20 \pm 0.10_{a}$	mucilageneous
	1.5	$0.50 \pm 0.10_{a}$	mucilageneous
	2.0	$7.00 \pm 0.90_{abc}$	mucilageneous
	2.5	$4.80 \pm 0.70_{ab}$	mucilageneous
1.5	0.5	$9.70 \pm 1.50_{abcd}$	Creamy white, globular, sticky
	1.0	$27.60 \pm 11.20_{d}$	Creamy white, globular, sticky
	1.5	$11.30 \pm 1.30_{abcd}$	Creamy white, globular, sticky
	2.0	$22.10 \pm 3.00_{bcd}$	Creamy white, globular, sticky
	2.5	$19.00 \pm 9.30_{abcd}$	Creamy white, globular, sticky
2.0	0.5	$15.00 \pm 6.50_{abcd}$	Creamy white, globular, sticky
	1.0	$13.50 \pm 3.01_{abcd}$	Creamy white, globular, sticky
	1.5	$26.30 \pm 4.20_{cd}$	Creamy white, globular, sticky
	2.0	$28.10 \pm 18.40_{d}$	Creamy white, globular, sticky
	2.5	$4.40 \pm 0.50_{ab}$	Creamy white, globular, sticky
2.5	0.5	$13.00 \pm 4.50_{abcd}$	Creamy white, globular, sticky
	1.0	$1.310 \pm 1.20_{abcd}$	Creamy white, globular, sticky
	1.5	$12.5\ 0 \pm 2.70_{abcd}$	Creamy white, globular, sticky
	2.0	$16.2 \pm 2.70_{abcd}$	Creamy white, globular, sticky

Mean values with different letters in a column are significantly different at $p<0.05$. (NR: no response)



(a)

(b)

Figure 4.1: Cream colored-calli from stem explant of *Oryza sativa* L. cv. MRQ 74 cultured on MS media supplemented with 1.5 mg/L 2,4-D (a), Cream colored-calli from root explant of *Oryza sativa* L. cv. MRQ 74 cultured on MS media supplemented with 0.5 mg/L 2,4-D (b).

Figure 4.2: Plantlets regenerated from some of the calli induced from stem explant cultured on MS media supplemented with 0.5 mg/L 2,4-D in combination with 2.0 mg/L BAP.

4.4 SUMMARY OF RESULTS

1. Callus induction from stem and root explants have been successfully achieved in *Oryza sativa* L. cv. MRQ 74. Leaf explants did not show any response on all callus induction media tested.
2. The mean callus dry weight derived from stem explants was significantly higher (71.60 ± 6.40 mg) in MS media supplemented with single hormone, 0.5 mg/L 2,4-D.
3. Callus induction from root explants was higher in MS media supplemented with 0.5 mg/L BAP in combination with 0.5 mg/L 2,4-D, as compared with other hormone combinations.
4. Shoots and roots formation were observed on hormone-free MS media.
5. Two different morphological callus types derived from stem explants were observed; creamy white, globular, compact and creamy white, globular, friable. Meanwhile, mucilaginous or creamy white, globular, and sticky callus were observed on callus derived from root explants.
6. Plantlets regenerated was observed from stem-derived callus suggesting that the produced calli were embryogenic.

CHAPTER 5

SOMATIC EMBRYOGENESIS OF *Oryza sativa* L. cv. MRQ 74

5.1 EXPERIMENTAL AIMS

Somatic embryogenesis is a process whereby a plant or embryo is derived from a single somatic cell or group of somatic cells. Somatic embryos are formed from plant cells that are not normally involved in the development of embryos, i.e. reproductive plant tissue. No endosperm or seed coat is formed around a somatic embryo. Applications of this process include: clonal propagation of genetically uniform plant material; elimination of viruses; provision of source tissue for genetic transformation; generation of whole plants from single cells called protoplasts; development of synthetic seed technology. Somatic embryogenesis has been described to occur in two ways: directly or indirectly. Direct embryogenesis occurs when embryos are started directly from explant tissue creating an identical clone while indirectly occurs from unorganized tissue (callus).

There are several factors that influence the induction of somatic embryogenesis such as genotype, type of explants, type of plant growth regulators added in the culture medium, age and developmental stage of an explant, physiological state of an explant-donor plant and physical culture conditions such as light and temperature. Somatic embryogenesis has been reported in rice cultures using various explants such as scutellar embryo (Syaiful Bahri *et al*., 2009), mature seeds (Zuraida *et al*., 2010) anthers (Chu *et al*., 1997), mature embryo-derived protoplast (Moura *et al*., 1997) and coleoptile tissues (Chand and Sahrawat, 1997).

Type of explant seems to be the most important factor which determines the embryogenic capacity of the culture. Different types of explants used to induce somatic embryogenesis displayed the highest response at a certain age, as was documented in

the culture of African and Indian Cassava cultivars (Beena *et al.,* 2014) cotyledonary leaf explants of *Solanum trilobatum* L. (Chakravarthi Dhavala *et al*. 2009) and flower explants of Cavendish banana cultivars (*Musa acuminata Colla*, AAA) (Youssef *et al*., 2010). Generally, young explants showed a better response for callus induction (Mohebodini *et al*., 2011). Even though root was found to be the responsive explants for callus induction of *Oryza sativa* L. cv. MRQ 74 in the current work, however, stem explants was chosen due to morphological characteristics and the ability of the produced callus to regenerate.

Plant growth regulators such as auxins and cytokinins have been the most frequently considered external factor that induces somatic embryogenesis. Among different auxins, 2,4-D was the most commonly applied for somatic embryogenesis either it was applied singly or in combination with other hormones. The high efficiency of 2,4-D for induction of embryogenic response found in different *in vitro* systems and plant species indicates a specific and unique character of this hormone. Therefore, in the present study, the most suitable type and concentration of plant growth regulator for somatic embryogenesis induction from stem derived callus of *Oryza sativa* L. cv. MRQ 74 was determined. The effects of amino acids presence in the somatic embryogenesis induction medium were also investigated.

5.2 MATERIALS AND METHODS

5.2.1 Source of Explants

Mature seeds of rice obtained from MARDI were surface sterilized and cultured as described in section 4.2.1.

5.2.2 Induction of Embryogenic Callus

Stem explants were approximately excised into 5.0 – 10.0 mm segments and cultured onto MS media supplemented with 30 g/L sucrose, 8 g/L technical agar and fortified with 2.0 mg/L BAP in combination with 1.0 mg/L 2,4-D for callus induction. This media was selected due to high amount of callus produced with morphological characteristics of embryogenic callus as obtained previously (Chapter 4). The pH of the media employed in this experiment was adjusted to 5.8 prior to autoclaving process at 121 ºC, 105 kPa for 21 minutes. The cultures were maintained in the culture room at 25 ± 1 ºC under 16 hours and 8 hours dark with 1000 lux of light intensity for 4 weeks.

5.2.3 Identification of Embryogenic Callus

In order to distinguish the embryogenic and non embryogenic callus, the callus produced was tested using Double Staining Technique. Prior to this, acetocarmine and Evan's blue solutions were prepared as below;

Preparation of 2 % Acetocarmine.

Approximately 45 ml of glacial acetic acid was added into a beaker containing 55 ml of distilled water to give a 45 % acid solution. The beaker containing the solution was placed on a stir plate in a fume hood and boiled gently for 5 minutes on highest setting, stirred, cooled and filtered by funnel using Whatman filter paper. Finally it was stored at room temperature.

Preparation of 0.5 % Evan's blue

Approximately 0.5 g of Evan's blue was added to 100 ml distilled water in a flask. The solution was stirred to mix properly and stored at room temperature. Small pieces of callus (3-5 mm) were placed on clean glass slides. A few drops of acetocarmine was added until all callus were submerged. The callus was gently divided with forceps into very small pieces in the acetocarmine solution. The specimens were flamed or heated gently for 2 minutes without boiling it. The callus was washed for 2 to 3 times with distilled water to remove all liquid of acetocarmine. Approximately 2 drops of 0.5 % Evan's blue was added to acetocarmine stained cells. After 30 seconds, the slides were washed 2 to 3 times with water. One to two drops of glycerol was added to the stained cells to prevent drying. The slides were then observed under light microscope and the embryogenic and non embryogenic callus were identified.

5.2.4 Induction of Somatic Embryos

Approximately 10 mg of embryogenic calli produced from section 5.2.2 were transferred onto MS media containing various concentrations of ABA and kinetin. Below is the list of MS media with different combinations and concentrations of ABA and kinetin that were used in this study.

1. MS + 1.0 mg/L ABA
2. MS + 1.5 mg/L ABA
3. MS + 2.0 mg/L ABA
4. MS + 2.5 mg/L ABA
5. MS + 3.0 mg/L ABA
6. MS + 0.5 mg/L ABA + 0.5 mg/L kinetin
7. MS + 0.5 mg/L ABA + 1.0 mg/L kinetin

8. MS + 0.5 mg/L ABA + 2.0 mg/L kinetin
9. MS + 1.0 mg/L ABA + 0.5 mg/L kinetin
10. MS + 1.0 mg/L ABA + 1.0 mg/L kinetin
11. MS + 1.0 mg/L ABA + 2.0 mg/L kinetin
12. MS + 1.5 mg/L ABA + 0.5 mg/L kinetin
13. MS + 1.5 mg/L ABA + 1.0 mg/L kinetin
14. MS + 1.5 mg/L ABA + 2.0 mg/L kinetin
15. MS + 2.0 mg/L ABA + 0.5 mg/L kinetin
16. MS + 2.0 mg/L ABA + 1.0 mg/L kinetin
17. MS + 2.0 mg/L ABA + 2.0 mg/L kinetin

Subsequently, the calli formed were examined microscopically to identify the formation and stages of embryogenic bodies such as globular, scutellar and coleoptilar.

5.2.5 The Effect of L - Proline on Somatic Embryogenesis

Besides ABA and kinetin, the effect of additives such as L-Proline on somatic embryogenesis of *Oryza sativa* L. cv. MRQ 74 was also tested. Approximately 10 mg of embryogenic calli produced in section 5.2.2 were transferred onto MS media supplemented with 1.5 mg/L ABA in combination with 1.0 mg/L kinetin and fortified with various concentrations of L-Proline (100, 200, 300, 400 and 500 mg/L). The number of somatic embryos produced was examined after 4 weeks of subculture.

5.2.6 Statistical Analysis

Data obtained were analysed using the IBM SPSS Statistics 20 software (International Business Machines Corp., Armonk, NY). Statistical variance analysis was conducted using ANOVA (Duncan's Multiple Range Test or DMRT) at 5 % significance level where thirty replicates were used for each treatment. The effect of

plant growth hormones on shoots and roots formation was quantified as mean ± SE (standard error).

5.3 RESULTS

5.3.1 Induction and Identification of Embryogenic Callus

Based on the results obtained from callus induction experiment (Chapter 4), MS media supplemented with 1.0 mg/L 2,4-D in combination with 2.0 mg/L BAP was chosen as callus induction media due to the quality of callus produced that were creamy white, globular and friable. The Double Staining Technique confirmed that the produced callus was embryogenic. The embryogenic callus was stained red in acetocarmine solution (Figure 5.1), while the non-embryogenic callus was stained blue (Figure 5.2).

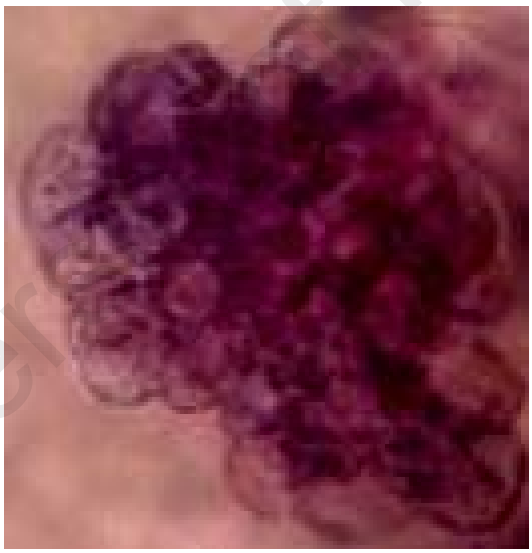


Figure 5.1: Embryogenic callus cells stained red with acetocarmine. Magnification 100X.

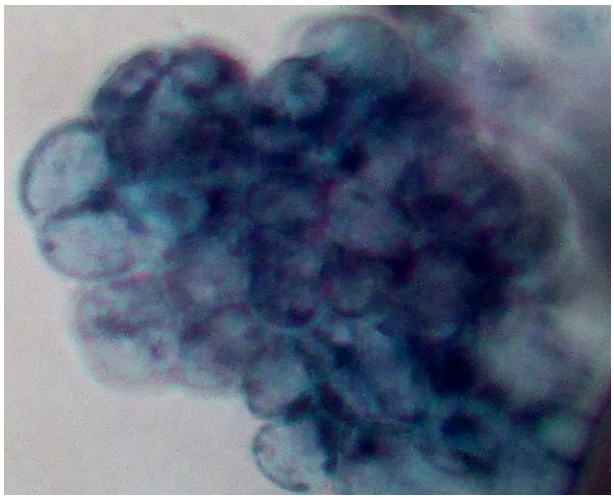


Figure 5.2: Non-embryogenic callus cells stained blue with Evan's blue stain. Magnification 100X.

5.3.2 Effects of ABA and Kinetin on Somatic Embryos Induction

After 4 weeks of incubation, approximately 10 mg of embrogenic calli were transferred onto MS media containing various concentrations of ABA either applied singly or in combinations with kinetin. The mean number of somatic embryos formation is presented in Table 5.1. The presence of ABA singly in the somatic embryo induction medium produced lower mean number of somatic embryos as compared with ABA in combinations with kinetin. The highest mean number of somatic embryos was recorded on MS media supplemented with 2.0 mg/L ABA, meanwhile MS media supplemented with 3.0 mg/L ABA showed the lowest mean number of somatic embryos, 11.50 ± 0.30 and 8.33 ± 0.28, respectively.

Table 5.1: Mean no. of somatic embryos of *Oryza sativa* L. cv. MRQ 74 from embryogenic calli subcultured on MS media supplemented with different concentrations of ABA and kinetin.

MS medium + Hormone mg/L		No. of somatic embryos (Mean ± SE)
ABA	kinetin	
1.0	0	$8.53 \pm 0.24_{bc}$
1.5	0	$10.27 \pm 0.25_{e}$
2.0	0	$11.50 \pm 0.30_{f}$
2.5	0	$9.83 \pm 0.22_{de}$
3.0	0	$8.33 \pm 0.28_{b}$
0.5	0.5	$11.40 \pm 0.29_{f}$
0.5	1.0	$12.00 \pm 0.24_{fg}$
0.5	2.0	$9.67 \pm 0.21_{de}$
1.0	0.5	$12.13 \pm 0.25_{fg}$
1.0	1.0	$14.33 \pm 0.27_{i}$
1.0	2.0	$10.17 \pm 0.27_{e}$
1.5	0.5	$8.67 \pm 0.21_{bc}$
1.5	1.0	$15.47 \pm 0.24_{j}$
1.5	2.0	$12.30 \pm 0.26_{g}$
2.0	0.5	$7.50 \pm 0.23_{a}$
2.0	1.0	$13.27 \pm 0.27_{h}$
2.0	2.0	$9.23 \pm 0.21_{cd}$

Mean values with different letters in a column are significantly different at $p<0.05$.

5.3.3 The Effect of L - Proline on Somatic Embryos Induction.

In order to study the effect of amino acids on somatic embryos induction, approximately 10 mg of embryogenic calli were cultured on MS media supplemented with 1.5 mg/L ABA in combination with 1.0 mg/L kinetin and fortified with various concentrations of L-Proline. The concentrations of ABA and kinetin applied in this experiment were based on the highest mean number of somatic embryos obtained in the previous experiment (Table 5.1). The results showed that the mean number of somatic embryos were significantly higher at 300 mg/L and 400 mg/L of L-Proline which were

15.33 ± 0.59 and 17.37 ± 0.66, respectively. However, somatic embryo formation decreased when the concentration of L-Proline increased to 500 mg/L (Table 5.2).

Table 5.2: Effect of L-Proline along with 1.5 mg/L ABA in combination with 1.0 mg/L kinetin on somatic embryos induction from stem derived callus of *Oryza sativa* L. cv. MRQ 74.

L-Proline (mg/L)	No. of somatic embryos (Mean ± SE)
100	$12.70 \pm 0.47_{ab}$
200	$11.30 \pm 0.39_{a}$
300	$15.33 \pm 0.59_{c}$
400	$17.37 \pm 0.66_{d}$
500	$13.17 \pm 0.69_{b}$

Mean values with different letters within a column are significantly different at $p<0.05$.

5.3.4 Somatic Embryos Development and Organogenesis

Somatic embryogenesis of *Oryza sativa* L. cv. MRQ 74 was successfully achieved in this experiment. The developmental stages of somatic embryo of *Oryza sativa* L. Cv. MRQ 74 was clearly observed, as shown in Figure 5.3. The globular somatic embryos elongate to form scutellar and subsequently coleoptilar stages. The formation of multiple microshoots and microshoots elongation was also observed when embryogenic callus derived from stem explants that were cultured on MS media supplemented with 1.0 mg/L 2,4-D in combination with 2.0 mg/L BAP, was subcultured on MS media supplemented with 2.0 mg/L ABA (Figures 5.4, 5.5 and 5.6). Microshoots were also formed when embryogenic callus was subcultured on MS media containing 0.5 mg/L kinetin in combination with 1.0 mg/L ABA.

Indirect organogenesis of *Oryza sativa* L. cv. MRQ 74 was observed in the present study. Roots were produced when embryogenic calli were subcultured on MS media supplemented with 1.0 mg/L kinetin in combination with 2.0 mg/L ABA (Figure 5.7).

Hairy roots were formed when embryogenic calli were subcultured on MS media supplemented with 1.0 mg/L kinetin in combination with 0.5 mg/L ABA (Figure 5.8).

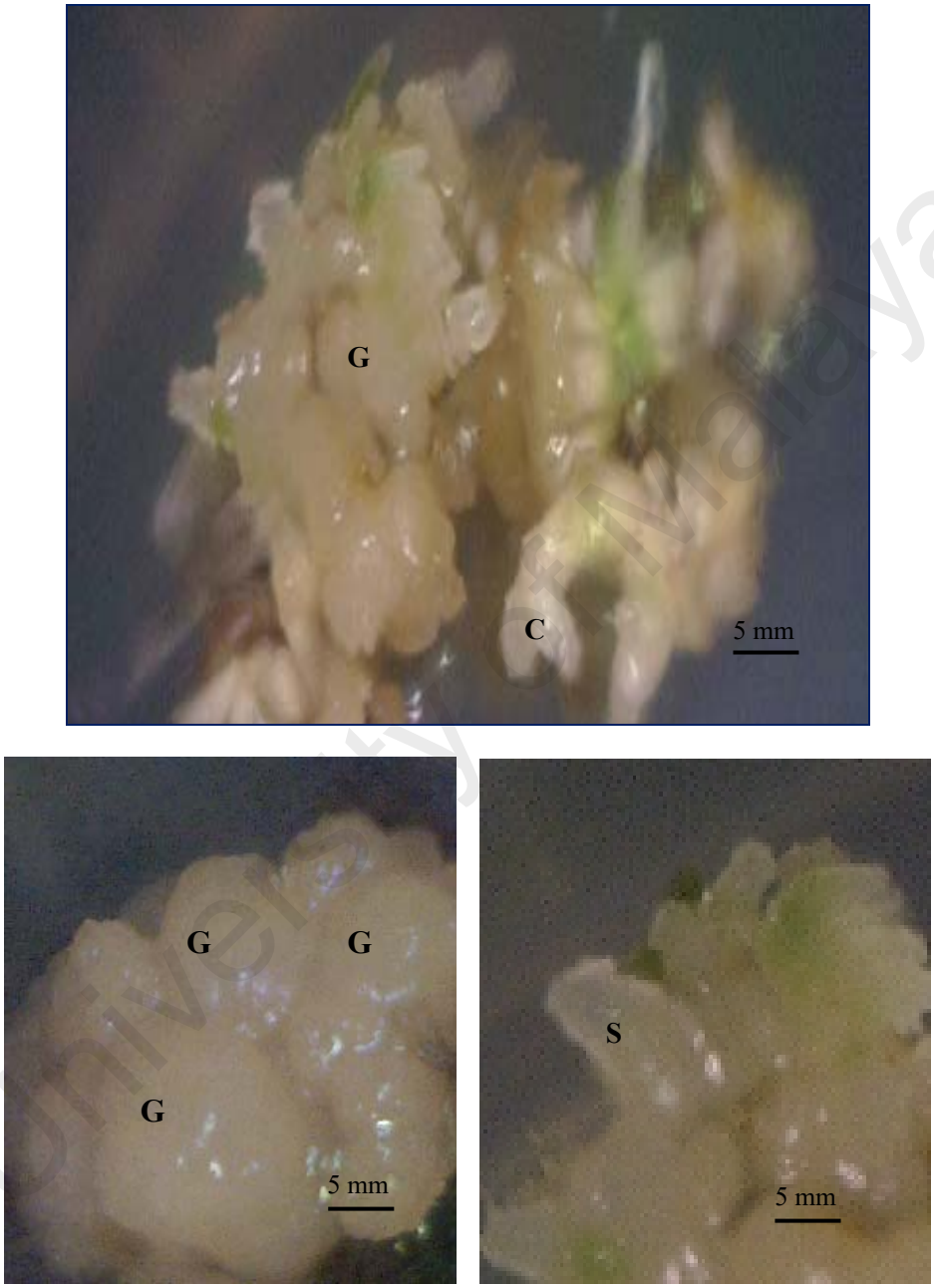


Figure 5.3: Embryogenic callus subcultured on MS media supplemented with 2.0 mg/L ABA, at the globular (G), scutellar (S) and coleoptilar (C) stages.

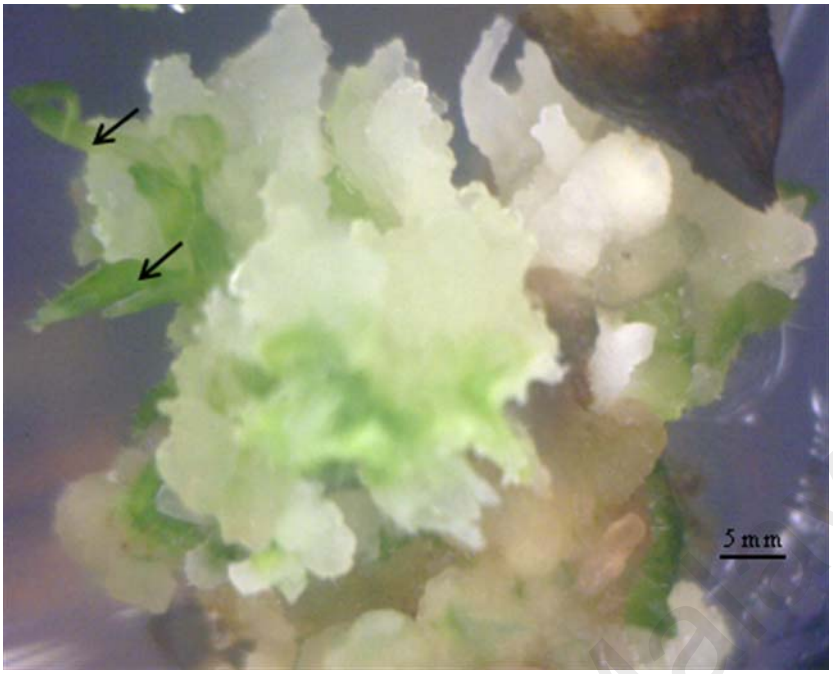


Figure 5.4: Development of microshoots from embryogenic calli that were subcultured on MS media supplemented with 0.5 mg/L kinetin + 1.0 mg/L ABA.

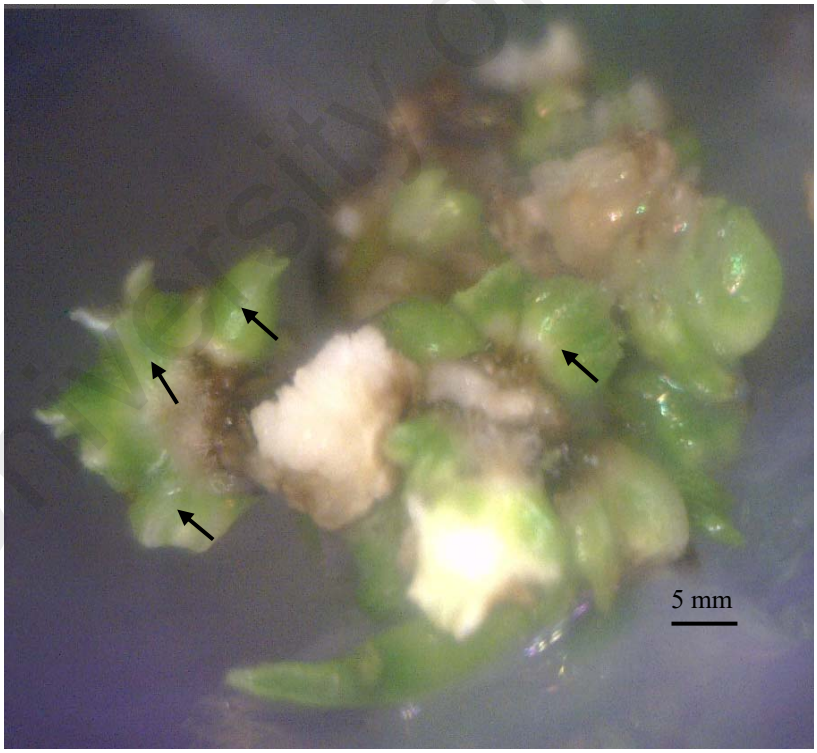


Figure 5.5: Development of microshoots from somatic embryos of *Oryza sativa* L. cv. MRQ 74, from stem explants subcultured on MS media supplemented with 2.0 mg/L ABA.

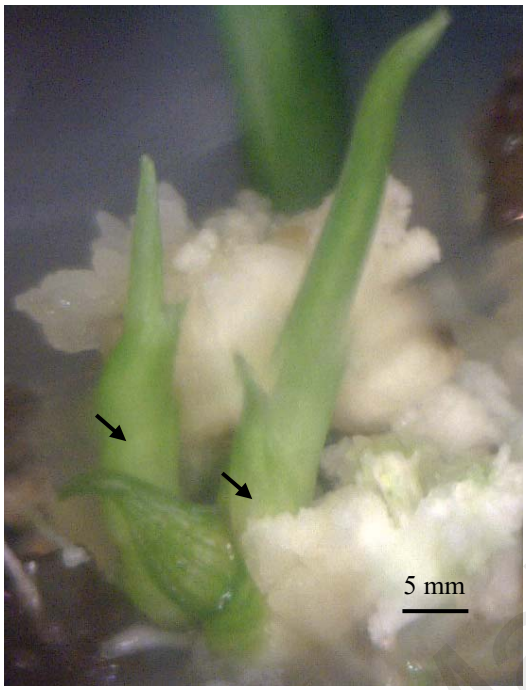


Figure 5.6: Further development of the microshoots from somatic embryo of *Oryza sativa* L. cv. MRQ 74, from stem derived callus subcultured on MS media supplemented with 2.0 mg/L ABA.

Figure 5.7: Formation of roots from embryogenic calli that were subcultured on MS media supplemented with 1.0 mg/L kinetin + 2.0 mg/L ABA.

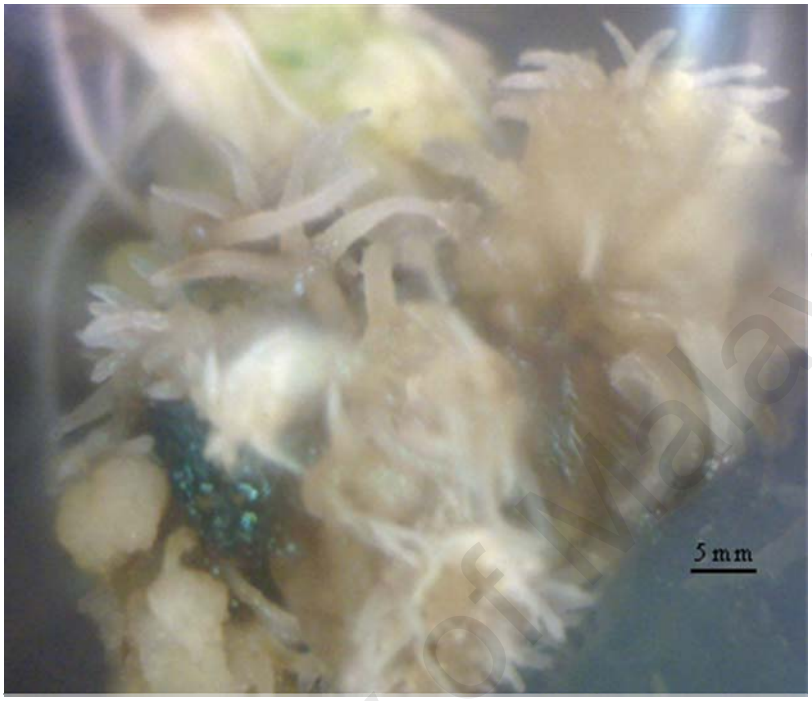


Figure 5.8: Formation of hairy roots from embryogenic callus, derived from stem explants subcultured on MS media supplemented with 1.0 mg/L kinetin + 0.5 mg/L ABA.

5.4 SUMMARY OF RESULTS

1. Somatic embryogenesis was successfully induced from stem explants of *Oryza sativa* L. cv. MRQ 74.
2. The presence of ABA in MS media significantly increased the mean number of somatic embryos.
3. The highest mean number of somatic embryos (15.47 ± 0.24) was observed on MS media supplemented with 1.5 mg/L ABA in combination with 1.0 mg/L kinetin.
4. The addition of L-Proline at the concentration of 400 mg/L resulted significantly higher mean number of somatic embryos (17.37 ± 0.66).
5. MS media supplemented with 1.5 mg/L ABA in combination with 1.0 mg/L kinetin and 400 mg/L L-Proline was the best induction medium for somatic embryogenesis of *Oryza sativa* L. cv. MRQ 74.
6. The formation of multiple microshoots were observed when embryogenic calli derived from stem explants cultured on MS media supplemented with 2.0 mg/L BAP in combination with 1.0 mg/L 2,4-D were subcultured on MS media supplemented with 2.0 mg/L ABA.
7. Organogenesis was also observed, whereby roots were produced when embryogenic calli were subcultured on MS media supplemented with 1.0 mg/L kinetin in combination with 2.0 mg/L ABA.
8. Hairy roots were produced when the embryogenic calli were subcultured on MS media supplemented with 1.0 mg/L kinetin in combination with 0.5 mg/L ABA.

CHAPTER 6

IN VITRO REGENERATION OF *Oryza sativa* L. cv. MRQ 74

6.1 EXPERIMENTAL AIMS

Micropropagation is an alternative method of propagation and is widely used for the commercial propagation of a large number of plant species. Nowadays, a wide range of plant tissue culture techniques have been developed for shoot multiplication of rice. However, *in vitro* propagation of different cultivars required different culture media components for shoot proliferation and root differentiation. The types of plant growth hormones and explants are among the factors influencing *in vitro* propagation of a species. The presence of the most suitable type of plant growth hormones with optimal concentration in culture medium will give maximum shoot multiplication and root induction of a species. Among the widely used plant growth hormones, 6-benzyladenine purine (BAP), kinetin and thidiazuron (TDZ) were reported as very effective cytokinins in shoot multiplication of many plant species such as *Plumbago zeylanica* L. (Ashok and Bashir, 2011) and *Bacopa chamaedryoides* (Kunth) Wettst (Sk Moquammel and Biswajit, 2013).

In vitro plant regeneration of rice have been reported using various explants such as protoplasts derived from cell suspension (Tang *et al*., 2001), mature seeds (Saharan *et al*., 2004; Bano *et al*., 2005), radicals (Mikami and Kinoshita, 1988), coleoptiles and mature embryos (Khanna and Raina, 1998), root segments (Hoque and Mansfield, 2004) and microspores (Genovesi and Magill, 1982). However, very few plant regeneration studies derived from stem explants have been reported on this economically important species. Hence, in the present study, besides young leaf and root segments, stem explants were also tested to compare the efficiency of shoot multiplication in *in vitro* regeneration of rice cultivar MRQ 74.

6.2 MATERIALS AND METHODS

6.2.1 Plant Materials

The mature seeds of *Oryza sativa* L. Cv MRQ 74 were obtained from Malaysia Agricultural Research and Development Institute (MARDI) Seberang Prai, Penang. The sterilised seeds were cultured on Murashige and Skoog (1962) media and one-month-old aseptic seedlings were used as source of explants.

6.2.2 Seeds Sterilization and Germination

The dehusked seeds were surface sterilized by soaking and shaking them in 70 % (v/v) Clorox with two dropes of 1 ml/L Tween 20 followed by 50 %, 30%, 20% and 10% (v/v) Clorox. Shaking the materials during sterilization will obviously enhance the effectiveness of the process. Each treatment lasted approximately one minute. The dehusked seeds were then rinsed three times in sterilised distilled water. Finally, the seeds were rinsed in 70 % (v/v) alcohol for one minute followed by three times in sterilised distilled water for complete removal of Clorox and ethanol in laminar air flow cabinet.

The sterilised seeds were then cultured onto MS media containing sucrose (30 g/L) and agar (8 g/L). Ten seeds were placed per culture tube. The cultures were incubated in the culture room at 25 ± 1 ºC under 16 hours light and 8 hours dark with 1000 lux of light intensity. The seeds started to germinate after 3 to 4 days to form seedlings. The aseptic seedlings were maintained in the culture room for 4 weeks as source of explants for *in vitro* plant regeneration.

6.2.3 Basal Medium and Culture Condition

For multiple shoots induction, MS media containing various concentrations of BAP, kinetin and TDZ applied singly or in combinations with NAA and IBA were used in this study. Below is the list of media with different combinations and concentrations of plant growth hormones that were utilised in the present study.

1. MS + 0.0 mg/L BAP + 0.0 mg/L NAA (as control)
2. MS + 0.1 mg/L BAP + 0.0 mg/L NAA
3. MS + 0.1 mg/L BAP + 0.1 mg/L NAA
4. MS + 0.1 mg/L BAP + 0.5 mg/L NAA
5. MS + 0.1 mg/L BAP + 1.0 mg/L NAA
6. MS + 0.5 mg/L BAP + 0.0 mg/L NAA
7. MS + 0.5 mg/L BAP + 0.1 mg/L NAA
8. MS + 0.5 mg/L BAP + 0.5 mg/L NAA
9. MS + 0.5 mg/L BAP + 1.0 mg/L NAA
10. MS + 1.0 mg/L BAP + 0.0 mg/L NAA
11. MS + 1.0 mg/L BAP + 0.1 mg/L NAA
12. MS + 1.0 mg/L BAP + 0.5 mg/L NAA
13. MS + 1.0 mg/L BAP + 1.0 mg/L NAA
14. MS + 1.5 mg/L BAP + 0.0 mg/L NAA
15. MS + 1.5 mg/L BAP + 0.1 mg/L NAA
16. MS + 1.5 mg/L BAP + 0.5 mg/L NAA
17. MS + 1.5 mg/L BAP + 1.0 mg/L NAA
18. MS + 0.5 mg/L kinetin + 0.0 mg/L NAA
19. MS + 0.0 mg/L kinetin + 0.1 mg/L NAA
20. MS + 0.1 mg/L kinetin + 0.1 mg/L NAA
21. MS + 0.5 mg/L Kinetin + 0.1 mg/L NAA

22. MS + 0.0 mg/L kinetin + 0.5 mg/L NAA
23. MS + 0.1 mg/L kinetin + 0.5 mg/L NAA
24. MS + 0.5 mg/L kinetin + 0.5 mg/L NAA
25. MS + 0.0 mg/L kinetin + 1.0 mg/L NAA
26. MS + 0.1 mg/L kinetin + 1.0 mg/L NAA
27. MS + 0.5 mg/L kinetin + 1.0 mg/L NAA
28. MS + 0.1 mg/L TDZ + 0.0 mg/L BAP + 0.0 mg/L NAA
29. MS + 0.2 mg/L TDZ + 0.0 mg/L BAP + 0.0 mg/L NAA
30. MS + 0.3 mg/L TDZ + 0.0 mg/L BAP + 0.0 mg/L NAA
31. MS + 0.4 mg/L TDZ + 0.0 mg/L BAP + 0.0 mg/L NAA
32. MS + 0.5 mg/L TDZ + 0.0 mg/L BAP + 0.0 mg/L NAA
33. MS + 0.1 mg/L TDZ + 0.0 mg/L BAP + 0.1 mg/L NAA
34. MS + 0.2 mg/L TDZ + 0.0 mg/L BAP + 0.1 mg/L NAA
35. MS + 0.1 mg/L TDZ + 0.1 mg/L BAP + 0.1 mg/L NAA
36. MS + 0.0 mg/L BAP + 0.1 mg/L IBA
37. MS + 0.0 mg/L BAP + 0.5 mg/L IBA
38. MS + 0.0 mg/L BAP + 1.0 mg/L IBA
39. MS + 0.1 mg/L BAP + 0.1 mg/L IBA
40. MS + 0.1 mg/L BAP + 0.5 mg/L IBA
41. MS + 0.1 mg/L BAP + 1.0 mg/L IBA
42. MS + 0.5 mg/L BAP + 0.1 mg/L IBA
43. MS + 0.5 mg/L BAP + 0.5 mg/L IBA
44. MS + 0.5 mg/L BAP + 1.0 mg/L IBA
45. MS + 1.0 mg/L BAP + 0.1 mg/L IBA
46. MS + 1.0 mg/L BAP + 0.5 mg/L IBA
47. MS + 1.0 mg/L BAP + 1.0 mg/L IBA

6.2.4 Explants Culture

Root, leaf and stem (the lowest part) explants were approximately excised into 5.0 to 10.0 mm segments and cultured onto MS media supplemented with 30 g/L sucrose, 8 g/L technical agar and fortified with various concentrations of BAP (0.1, 0.5 and 1.0 mg/L) in combinations with NAA (0.1, 0.5 and 1.0 mg/L), kinetin (0.1 and 0.5 mg/L) in combinations with NAA (0.1, 0.5 and 1.0 mg/L), and BAP (0.1, 0.5 and 1.0 mg/L) in combinations with IBA (0.1, 0.5 and 1.0 mg/L). The explants were also cultured onto MS media containing TDZ (0.1, 0.2, 0.3, 0.4 and 0.5 mg/L) singly and in combinations with NAA and BAP at a concentration of 0.1 mg/L each. All cultures were maintained in the culture room at 25 ± 1 ºC, 16 hours light and 8 hours dark for two months. The number of shoots, roots and root length were counted from each treatment after two months of culture.

6.2.5 Statistical Analysis.

Data obtained were analysed using the IBM SPSS Statistics 20 software (International Business Machines Corp., Armonk, NY). Statistical variance analysis was conducted using ANOVA (Duncan's Multiple Range Test or DMRT) at 5 % significance level where thirty replicates were used for each treatment. The effect of plant growth hormones on shoots and roots formation was quantified as mean ± SE (standard error).

6.3 RESULTS

6.3.1 Effects of Different Concentrations of BAP and NAA on *In Vitro* Regeneration

Root, leaf and stem explants from one-month-old aseptic seedlings were cultured onto regeneration media augmented with nine combinations of BAP and NAA in order to explore its regeneration potential. BAP was also applied singly at the concentrations of 0.1, 0.5, 1.0 and 1.5 mg/L (Table 6.1). From three types of explants used, only stem explant was responsive. The results showed that the presence of BAP in MS media induced multiple shoots formation from stem explants of *Oryza sativa* L. cv. MRQ 74. The number of multiple shoots produced varied with hormone concentrations. The presence of BAP alone in the culture media at the highest concentration (1.5 mg/L) showed the best response for shoot proliferation (Figure 6.1 A). The maximum mean number of shoots per explant (4.03 ± 0.3) was observed at this concentration. There were no significant differences in mean number of shoots per explant at the concentrations of 0.5 mg/L BAP in combination with 1.0 mg/L NAA and 1.5 mg/L BAP in combination with 0.1 mg/L NAA as compared with control. The lowest mean number of shoots per explant (1.97 ± 0.16) was recorded on MS media supplemented with 0.1 mg/L BAP and 0.5 mg/L NAA. The combinations of BAP and NAA at all concentrations applied, except for the concentrations of 1.0 mg/L BAP in combination with 1.0 mg/L NAA and 0.5 mg/L BAP in combination with 0.5 mg/L NAA produced significantly lower mean number of shoots per explant as compared with application of BAP singly.

On the other hand, different effects were observed for roots formation when both BAP and NAA were supplemented in the culture media (Table 6.2). The highest mean number of roots per explant (25.53 ± 1.89) was found on MS media supplemented with

0.1 mg/L BAP and 0.1 mg/L NAA with the longest root length ranged from 0.5 to 7.0 cm. MS media fortified with 0.5 mg/L BAP showed the lowest percentage of explants produced roots which was 63.33%. The addition of BAP singly in MS media resulted lower mean number of roots produced. This scenario was observed on MS media supplemented with 0.5, 1.0 and 1.5 mg/L BAP. At these concentrations, the mean number of roots per explant were significantly lower, which were 1.23 ± 0.25, 1.27 ± 0.25 and 1.37 ± 0.24, respectively.

Table 6.1: Percentage of explants produced shoots and mean no. of shoots per explant obtained from stem explants of *Oryza sativa* L. cv. MRQ 74 cultured on MS media supplemented with different concentrations of BAP and NAA.

MS + Hormone mg/L		Percentage of explants produced shoots (%)	No. of shoots per explant (Mean ± SE)
BAP	NAA		
0.0	0.0	$100.00 \pm 0.00_{a}$	$2.30 \pm 0.16_{abc}$
0.1	0.0	$100.00 \pm 0.00_{a}$	$3.00 \pm 0.27_{cde}$
	0.1	$100.00 \pm 0.00_{a}$	$2.80 \pm 0.23_{bcde}$
	0.5	$100.00 \pm 0.00_{a}$	$1.97 \pm 0.16_{a}$
	1.0	$100.00 \pm 0.00_{a}$	$2.13 \pm 0.21_{ab}$
0.5	0.0	$100.00 \pm 0.00_{a}$	$3.13 \pm 0.23_{de}$
	0.1	$100.00 \pm 0.00_{a}$	$2.37 \pm 0.15_{abcd}$
	0.5	$100.00 \pm 0.00_{a}$	$3.03 \pm 0.24_{cde}$
	1.0	$100.00 \pm 0.00_{a}$	$2.30 \pm 0.18_{abc}$
1.0	0.0	$100.00 \pm 0.00_{a}$	$3.13 \pm 0.26_{de}$
	0.1	$100.00 \pm 0.00_{a}$	$2.70 \pm 0.25_{abcd}$
	0.5	$100.00 \pm 0.00_{a}$	$2.57 \pm 0.22_{abcd}$
	1.0	$100.00 \pm 0.00_{a}$	$3.53 \pm 0.43_{ef}$
1.5	0.0	$100.00 \pm 0.00_{a}$	$4.03 \pm 0.31_{f}$
	0.1	$100.00 \pm 0.00_{a}$	$2.30 \pm 0.28_{abc}$
	0.5	$100.00 \pm 0.00_{a}$	$2.67 \pm 0.24_{abcd}$
	1.0	$100.00 \pm 0.00_{a}$	$2.87 \pm 0.18_{bcde}$

Mean values with different letters in a column are significantly different at $p<0.05$.

Table 6.2: Percentage of explants produced roots and mean no. of roots per explant obtained from stem explants of *Oryza sativa* L. cv MRQ 74 cultured on MS media supplemented with different concentrations of BAP and NAA.

MS + Hormone mg/L		Percentage of explants produced roots (%)	No. of roots per explant (Mean ± SE)	Root length (cm)
BAP	NAA			
0.0	0.0	$96.67 \pm 0.48_{h}$	$5.27 \pm 0.76_{d}$	0.2 - 4.0
0.1	0.0	$86.67 \pm 0.48_{f}$	$5.83 \pm 1.18_{d}$	0.3 - 4.0
	0.1	$100.00 \pm 0.00_{i}$	$25.33 \pm 1.89_{f}$	0.5 - 7.0
	0.5	$93.33 \pm 0.48_{g}$	$5.37 \pm 0.74_{d}$	0.2 - 4.5
	1.0	$100.00 \pm 0.00_{i}$	$6.80 \pm 0.70_{d}$	0.4 - 4.5
0.5	0.0	$63.33 \pm 0.73_{a}$	$1.23 \pm 0.25_{a}$	0.4 - 2.7
	0.1	$100.00 \pm 0.00_{i}$	$5.53 \pm 0.86_{d}$	0.3 - 4.2
	0.5	$93.33 \pm 0.48_{g}$	$2.57 \pm 0.27_{abc}$	0.2 - 0.7
	1.0	$93.33 \pm 0.48_{g}$	$4.80 \pm 0.58_{cd}$	0.3 - 1.7
1.0	0.0	$73.33 \pm 0.82_{c}$	$1.27 \pm 0.25_{a}$	0.3 - 2.5
	0.1	$100.00 \pm 0.00_{i}$	$5.13 \pm 0.66_{d}$	0.3 - 4.3
	0.5	$93.33 \pm 0.48_{g}$	$9.80 \pm 1.35_{e}$	0.4 - 5.0
	1.0	$96.67 \pm 0.48_{h}$	$4.40 \pm 0.69_{bcd}$	0.2 - 2.5
1.5	0.0	$70.00 \pm 0.75_{b}$	$1.37 \pm 0.24_{a}$	0.2 - 2.5
	0.1	$76.67 \pm 0.68_{d}$	$2.67 \pm 0.56_{abc}$	0.3 - 2.0
	0.5	$93.33 \pm 0.48_{g}$	$2.13 \pm 0.26_{ab}$	0.2 - 2.3
	1.0	$80.00 \pm 0.68_{e}$	$1.87 \pm 0.28_{a}$	0.2 - 1.1

Mean values with different letters in a column are significantly different at $p<0.05$.

6.3.2 Effects of Different Concentrations of Kinetin and NAA on *In Vitro* Regeneration

The effects of kinetin and NAA on shoot multiplication is presented in Table 6.3. The results showed that all cultures developed shoots regardless of the types and concentrations used in the culture media. Among these, MS media supplemented with 0.5 mg/L kinetin and 1.0 mg/L NAA in combination with 0.5 mg/L kinetin produced the maximum number of shoots which were 2.57 ± 0.20 and 2.60 ± 0.18, respectively.

In general, it was observed that the mean number of roots per explant was significantly higher in MS media supplemented with NAA and kinetin compared to control except for 0.1 mg/L NAA in combination with 0.5 mg/L kinetin and 0.5 mg/L NAA in combination with 0.5 mg/L kinetin (Table 6.4). All the cultured stem explants produced roots except for the stem explants cultured on MS media supplemented with 0.1 mg/L NAA in combination with 0.5 mg/L kinetin, 0.5 mg/L NAA in combination with 0.5 mg/L kinetin and control. The percentage of explants produced roots were 96.67 ± 0.48 and 93.33 ± 0.48, respectively. The best result for rooting (24.37 ± 1.91) was found on MS media supplemented with NAA singly at the concentration of 0.1 mg/L (Figure 6.1 B). The presence of kinetin alone in MS media resulted in the longest root length that ranged from 0.7 – 9.0 cm.

Table 6.3: Percentage of explants produced shoots and mean no. of shoots per explant obtained from stem explants of *Oryza sativa* L. cv MRQ 74 cultured on MS media supplemented with different concentrations of NAA and kinetin.

MS + Hormone mg/L		Percentage of explants produced shoots (%)	No. of shoots per explant (Mean ± SE)
NAA	kinetin		
0.0	0.0	$100.00 \pm 0.00_{a}$	$2.30 \pm 0.16_{bc}$
0.0	0.5	$100.00 \pm 0.00_{a}$	$2.57 \pm 0.20_{c}$
0.1	0.0	$100.00 \pm 0.00_{a}$	$1.63 \pm 0.21_{a}$
	0.1	$100.00 \pm 0.00_{a}$	$2.40 \pm 0.20_{bc}$
	0.5	$100.00 \pm 0.00_{a}$	$2.03 \pm 0.18_{abc}$
0.5	0.0	$100.00 \pm 0.00_{a}$	$2.10 \pm 0.19_{abc}$
	0.1	$100.00 \pm 0.00_{a}$	$2.00 \pm 0.19_{abc}$
	0.5	$100.00 \pm 0.00_{a}$	$2.03 \pm 0.19_{abc}$
1.0	0.0	$100.00 \pm 0.00_{a}$	$1.80 \pm 0.16_{ab}$
	0.1	$100.00 \pm 0.00_{a}$	$2.07 \pm 0.19_{abc}$
	0.5	$100.00 \pm 0.00_{a}$	$2.60 \pm 0.18_{c}$

Mean values with different letters in a column are significantly different at $p<0.05$.

Table 6.4: Percentage of explants produced roots and mean no. of roots per explant obtained from stem explants of *Oryza sativa* L. cv. MRQ 74 cultured on MS media supplemented with different concentrations of NAA and kinetin.

MS + Hormone mg/L		Percentage of explants produced roots (%)	No. of roots per explant (Mean ± SE)	Root length (cm)
NAA	kinetin.			
0.0	0.0	$96.67 \pm 0.48_{ab}$	$5.27 \pm 0.76_{a}$	0.2 - 4.0
0.0	0.5	$100.00 \pm 0.00_{b}$	$6.00 \pm 0.58_{ab}$	0.7 - 9.0
0.1	0.0	$100.00 \pm 0.00_{b}$	$24.37 \pm 1.91_{e}$	0.3 - 3.0
	0.1	$100.00 \pm 0.00_{b}$	$8.63 \pm 0.83_{bc}$	0.2 - 3.2
	0.5	$96.67 \pm 0.48_{ab}$	$5.27 \pm 0.69_{a}$	0.2 - 2.0
0.5	0.0	$100.00 \pm 0.00_{b}$	$9.90 \pm 1.03_{cd}$	0.3 - 5.5
	0.1	$100.00 \pm 0.00_{b}$	$9.73 \pm 0.79_{cd}$	0.4 - 5.0
	0.5	$93.33 \pm 0.48_{a}$	$5.10 \pm 0.62_{a}$	0.2 - 2.5
1.0	0.0	$100.00 \pm 0.00_{b}$	$12.17 \pm 0.87_{d}$	0.2 - 2.8
	0.1	$100.00 \pm 0.00_{b}$	$10.30 \pm 1.51_{cd}$	0.3 - 5.7
	0.5	$100.00 \pm 0.00_{b}$	$8.77 \pm 1.00_{bc}$	0.4 - 3.0

Mean values with different letters in a column are significantly different at $p<0.05$.

6.3.3 Effects of Different Concentrations of TDZ on *In Vitro* Regeneration.

The highest mean number of shoots per explant (8.23 ± 1.09) was obtained when stem explants were cultured on MS media supplemented with 0.1 mg/L TDZ (Figure 6.1C), followed by 6.47 ± 0.86, 5.40 ± 0.73, 5.40 ± 0.60 and 4.67 ± 0.66 shoots per explant at the concentrations of 0.2, 0.3, 0.4 mg/L TDZ and 0.2 mg/L TDZ in combination with 0.1 mg/L NAA, respectively (Table 6.5). The mean number of shoots per explant decreased significantly with increase concentrations of this hormone, 0.2 to 0.5 mg/L. The plantlets produced at the concentrations of 0.3 to 0.5 mg/L TDZ showed abnormal growth (Figure 6.1D).

In order to promote higher number of shoots formation, NAA and BAP were added to MS media containing 0.1 and 0.2 mg/L TDZ. However, the results showed that an addition of these hormones produced no significant difference in mean number of shoots per explant. In fact, TDZ in combination with NAA and BAP at the

concentrations of 0.1 mg/L each yielded the least mean number of shoots per explant (2.57 ± 0.22) as compared to BAP alone, which were 3.00 ± 0.27, 3.13 ± 0.23, 3.13 ± 0.26 and 4.03 ± 0.31 at the concentrations of 0.1, 0.5. 1.0 and 1.5 mg/L BAP, respectively (Table 6.1).

In terms of number of roots produced, the lowest concentration of TDZ (0.1 mg/L) showed the highest mean number of roots per explant (0.30 ± 0.16) with root length ranged from 0.2 to 0.7 cm (Table 6.6). Similar results have been reported on regeneration of *Echinacea purpurea* L. by Jones *et al*., (2007). The plantlets of *E. purpurea* obtained from low concentration of TDZ were healthy and rooted earlier. However, in the present study, all treatments showed no significant difference in mean numbers of roots per explant, but there were significantly different ($P<0.05$) as compared to control (5.27 ± 0.76). The highest percentage of explants produced roots was achieved on MS media supplemented with 0.1 mg/L TDZ. The percentage was significantly lower (0.30 ± 0.16 %) as compared to control (96.67 ± 0.03 %).

Table 6.5: Percentage of explants produced shoots and mean no. of shoots per explant of *Oryza sativa* L. cv. MRQ 74 cultured on MS media supplemented with different concentrations of TDZ, NAA and BAP.

MS + Hormone (mg/L)			Percentage of explants produced shoots (%)	No. of shoots per explant (Mean ± SE)
TDZ	NAA	BAP		
0.0	0.0	0.0	$100.00 \pm 0.00_{a}$	$2.30 \pm 0.16_{a}$
0.1			$100.00 \pm 0.00_{a}$	$8.23 \pm 1.09_{e}$
0.2			$100.00 \pm 0.00_{a}$	$6.47 \pm 0.86_{d}$
0.3			$100.00 \pm 0.00_{a}$	$5.40 \pm 0.73_{cd}$
0.4			$100.00 \pm 0.00_{a}$	$5.40 \pm 0.60_{cd}$
0.5			$100.00 \pm 0.00_{a}$	$4.20 \pm 0.33_{abc}$
0.1	0.1		$100.00 \pm 0.00_{a}$	$3.17 \pm 0.41_{ab}$
0.2	0.1		$100.00 \pm 0.00_{a}$	$4.67 \pm 0.66_{bcd}$
0.1	0.1	0.1	$100.00 \pm 0.00_{a}$	$2.57 \pm 0.22_{a}$

Mean values with different letters in a column are significantly different at $p<0.05$.

Table 6.6: Percentage of explants produced roots and mean no. of roots per explant of *Oryza sativa* L. cv. MRQ 74 cultured on MS media supplemented with different concentrations of TDZ, NAA and BAP.

MS + Hormone (mg/L)			Percentage of explants produced roots (%)	No. of roots per explant (Mean ± SE)	Root length (cm)
TDZ	NAA	BAP			
0.0	0.0	0.0	$96.67 \pm 0.03_{c}$	$5.27 \pm 0.76_{b}$	0.2 - 4.0
0.1			$13.33 \pm 0.06_{b}$	$0.30 \pm 0.16_{a}$	0.2 - 0.7
0.2			NR	NR	NR
0.3			NR	NR	NR
0.4			NR	NR	NR
0.5			$3.33 \pm 0.03_{a}$	$0.03 \pm 0.03_{a}$	0.2
0.1	0.1		$3.33 \pm 0.03_{a}$	$0.03 \pm 0.03_{a}$	0.4
0.2	0.1		NR	NR	NR
0.1	0.1	0.1	$6.67 \pm 0.05_{ab}$	$0.07 \pm 0.05_{a}$	0.2 - 0.5

Mean values with different letters in a column are significantly different at $p<0.05$. (NR: no response)

6.3.4 Effects of IBA on Rooting

The maximum number of shoots (4.40 ± 0.53) regenerated on MS media containing 0.5 mg/L BAP in combination with 1.0 mg/L IBA, followed by 0.5 mg/L IBA, 0.5 mg/L BAP in combination with 0.5 mg/L IBA, 0.1 mg/L BAP in combination with 1.0 mg/L IBA, 1.0 mg/L BAP in combination with 0.1 mg/L IBA, whereby the mean number of shoots induction were 3.37 ± 0.31, 3.23 ± 0.27, 3.17 ± 0.22 and 3.17 ± 0.30, respectively (Table 6.7). The lowest mean number of shoots per explant (1.53 ± 0.13) was observed on MS media supplemented with 0.1 mg/L IBA.

A significant effect was noticed on number of roots per explant at different concentrations and combinations of BAP and IBA. The presence of IBA singly in the culture media produced lower mean number of roots per explant. The mean number of roots produced were 5.27 ± 0.50, 4.50 ± 0.45 and 6.33 ± 0.54 on MS media supplemented with 0.1 mg/L IBA, 0.5 mg/L IBA in combination with 1.0 mg/L IBA, respectively (Table 6.8). On the other hand, IBA in combination with a low concentration of BAP (0.1 mg/L) was found to be the most effective for root induction

of this species. The highest mean number of roots per explant (11.07 ± 0.78) was recorded on MS media supplemented with 0.1 mg/L BAP in combination with 1.0 mg/L IBA. Increased in BAP concentrations to 0.5 mg/L and 1.0 mg/L resulted in decreased in root induction. The combination of 1.0 mg/L BAP with 0.5 mg/L IBA produced the lowest mean number of roots which was 2.10 ± 0.41.

Table 6.7: Percentage of explants produced shoots and mean no. of shoots per explant of *Oryza sativa* L. cv. MRQ 74 cultured on MS media supplemented with different concentrations of BAP and IBA.

MS + Hormone mg/L		Percentage of explants produced shoots (%)	No. of shoots per explant (Mean ± SE)
BAP	IBA		
0.0	0.0	$100.00 \pm 0.00_{a}$	$2.23 \pm 0.15_{abc}$
	0.1	$100.00 \pm 0.00_{a}$	$1.53 \pm 0.13_{a}$
	0.5	$100.00 \pm 0.00_{a}$	$3.37 \pm 0.31_{d}$
	1.0	$100.00 \pm 0.00_{a}$	$2.10 \pm 0.16_{ab}$
0.1	0.1	$100.00 \pm 0.00_{a}$	$2.47 \pm 0.27_{bcd}$
	0.5	$100.00 \pm 0.00_{a}$	$2.87 \pm 0.29_{bcd}$
	1.0	$100.00 \pm 0.00_{a}$	$3.17 \pm 0.22_{d}$
0.5	0.1	$100.00 \pm 0.00_{a}$	$2.80 \pm 0.21_{bcd}$
	0.5	$100.00 \pm 0.00_{a}$	$3.23 \pm 0.27_{d}$
	1.0	$100.00 \pm 0.00_{a}$	$4.40 \pm 0.53_{e}$
1.0	0.1	$100.00 \pm 0.00_{a}$	$3.17 \pm 0.30_{d}$
	0.5	$100.00 \pm 0.00_{a}$	$3.10 \pm 0.29_{cd}$
	1.0	$100.00 \pm 0.00_{a}$	$2.80 \pm 0.22_{bcd}$

Mean values with different letters in a column are significantly different at $p<0.05$.

Table 6.8: Percentage of explants produced roots and mean no. of roots per explant of *Oryza sativa* L. cv. MRQ 74 cultured on MS media supplemented with different concentrations of BAP and IBA.

MS + Hormone mg/L		Percentage of explants produced roots (%)	No. of roots per explant (Mean ± SE)	Root length (cm)
BAP	IBA			
0.0	0.0	$96.67 \pm 0.03_{b}$	$5.10 \pm 0.76_{cd}$	0.2 - 4.0
	0.1	$100.00 \pm 0.00_{b}$	$5.27 \pm 0.50_{cd}$	0.3 - 15.0
	0.5	$96.67 \pm 0.03_{b}$	$4.50 \pm 0.45_{bcd}$	0.3 - 4.5
	1.0	$100.00 \pm 0.00_{b}$	$6.33 \pm 0.54_{d}$	0.3 - 5.0
0.1	0.1	$100.00 \pm 0.00_{b}$	$9.20 \pm 0.72_{e}$	0.5 - 5.5
	0.5	$100.00 \pm 0.00_{b}$	$6.03 \pm 0.73_{d}$	0.3 - 5.8
	1.0	$100.00 \pm 0.00_{b}$	$11.07 \pm 0.78_{f}$	0.5 - 4.0
0.5	0.1	$100.00 \pm 0.00_{b}$	$5.03 \pm 0.49_{bcd}$	0.3 - 3.0
	0.5	$90.00 \pm 0.06_{ab}$	$3.97 \pm 0.54_{bc}$	0.2 - 3.7
	1.0	$90.00 \pm 0.06_{ab}$	$3.87 \pm 0.53_{abc}$	0.3 - 4.0
1.0	0.1	$80.00 \pm 0.07_{a}$	$3.17 \pm 0.55_{ab}$	0.5 - 3.0
	0.5	$80.00 \pm 0.07_{a}$	$2.10 \pm 0.41_{a}$	0.3 - 1.5
	1.0	$93.33 \pm 0.05_{b}$	$3.20 \pm 0.54_{ab}$	0.3 - 3.0

Mean values with different letters in a column are significantly different at $p<0.05$.

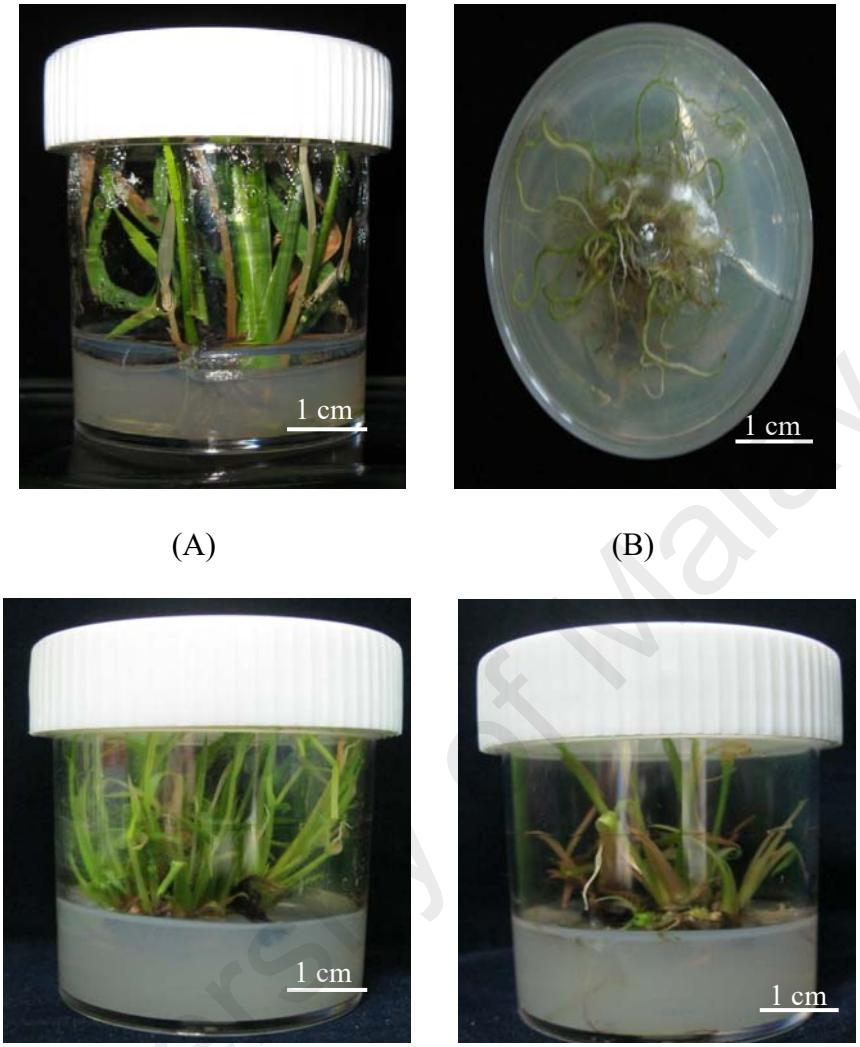


Figure 6.1: Plantlets produced from stem explants of *Oryza sativa* L. cv. MRQ 74 cultured on MS media supplemented with 1.5 mg/L BAP (A); roots from stem explants cultured on MS media supplemented with 0.1 mg/L NAA (B); plantlets produced from stem explants cultured on MS media supplemented with 0.1 mg/L TDZ (C) and 0.5 mg/L TDZ (D).

6.4 SUMMARY OF RESULTS

1. Stem explants was the most responsive for *in vitro* regeneration of *Oryza sativa* L. cv. MRQ 74.
2. BAP was found to be the most effective plant growth hormone for shoots induction. Meanwhile, NAA was the most suitable hormone for roots induction.
3. MS media containing 1.5 mg/L BAP produced the highest mean number of shoots per explant (4.03 ± 0.31).
4. The highest mean number of roots was recorded on MS media supplemented with 0.1 mg/L NAA (24.37 ± 1.91).
5. Based on the number of shoots and roots produced, it can be concluded that the optimum medium for *in vitro* regeneration of *Oryza sativa* L. cv. MRQ 74 was MS media supplemented with 0.1 mg/L BAP in combination with 0.1 mg/L NAA.
6. Direct *in vitro* regeneration was observed in all treatments (BAP in combination with NAA, kinetin in combination with NAA, BAP in combination with NAA and TDZ) including control. All cultures produced shoots regardless of the types and concentrations of hormones used.

CHAPTER 7

SYNTHETIC SEED PRODUCTION OF *Oryza sativa* L. cv. MRQ 74

7.1 EXPERIMENTAL AIMS

The encapsulation technique for producing synthetic seeds has become an important asset in micropropagation. The main objective of micropropagation technique is to ensure continuous supply of desired plant species. Development of artificial seed production is effective and acts as an important method of propagation in several commercially important plant species with high commercial values. Synthetic seed production has many advantages over conventional propagation. Plants could be produced in large scale with high volumes. Consequently genetic uniformity and stability of the plant could be maintained. Due to the sterility of the synthetic seeds, it could be easily transported from one country to another and has potential for long term storage without losing its viability.

Plant tissues such as somatic embryos, apical shoot tips, axillary shoot buds, embryogenic calli and protocom-like bodies are potential micropropagules that have been considered for creating synthetic seeds. Encapsulation of somatic embryos, apical and axillary shoot buds, and regeneration of whole plants from them has been reported for a number of plant species (Redenbaugh *et al*., 1986: Mathur *et al*., 1989; Ganapathi *et al*., 1992; Lulsdorf *er al*., 1993). The use of unipolar axillary shoot buds and apical shoot tips in creating the synthetic seeds have been reported in many plants species such as *Actinidia deliciosa*, *Brassica campestris*, *Malus pumila* Mill, *Zingiber officinale* Rose and *Syringa vulgaris* L. (Ara *et al*., 2000). Axillary shoot buds and apical shoot tips are suitable for encapsulation studies of artificial seeds as they possess great potential for plant development from pre-existing meristematic tissue.

In addition, the use of axillary shoot buds and apical shoot tips would also ensure a genetic uniformity and stability in the regenerants. However, information about production of artificial seeds from apical shoot tips or microshoots in rice is extremely limited. Therefore, in the present study, different encapsulation matrix and efficacy in the plantlet regeneration of encapsulated microshoots of *Oryza sativa* L. cv. MRQ 74 were investigated. Comparison on morphological structure such as stomata density between intact plant, *in vitro* plantlet and plantlet derived from synthetic seeds were also carried out.

7.2 MATERIALS AND METHODS

7.2.1 Source of Microshoots Explants

The explants source consisting of small pieces of stems derived from aseptic seedlings were used in this study (4.2.1). The stem explants were approximately excised into 5.0 -10.0 mm segments and cultured onto MS media supplemented with 30 g/L sucrose and 8 g/L technical agar fortified with 1.5 mg/L BAP for microshoots induction. The pH of the medium employed in this experiment was adjusted to 5.8 prior to autoclaving process at 121 ºC, 105 kPa for 21 minutes. The cultures were maintained in the culture room at 25 ± 1 ºC under 16 hours light and 8 hours dark with 1000 lux of light intensity. Microshoots (approximately 5.0 mm in length) were excised from cultures after 2 weeks in culture. The microshoots were carefully isolated and encapsulated.

7.2.2 Preparation of Encapsulation Matrix

Microshoots (5.0 mm in length) were used as explant sources for synthetic seeds creation. Different encapsulation matrices were evaluated; (1) Ca-free MS + distilled water, (2) Ca-Free MS + 30 g/L sucrose, and (3) Ca-free MS + 30 g/L sucrose + 0.1 mg/L BAP + 0.1 mg/L NAA. Microshoots were mixed in the encapsulation matrix consisted of 3 % sodium alginate, added with MS basal liquid medium with or without 30 g/L sucrose and plant growth hormone. For complexation, 0.2 M of calcium chloride solution ($CaCl_2.2H_2O$) was prepared in distilled water. The gel matrix and the complexing agent were autoclaved after adjusting the pH to 5.8. The microshoots were drawn up with some encapsulation matrix and dropped into $CaCl_2.2H_2O$ solution using sterilized micro pipette. The encapsulated microshoots were left for 30 minutes for hardening. The beads containing one microshoot each were washed in sterilized distilled water to avoid sticking together and were retrieved using nylon mesh.

7.2.3 Preparation of Germination Medium/Substrate

The substrates purchased from Warisan Alam Trading and Services were used in the present study. The beads were germinated on various germination media and substrates; (1) MS basal medium + 3 g/L sucrose + 8 g/L agar (MSO), (2) MS + 30 g/L sucrose + 8 g/L agar + 0.1 mg/L BAP, (3) MS + 8 g/L agar, (4) Tap water + 8 g/L agar, (5) Topsoil, (6) Topsoil + tap water, and (7) Topsoil + ½ strength (2.2 g/L) MS + 30 g/L sucrose. All the culture media and substrates were autoclaved at 121 ºC, 105 kPa for 21 minutes prior to be used. In testing the best encapsulation matrix, the non encapsulated microshoots were used as control. The encapsulated and non encapsulated microshoots were cultured onto MS basal media. Meanwhile, in determining the best culture medium and culture substrate, MS basal media with 30 g/L sucrose and 8 g/L agar was used as control and Ca-free MS + 30 g/L sucrose + 3 % sodium alginate + 0.1 mg/L BAP + 0.1 mg/L NAA as encapsulation matrix. Thirty replicates were used for each treatment. All the cultures were maintained in the culture room at 25 ± 1 ºC, 16 hours light and 8 hours dark. The germination rate of the synthetic seeds and plantlets survival rate were recorded after 10 and 30 days of culture.

7.2.4 Storage Period

The beads were also cold-stored in the incubator at 4 ºC prior to germination process. Thirty seeds were sown in MS basal medium for every 15 days interval. The germination rates were recorded after 4 weeks of sowing.

7.2.5 Microscopic Studies (Scanning Electron Microscopy-SEM)

Scanning electron microscope (SEM) was used to view the differences between leaves of *in vivo* (intact) and *in vitro* grown plants. The leaves were excised (5-10 mm) and treated with different types of chemicals. The samples were incubated in 30 ml of gluteraldehyde mixed with 30 ml of phosphate buffer for 1 hour at room temperature.

The samples were then rinsed with phosphate buffer solution and distilled water in 1: 1 mixture. The samples were incubated in Osmium (4%) and distilled water in 1:1 mixture for 14 hours at 4 ^{o}C. The samples were rinsed with distilled water and fixed using the ethyl alcohol series (10% to 100%) for 15 minutes each step followed by ethyl alcohol and acetone in 3:1, 1:1 and 1:3 mixtures for 20 minutes each. The samples were then incubated in acetone (100%) for 20 minutes and this step was repeated four times.

The samples were dried up in the liquid carbon dioxide for several times using critical point drying (CPD) equipment to replace the acetone with carbon dioxide. The temperature was increased until liquid carbon dioxide changed to gas to avoid surface tension of the sample. The samples were attached to special aluminum pin using conductive carbon cement. The pin was then placed inside of SPI-Module Sputter Coaster Chamber. The air of the chamber was placed with argon gas using a pump, which has been attached to the gold coater equipment. The samples were coated with gold particle for 60 seconds. Finally, the specimens were observed using SEM (JEOL, JSM. 6400, Tokyo, Japan). Observations and comparisons were made on the differences of number of stomata and trichomes.

7.2.6 Statistical Analysis

Data obtained were analyzed using the IBM SPSS Statistics 20 software (International Business Machines Corp., Armonk, NY). Statistical variance analysis was conducted using ANOVA (Duncan's Multiple Range Test or DMRT) at 5 % significance level where thirty replicates were used for each treatment. The effect of different treatments was quantified as mean ± SE (standard error).

7.3 RESULTS

7.3.1 Encapsulation Matrix

Based on the results obtained from the previous experiment (Chapter 6), MS media supplemented with 1.5 mg/L BAP was selected as microshoots induction medium due to the highest (4.03 ± 0.31) mean number of microshoots produced. In the present study, the encapsulated and non encapsulated microshoots showed 100 % germination rate and had a high potential to be converted into plantlets on the cultured medium (Table 7.1). Since the optimum regeneration media of *Oryza sativa* L. cv. MRQ 74 was MS supplemented with 0.1 mg/L BAP + 0.1 mg/L NAA (Chapter 6, Table 6.1), the same types and concentrations of hormones were added in encapsulation matrix to stimulate the emergence of shoots and roots of encapsulated microshoots (Figure 7.1). However, the use of these hormones had no significant effect on synthetic seeds germination. All treatments with and without hormone showed 100 % germination rate.

The highest survival rate (100 %) was found using Ca-free MS + 30 g/L sucrose compared to 96.67 ± 0.48 % of hormone treatment. The non encapsulated microshoots (control) showed the lowest survival rate of plantlets (90.0 ± 0.55 %). The survival rate of the plantlets significantly reduced (93.33 ± 0.48 %) when the encapsulation matrix only contained 3 % sodium alginate with the addition of MS basal (free calcium) and distilled water.

Synthetic seeds germination was affected by sucrose concentration in the encapsulation matrix. Synthetic seeds with no sucrose in the encapsulation matrix (Ca-free MS + distilled water) had significantly lower plantlets survival rate (93.33 ± 0.48 %) as compared to synthetic seeds with sucrose in encapsulation matrices (Ca-free MS + 30 g/L sucrose and Ca-free MS + 30 g/L sucrose + 0.1 mg/L BAP + 0.1 mg/L NAA), 96.67 ± 0.48 % and 100.00 ± 0.00 %, respectively. These results indicate that coating

material and the concentration of the coating material are important limiting factors for the synthetic seed technology.

Table 7.1: Growth response of encapsulated microshoots of *Oryza sativa* L. cv. MRQ 74 in different encapsulation matrices after being transplanted onto MS media.

Encapsulation matrix	Germination rate (after 10 days) (% ± SE)	Survival rate (after 30 days) (% ± SE)
Control (non encapsulated)	$100.00 \pm 0.00_a$	$90.00 \pm 0.55_a$
Ca-free MS + distilled water	$100.00 \pm 0.00_a$	$93.33 \pm 0.48_b$
Ca-free MS + 30 g/L sucrose	$100.00 \pm 0.00_a$	$100.00 \pm 0.00_d$
Ca-free MS + 30 g/L sucrose + 0.1 mg/L BAP + 0.1 mg/L NAA	$100.00 \pm 0.00_a$	$96.67 \pm 0.48_c$

Mean values with different letters in a column are significantly different at $p<0.05$.

7.3.2 Germination Medium/Substrate

The effect of different germination substrates on germination rate of synthetic seeds of *Oryza sativa* L. cv. MRQ 74 is presented in Table 7.2. The highest germination rate (100 %) was recorded on MS basal media (Figures 7.1 and 7.2), MS media supplemented with 0.1 mg/L BAP (Figure 7.3), tap water + 8 g/L agar (Figure 7.4), topsoil + tap water (Figure 7.5) and topsoil + ½ strength MS + 30 g/L sucrose. Topsoil showed the least preferred germination substrate with 6.67 % germination rate. However, the survival rate of plantlets varied from 0 % to 100 %. The maximum survival rate (100 %) was observed on MS media with and without 0.1 mg/L BAP, followed by tap water + 8 g/L agar (93.33 ± 0.48 %), MS + 8 g/L agar (90.0 ± 0.55 %), topsoil + tap water (36.67 ± 0.36 %), topsoil + ½ strength MS + 30 g/L sucrose (30.0 ± 0.55 %) and the least topsoil (0.00 ±0.00 %).

The use of tap water in the culture medium and substrate (topsoil) gave 100 % germination rate. However, plantlets survival rates dropped to 36.67 ± 0.36 %, 30.00 ± 0.55 % and 0.0 % in topsoil + tap water, topsoil + ½ strength MS + 30 g/L sucrose and topsoil only, respectively after 30 days. The most abundant minerals dissolved in water are salts of calcium, magnesium, ferrous iron and manganese. Among the macronutrients required for plant cell and tissue growth are calcium and magnesium. In this study, plantlet survival rate was significantly higher in topsoil + tap water (36.67 ± 0.36 %) compared to topsoil + ½ strength MS + 30 g/L sucrose (30.00 ± 0.55 %). However, the mineral content of tap water used in this study was not determined.

Table 7.2: Effect of different germination media/substrates on germination rate of synthetic seeds of *Oryza sativa* L. cv. MRQ 74.

Germination medium/substrate	Germination rate (after 10 days) (% ± SE)	Survival rate (after 30 days) (% ± SE)
MS basal medium + 30 g/L sucrose + 8 g/L agar (control)	$100.00 \pm 0.00_{c}$	$100.00 \pm 0.00_{f}$
MS + 30 g/L sucrose + 8 g/L agar + 0.1 mg/L BAP	$100.00 \pm 0.00_{c}$	$100.00 \pm 0.00_{f}$
MS + 8 g/L agar	$96.67 \pm 0.48_{b}$	$90.00 \pm 0.55_{d}$
Tap water + 8 g/L agar	$100.00 \pm 0.00_{c}$	$93.33 \pm 0.48_{e}$
Topsoil	$6.67 \pm 0.48_{a}$	$0.00 \pm 0.00_{a}$
Topsoil + tap water	$100.00 \pm 0.00_{c}$	$36.67 \pm 0.36_{c}$
Topsoil + ½ strength MS + 30 g/L sucrose	$100.00 \pm 0.00_{c}$	$30.00 \pm 0.55_{b}$

Mean values with different letters in a column are significantly different at $p < 0.05$.

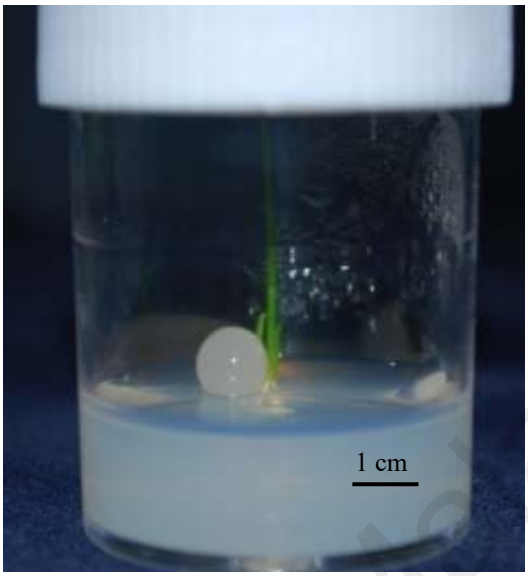


Figure 7.1: Two-week-old synthetic seed germinating on MS basal medium.

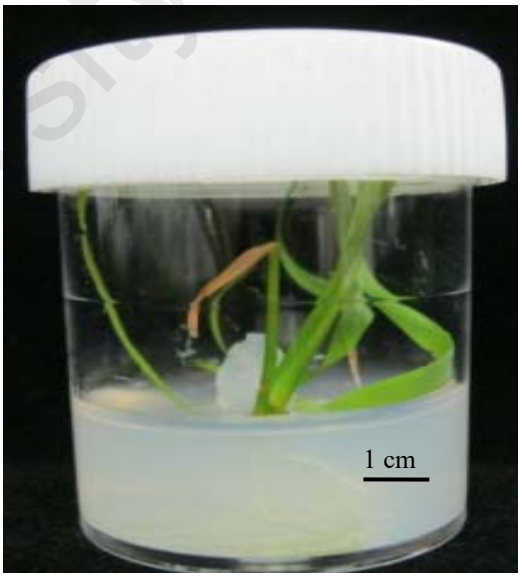


Figure 7.2: Synthetic seed germination on MS basal medium after one month.

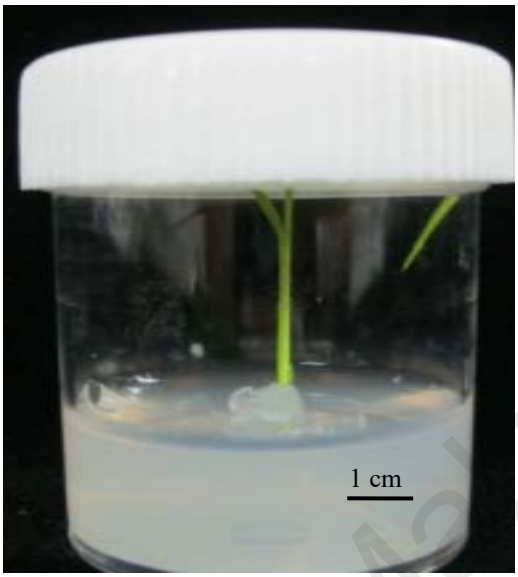


Figure 7.3: Synthetic seed germination on MS media + 0.1 mg/L BAP after one month.

Figure 7.4: Synthetic seed germination on tap water + agar after two months.

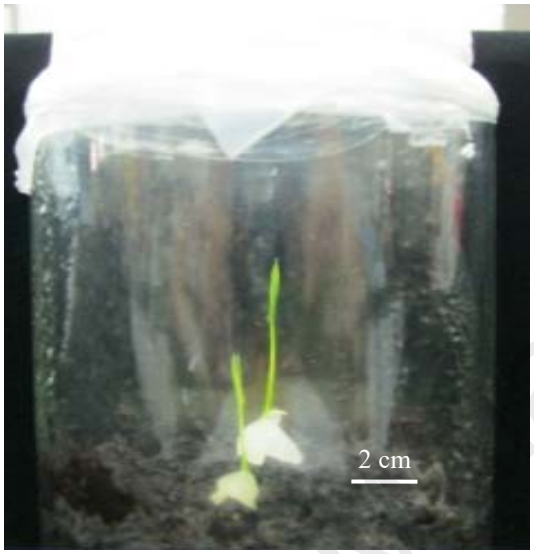


Figure 7.5: Encapsulated microshoot containing 0.1 mg/L BAP + 0.1 mg/L NAA in the encapsulation matrix was germinated on topsoil + tap water.

7.3.3 Storage Period

Storage conditions such as temperature and period of storage are important factors to determine the regeneration frequency of the stored encapsulated propagules. The effect of storage period on germination of synthetic seeds produced is presented in Table 7.3. The encapsulated microshoots with MS media supplemented with 0.1 mg/L BAP + 0.1 mg/L NAA, 30 g/L sucrose and 8 g/L agar gave 93.33 ± 0.48 % germination without storage. A low germination rate (16.67 ± 0.42 %) was recorded after 2 weeks of storage. The viability of seeds had fallen from 93.33 ± 0.48 % to 3.33 ± 0.36 % after one month storage at 4 ^{o}C.

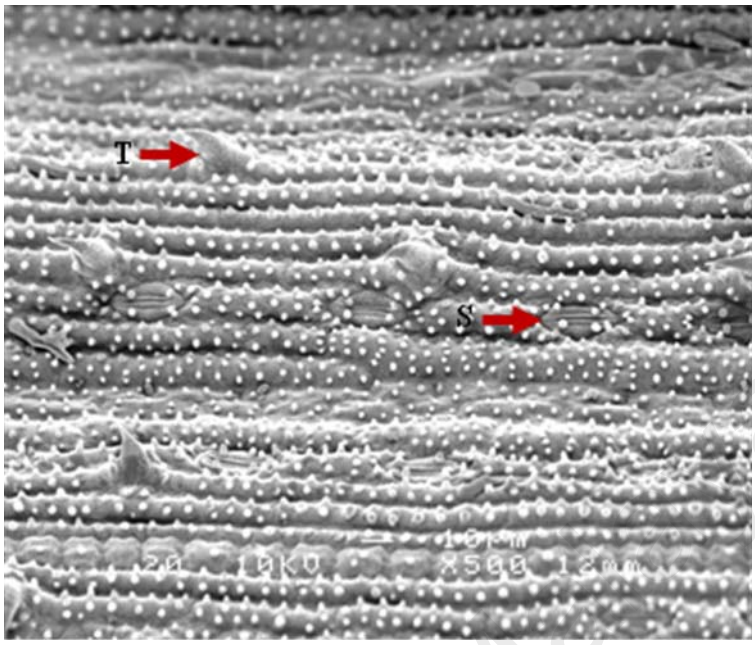
Table 7.3: Effect of storage period at 4 ± 1 ºC on germination of synthetic seeds of *Oryza sativa* L. cv. MRQ 74 on MS basal medium.

Storage period (day)	No. of synthetic seeds	No. germinated	Germination rate (% ± SE)
0	30	28	$93.33 \pm 0.48_{d}$
15	30	5	$16.67 \pm 0.42_{c}$
30	30	1	$3.33 \pm 0.36_{b}$
45	30	0	$0.00 \pm 0.00_{a}$

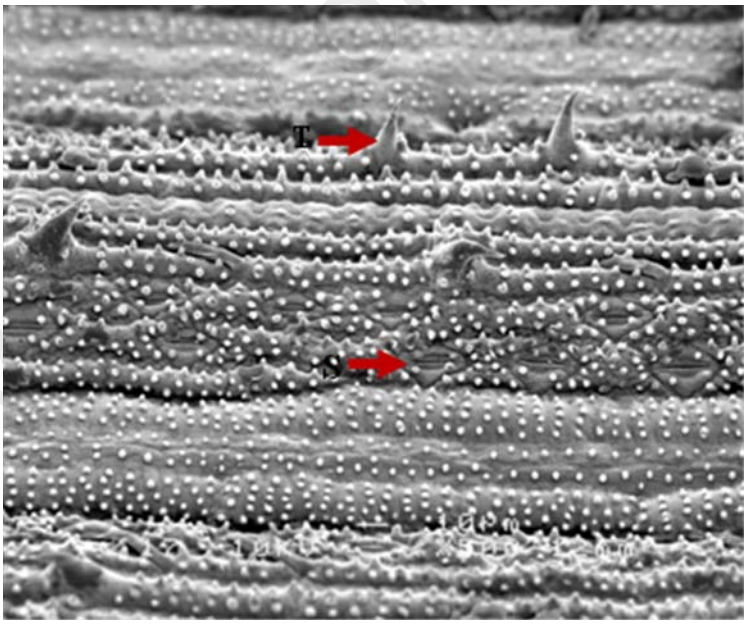
Mean values with different letters in a column are significantly different at $p<0.05$

7.3.4 Microscopic Studies (Scanning Electron Microscopy)

The microscopic studies of the structure of *in vitro* (intact) and *in vivo* leaves showed that the type of stomata was paracytic. The stomata guard cells are dumb bell shaped (Figures 7.6, 7.7 and 7.8). The presence of stomata was observed on both surfaces of all leaf samples. However, a comparable number of stomata were observed on abaxial and adaxial surfaces of leaf from MS media fortified with 0.1 mg/L BAP + 0.1 mg/L NAA. More trichomes were seen on adaxial surface of intact leaf compared with *in vitro* leaf, grown on MS media supplemented with 0.1 mg/L BAP + 0.1 mg/L NAA.

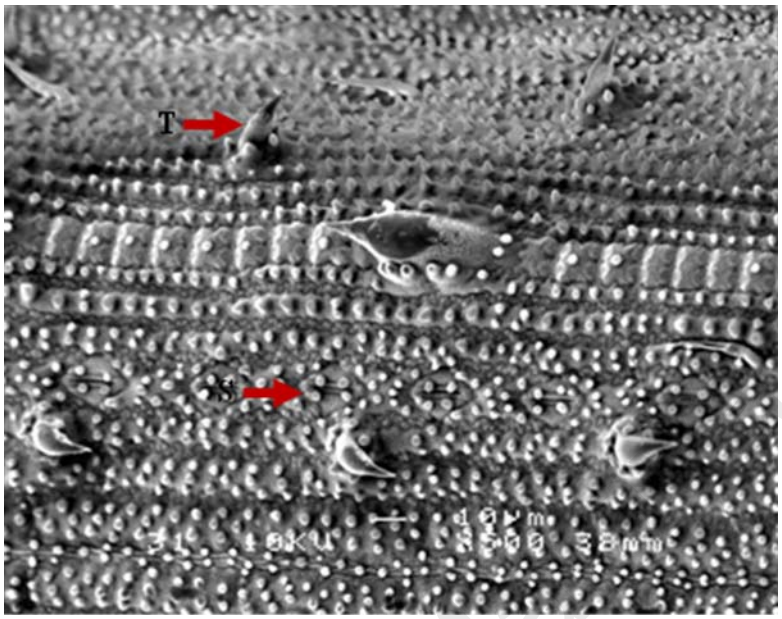


(a)

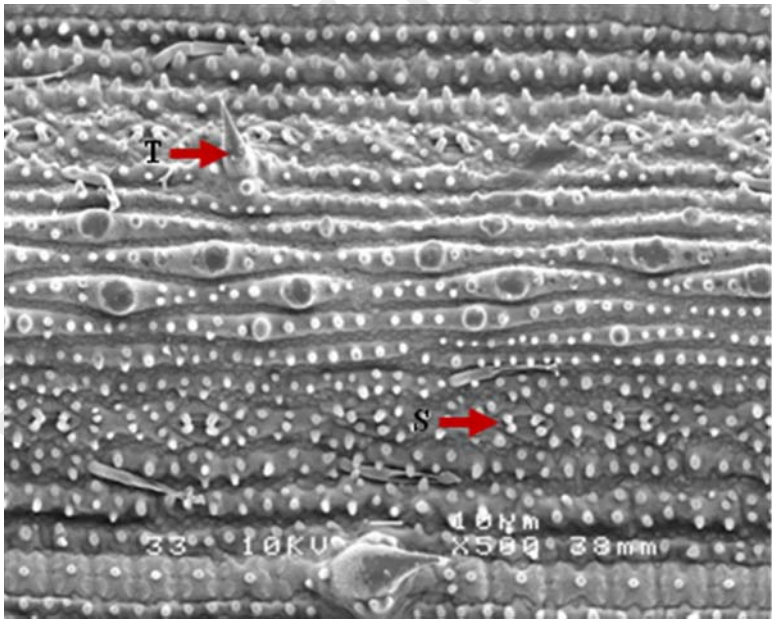


(b)

Figure 7.6 : Scanning electron micrograph showing adaxial (a) and abaxial (b) surfaces of *in vitro* leaf of plantlet from synthetic seed of *Oryza sativa* L. cv. MRQ 74. S: Stomata, T: Trichomes. Bar represents 10 µm.

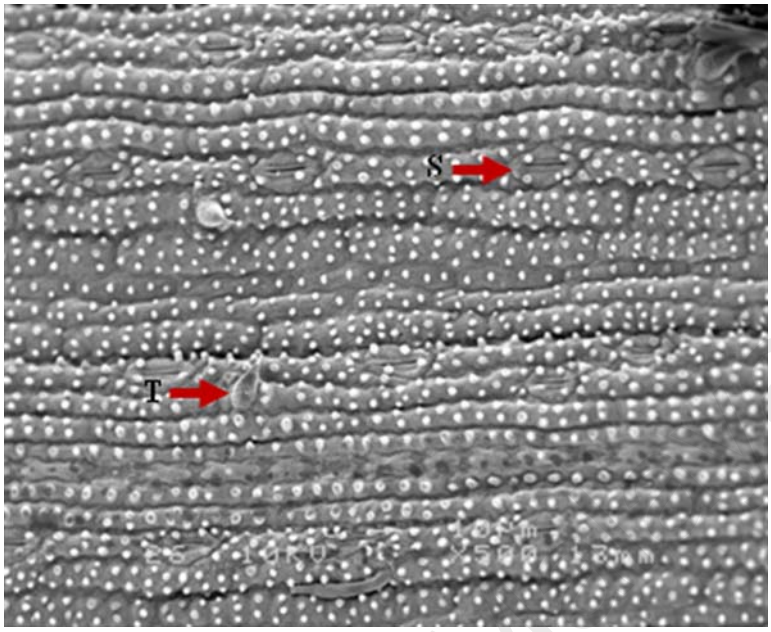


(a)

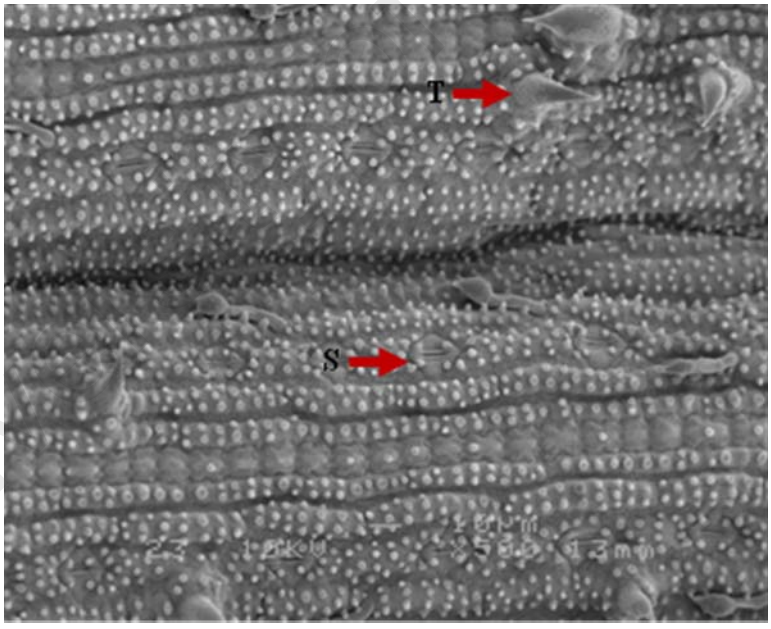


(b)

Figure 7.7: Scanning electron micrograph showing adaxial (a) and abaxial (b) surfaces of leaf from *in vivo* (intact) of *Oryza sativa* L. cv. MRQ 74. S: Stomata, T: Trichomes. Bar represents 10 µm.



(a)



(b)

Figure 7.8: Scanning electron micrograph showing adaxial (a) and abaxial (b) surfaces of *in vitro* leaf of plantlet from MS media containing 0.1 mg/L BAP + 0.1 mg/L NAA of *Oryza sativa* L. cv. MRQ 74. S: Stomata, T: Trichomes. Bar represents 10 µm.

7.4 SUMMARY OF RESULTS

1. The most suitable encapsulation matrix was Ca-Free MS + 30 g/L sucrose.
2. The maximum germination rate (100 %) and plantlets survival rate of 100 % was achieved on MS medium + 30 g/L sucrose with and without 0.1 mg/L BAP.
3. The encapsulated and non-encapsulated microshoots developed into plantlets on MS basal medium.
4. The preferred germination substrates for synthetic seeds of *Oryza sativa* L. cv. MRQ 74 were MS basal and MS medium supplemented with 0.1 mg/L BAP.
5. The viability of the synthetic seeds decreased as the storage period increased.
6. Based on Scanning Electron Microscopic (SEM) studies, leaves derived from stem explants cultured on MS media supplemented with 0.1 mg/L BAP + 0.1 mg/L NAA showed higher stomata number on both surfaces.

CHAPTER 8

ACCLIMATIZATION OF MICROPROPOGATED PLANTLETS OF *Oryza sativa* L. cv. MRQ 74

8.1 EXPERIMENTAL AIMS

The ultimate success of micropropagation depends on the ability of transferred plants to grow and survive under new environmental conditions. This is due to altered morphological, anatomical and physiological characteristics of plantlets resulted from *in vitro* culture conditions such as low light intensity, high humidity, low temperature, limited growing space, etc. The retardation in development of cuticle, epicuticular waxes and functional stomatal apparatus during *in vitro* culture cause high stomatal and cuticular transpiration rates of leaves in plantlets when taken out of culture container. Therefore, it is not surprising that the plant is often associated with slow growth and significant plant losses. In order to increase growth and reduce mortality of plants during field transfer, the plants must undergo a process of hardening or acclimatization. The plantlets should be slowly transferred from high humidity to low humidity condition, example from culture room to green house rather than direct to field.

The aim of this experiment was to measure the survival rates of acclimatized plantlets derived from *in vitro* regeneration. In this study, there were two stages of acclimatization. The first stage was the acclimatized plantlets were kept in culture room for two months followed by the second stage maintaining the plantlets in green house for another two months. Crop agronomical parameters such as plant height, number of leaves, leaf length and number of seeds per stalk of acclimatized plantlets were measured and compared with *in vivo* plants. Apart from that, histological studies were also carried out to observe the ultra cellular structures of leaves and roots of acclimatized, *in vivo* and *in vitro* plantlets.

8.2 MATERIALS AND METHODS

8.2.1 Plant Materials and Culture conditions

Stem explants from aseptic seedlings as described in section 4.2.1 were approximately excised into 5.0-10.0 mm segments and cultured onto MS media supplemented with 30 g/L sucrose and 8 g/L technical agar fortified with 0.5 mg/L 2,4-D and MS media fortified with 0.1 mg/L BAP in combination with 0.1 mg/L NAA for *in vitro* regeneration. The pH of the medium employed in this experiment was adjusted to 5.8 prior to autoclaving process at 121 ºC, 105 kPa for 21 minutes. The cultures were maintained in the culture room at 25 ± 1 ºC under 16 hours light and 8 hours dark with 1000 lux of light intensity for two months. The regenerated plantlets were then transferred onto different growing substrates.

8.2.2 Growing Substrates and Acclimatization Conditions

Two-month-old *in vitro* plantlets with well rooted system were carefully taken out from the culture media and washed thoroughly under running tap water to remove all traces of medium attached to the roots. The plantlets were planted in plastic containers containing different types of soil (purchased from Warisan Alam Trading and Services), namely black soil, red soil and a mixture of black and red soil in the ratio of 1:1. The plastic containers were covered with plastic bag to maintain humidity and were kept in tissue culture room at 25 ± 0.1 ºC, 16 hours light and 8 hours dark for two months. The survival rates were recorded after 4 and 8 weeks of transfer.

The survived and established plantlets with new leaves were transplanted to plastic containers containing garden soil before they were transferred to greenhouse (29 ± 1 ºC, 12 hours light, 12 hours dark) for two months. The plantlets received irrigation on alternate days throughout the experiment. Fertilizers in form of liquid urea (50 g/L) were applied every three weeks after acclimatization commenced.

8.2.3 Measurement of Agronomic Parameters

Several agronomic parameters such as plant height, number of leaf, leaf length and number of seeds per stalk were measured after 8 weeks in the green house. Rice mature seeds were also germinated on garden soil as *in vivo* for a comparison.

8.2.4 Histological Studies on Leaf and Root of *In Vivo, In vitro* and Acclimatized Plants.

Leaf and root specimens were fixed in glutaraldehyde-paraformaldehyde-caffeine fixative for 24 to 48 hours at room temperature. After fixation, the specimens were then dehydrated in a series of ethanol, 30, 50, 70, 80, 90, 95, 100 % ethanol for 30, 45, 45, 60, 60, 60 and 60 minutes, respectively. After the dehydration process, the specimens were treated with xylene for clearing. Subsequently, the specimens were embedded in paraffin wax and sectioned at 35 um. The specimens were stained with Schiff's reaction and naphthol blue black. Finally, the specimens were mounted with Surgipath mounting medium and were dried for one day prior to observation.

8.2.5 Soil Analysis

The growing substrates used in this study were analysed using X-Ray Fluorescence (XRF)) method. The soil samples (red and black soil) were dried in an oven at 40 ºC for 24 hours. The dried soil samples were screened with a 2 mm mesh to remove large objects. The samples were then ground into powder form. Approximately 3 to 5 g of the dried samples were placed in an XRF sample cup for analysis.

8.2.6 Statistical Analysis

Data obtained were analyzed using the IBM SPSS statistical package version 20 software (International Business Machines Corp., Armonk, NY). Data were subjected to statistical analysis using Duncan's Multiple Range Test (DMRT) at 5 % significance

level. Data were also subjected to t-test (paired t-test) to compare whether the results obtained from two sets of experiments were significantly different. The effect of different treatments was quantified as mean ± SE (standard error).

8.3 RESULTS

8.3.1 The Effect of Different Growing Substrates on Acclimatization

The data recorded on the survival rate of the plantlets during acclimatization is presented in Table 8.1. The results showed that the plantlets derived from MS media supplemented with 0.5 mg/L 2,4-D were well adapted in two types of growing substrates after four weeks being acclimatized. The highest survival rate was obtained on the mixture of black and red soil (90.00 ± 1.53 %), followed by black soil (80.00 ± 2.52 %) and red soil (3.33 ± 0.33 %). However, the percentage of survival rate significantly declined in all treatments on the eight weeks, 83.33 ± 1.20 %, 40.00 ± 1.15 % and 0.00 ± 0.00 % in the mixture of black and red soil, black soil and red soil, respectively (Figure 8.1).

A similar pattern of the survival rate was observed for the plantlets derived from MS media containing 0.1 mg/L BAP in combination with 0.1 mg/L NAA (Table 8.2). Nevertheless, the percentage of the survival plantlets was significantly lower compared with the plantlets derived from MS media containing 0.5 mg/L 2,4-D (Table 8.1). The highest percentage was 10.00 ± 0.04 %, followed by 3.33 ± 0.03 % and 0.00 ± 0.00 % which were recorded on the fourth week on the mixture of black and red soil, black soil and red soil, respectively. None of the plantlets was able to survive until week eight.

Table 8.1: The survival rate of plantlets derived from MS media supplemented with 0.5 mg/L 2,4-D after 4 and 8 weeks being transferred to different types of growing substrates.

Acclimatized plantlets on different growing substrates	Survival rates of the plantlets (%)
4 weeks	
Black Soil	$80.00 \pm 2.52_b$
Red Soil	$3.33 \pm 0.33_a$
Black + red soil	$90.00 \pm 1.53_c$
8 weeks	
Black Soil	$40.00 \pm 1.15_b$
Red Soil	$0.00 \pm 0.00_a$
Black + red soil	$83.33 \pm 1.20_c$

Mean values with different letters in a column are significantly different at $p< 0.05$

Table 8.2: The survival rate of plantlets derived from MS media supplemented with 0.1 mg/L BAP in combination with 0.1 mg/L NAA after 4 and 8 weeks being transferred to different types of growing substrates.

Acclimatized plantlets on different growing substrates	Survival rates (%)
4 weeks	
Black Soil	$3.33 \pm 1.93_a$
Red Soil	$0.00 \pm 0.00_a$
Black + red soil	$10.00 \pm 1.91_b$
8 weeks	
Black Soil	$0.00 \pm 0.00_a$
Red Soil	$0.00 \pm 0.00_a$
Black + red soil	$0.00 \pm 0.00_a$

Mean values with different letters in a column are significantly different at $p< 0.05$.

8.3.2 Morphological Studies of *In Vivo*, *In Vitro* and Acclimatized Plantlets

After 8 weeks in the culture room, the survived plantlets from the mixture of red and black soil were then transferred to plastic containers containing garden soil and were maintained in the green house for further growth and development. An abnormal leaf morphology was observed from *in vitro* regenerated plantlet (Figure 8.2). The acclimatized plants produced new leaves and started to grow after 2 weeks in the green house. However, only 80 % of the plants managed to survive under new environmental conditions. Approximately, 60 % of the plants started fruiting after week 8 in the green house (Figure 8.3). The analysis of variance (t-test, $p<0.05$) showed that all the measured parameters were significantly higher for *in vivo* than that of acclimatized plants (Table 8.3). These results indicate that the growth rate of acclimatized plants was low as its need to adapt with low humidity, high light intensity and temperature.

Table 8.3: Performance of *in vivo* and acclimatized plantlets of *Oryza sativa* L. cv. MRQ 74 on a few agronomic parameters.

Parameters	*In vivo*	Acclimatized plantlets
Plant height (cm)	49.70 ± 1.08*	37.80 ± 0.91*
Number of leaves	8.07 ± 0.78*	5.37 ± 0.67*
Leaf length (cm)	40.83 ± 0.58*	26.80 ± 0.47*
Number of seeds per stalk	38.60 ± 2.07*	16.40 ± 0.81*

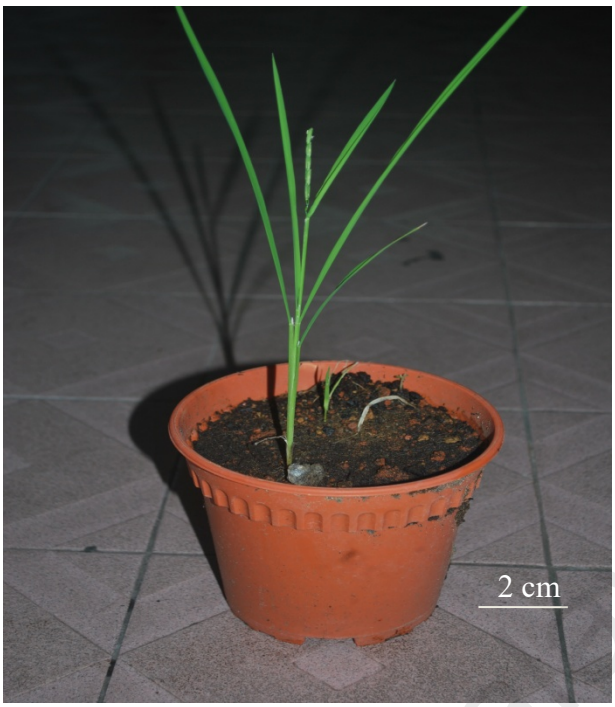
*Significant difference (t-test, $p<0.05$)

Figure 8.1: One-month-old plantlets derived from MS media supplemented with 0.5 mg/L 2,4-D grown in containers containing black soil and mixture of black and red soil (1:1 ratio) during hardening process in the culture room at 25 ± 1 ºC with 18 hours light and 6 hours dark.

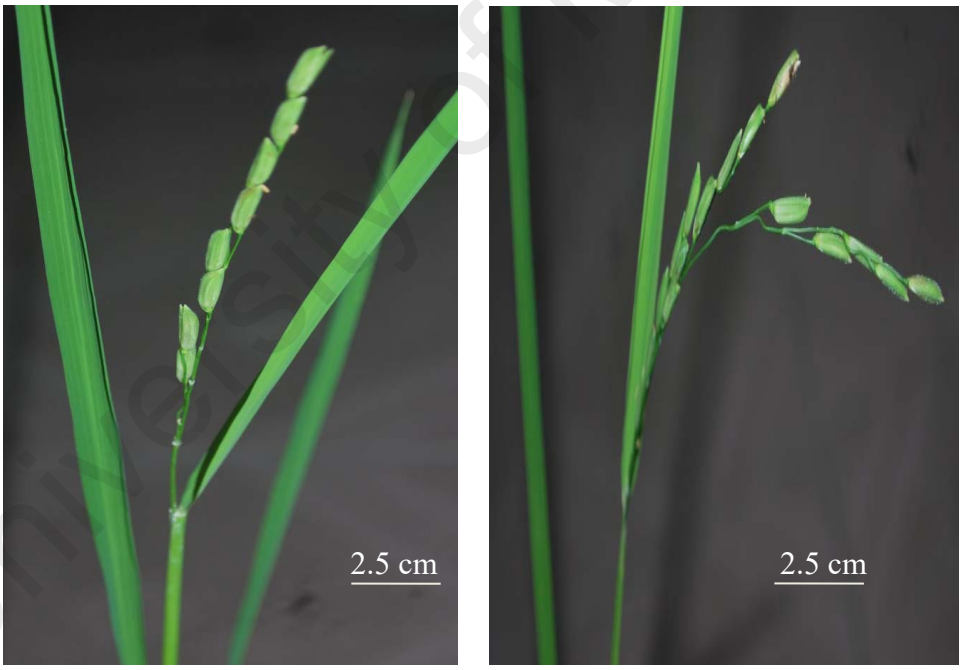
(a)

(b)

Figure 8.2: Two-month-old plantlets during hardening process (a) and plantlet with abnormal leaf structure (red arrow) (b) in the culture room.



(a)



(b)

Figure 8.3: Acclimatized plantlet at fruiting stage (a) and immature rice seeds (b).

8.3.3 Histological Studies of Leaf and Root of *In Vivo, In Vitro* and Acclimatized Plants.

In this experiment, *in vivo* leaf and root sections served as the reference for histology comparisons of acclimatized and *in vitro* leaf and root. The cross section of *in vitro* leaf showed single layered of upper and lower epidermis with almost no cuticle on both adaxial and abaxial leaf surfaces (Figure 8.4). The similar epidermal structure was observed in *in vivo* and acclimatized leaves (Figures 8.5 and 8.6). The bulliform cells were well developed on adaxial surfaces of all leaf samples (*in vitro*, *in vivo* and acclimatized). These cells facilitate leaf rolling in response to water stress. However, the bundle sheath cells of *in vitro* leaf could not be observed clearly. Infact, abnormal differentiation of cells in the bundle sheath had occurred. This caused the number of the cells to increase as compared to *in vivo* and acclimatized leaves. The vascular tissues, xylem and phloem can be seen apparently in all samples of leaves. Unlike vascular tissues, stoma and guard cells were almost absent. Compact mesophyll cells were clearly present between the epidermal layers and the vascular bundles.

Histological study of the root showed that the cross section of *in vivo* root consists of epidermis, cortex, endodermis, pericycle, xylem, phloem and pith (Figure 8.7). All these sections were also present in acclimatized root (Figure 8.8). However, the cortex of the root was poorly developed. Besides that, starch granules were also observed in all samples of roots (Figures 8.7 to 8.9).

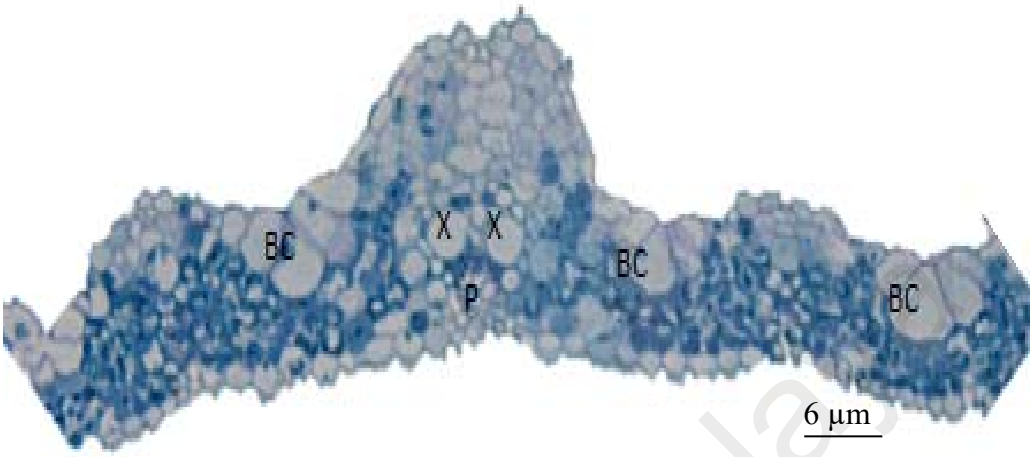


Figure 8.4: Cross-section of the *in vitro* leaf from plantlets regenerated on MS medium supplemented with 0.5 mg/L 2,4-D. BC: bulliform cell, X: xylem, P: pholem. Magnification 200x.

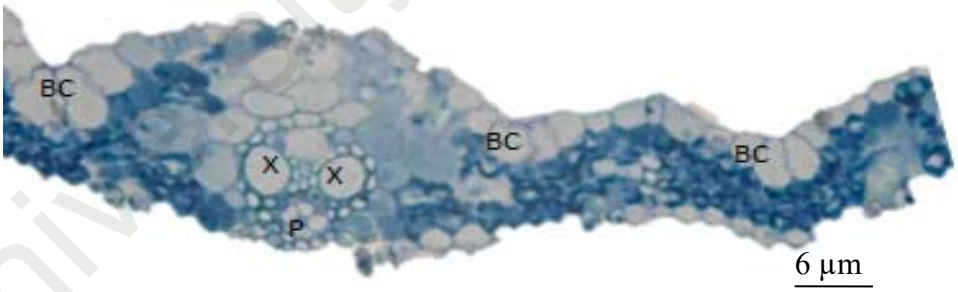


Figure 8.5: Cross-section of the *in vivo* leaf. BC: bulliform cell, X: xylem, P: pholem. Magnification 200x.

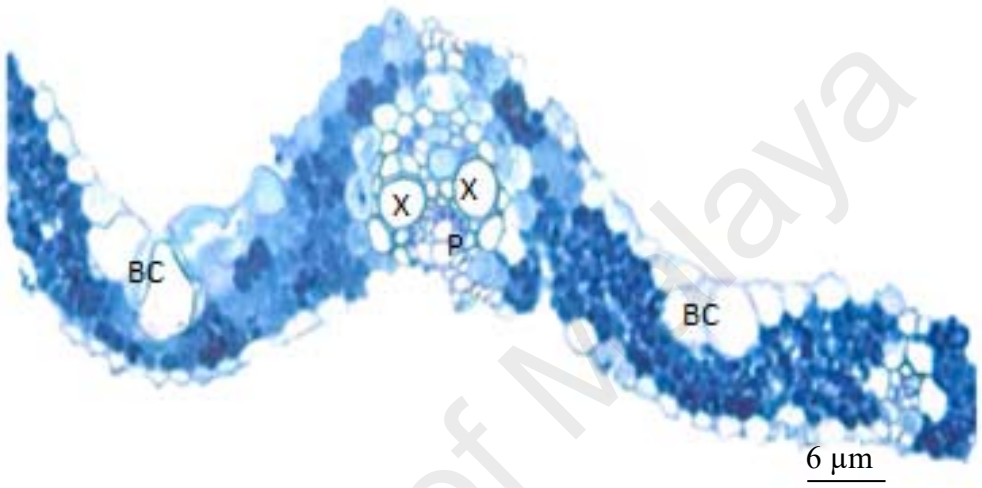


Figure 8.6: Cross-section of the acclimatized leaf. BC: bulliform cell, X: xylem, P: pholem. Magnification 200x.

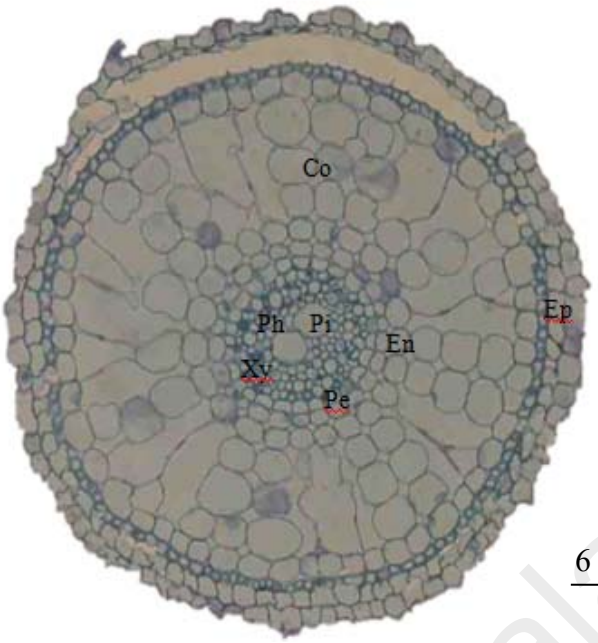


Figure 8.7 : Cross section of root from *in vivo* grown *Oryza sativa* L. cv. MRQ 74. Ep: epidermis, Co: cortex, En: endodermis, Pe: pericycle, Xy: xylem, Ph: phloem, Pi: pith. Magnification 100x.

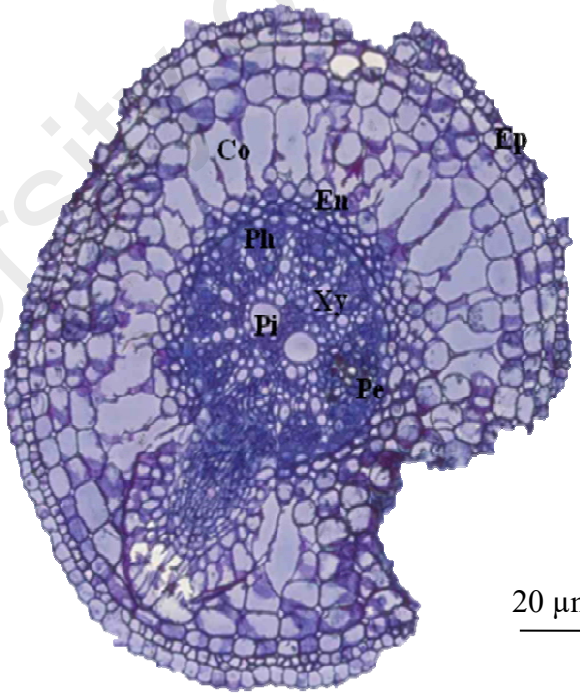


Figure 8.8 : Cross section of the acclimatized root of *Oryza sativa* L. cv. MRQ 74. Ep: epidermis, Co: cortex, En: Endodermis, Pe: pericycle, Xy: xylem, Ph: phloem, Pi: pith. Magnification 100x.

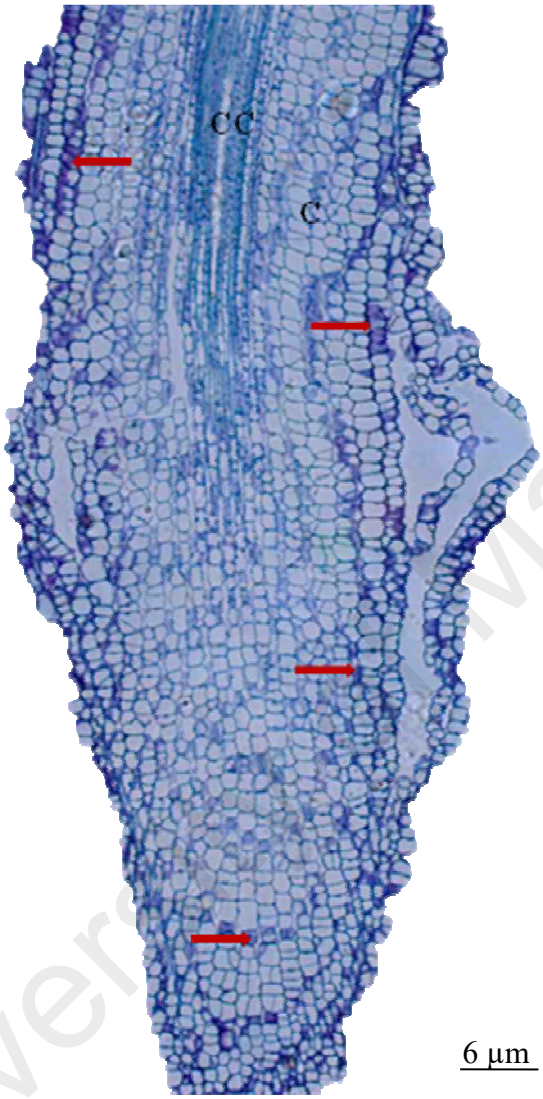


Figure 8.9: Longitudinal section of *in vitro* root of *Oryza sativa* L. cv. MRQ 74 showing cortex (C), central cylinder (CC) and starch granules (red arrows). Magnification 100x.

8.3.4 Soil Compounds

Results of XRF analysis showed that the values of compounds varied in different types of soil as presented in Tables 8.4 and 8.5. The major compounds in black soil are Calcium oxide (CaO), Silicon dioxide (SiO_2) and Aluminium oxide (Al_2O_3). Red soil containing the same major compounds, except Iron (III) oxide instead of Calcium oxide (CaO). Higher values of compounds containing important nutrients for plant growth and development such as phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg) and sulphur (S) were found in black soil as compared to red soil.

Table 8.4 : Types of compounds in black soil as identified by X-Ray Fluorescence (XRF) spectrometry.

Compound	Value (%)
Aluminium oxide (Al_2O_3)	9.158
Barium oxide (BaO)	0.167
Bromine (Br)	0.120
Calcium oxide (CaO)	37.584
Chloride (Cl)	0.795
Iron (III) oxide	5.234
Lead (II) oxide (PbO)	0.120
Magnesium oxide (MgO)	1.598
Manganese (II) oxide (MnO)	0.151
Phosphorus pentoxide (P_2O_5)	1.758
Potassium oxide (K_2O)	2.38
Silicon dioxide (SiO_2)	35.445
Sodium oxide (Na_2O)	0.286
Sulfur trioxide (SO_3)	5.162
Titanium dioxide (TiO_2)	1.094
Zinc oxide (ZnO)	0.080
Zirconium oxide (ZrO_2)	0.070

Table 8.5: Types of compounds in red soil as identified by X-Ray Fluorescence (XRF) spectrometry.

Compound	Value (%)
Aluminium oxide (Al_2O_3)	43.165
Calcium oxide (CaO)	0.420
Chloride (Cl)	0.024
Iron (III) oxide	6.830
Lead (II) oxide	0.010
Magnesium oxide (MgO)	0.000
Manganese (II) oxide (MnO)	0.021
Phosphorus pentoxide (P_2O_5)	0.108
Potassium oxide (K_2O)	0.447
Silicon dioxide (SiO_2)	47.823
Sodium oxide (Na_2O)	0.053
Sulfur trioxide (SO_3)	0.183
Titanium dioxide (TiO_2)	0.773
Zinc oxide (ZnO)	0.015
Zirconium oxide (ZrO_2)	0.052

8.4 SUMMARY OF RESULTS

1. The *in vitro* regenerated plantlets of *Oryza sativa* L. cv. MRQ 74 derived from direct regeneration of stem explants that were cultured on MS media supplemented with 0.5 mg/L 2,4-D were successfully acclimatized on a mixture of black and red soil.
2. The survival rate of the acclimatized plantlets decreased from 90.0 ± 1.53 % to 83.33 ± 1.20 % after 8 weeks of acclimatization.
3. None of the *in vitro* regenerated plantlets derived from MS media supplemented with 0.1 mg/L BAP in combination with 0.1 mg/L NAA survived after acclimatization.
4. Plant height, number of leaves, leaf length and number of seeds per stalk of acclimatized plants were significantly lower as compared to *in vivo* plants.
5. An abnormal leaf morphology (wavy leaf) was detected among the *in vitro* regenerated plantlets, which were cultured on MS media supplemented with 0.5 mg/L 2,4-D.
6. The micro-morphological features of *in vitro* grown *Oryza sativa* L. cv. MRQ 74 leaves as viewed using SEM revealed that cells in the bundle sheath increased in number and arranged randomly.
7. The major compounds in black soil are Calcium oxide (CaO), Silicon dioxide (SiO_2) and Aluminium oxide (Al_2O_3).
8. Red soil contains three major compounds that are Silicon dioxide (SiO_2), Aluminium oxide (Al_2O_3) and Iron (III) oxide (CaO).

CHAPTER 9

CELLULAR BEHAVIOUR OF *Oryza sativa* L. cv. MRQ 74 GROWN *IN VIVO* AND *IN VITRO*

9. 1 EXPERIMENTAL AIMS

Chromosomal instability is a feature of *in vitro* culture of plant cells which causes somaclonal variation that is genotypic and phenotypic changes in regenerants (Duncan, 1997) due to the presence of plant growth regulators in the culture medium (Nicuta *et al*., 2012). Among the groups of plant growth regulators, cytokinins have been reported to have important roles in plant growth and development by promoting cytokinensis, regulating cell division (Carle *et al*., 1998) and increasing mitotic activity (Tomaszewska-Sowa *et al*., 2002). Meanwhile, auxins are added for root induction. Simultaneously, it also plays an important role in the induction of cell division and control of cell-cycle progression. However, unbalanced concentrations of these two groups of hormones may induce polyploidy (Swartz, 1991). The presence of 2,4-D in culture medium is often associated with genetic abnormalities such as polyploidy and stimulation of DNA synthesis, which may result in endoreduplication (Ahmed *et al*., 2004; Mohanty *et al*., 2008).

Other factors that affect genetic stability in tissue culture system are duration of culture, genotype and type of explants. According to Bairu *et al*., (2006), the frequency of somaclonal variation increases as the number of subcultures and their duration increases, especially in callus and cell suspensions cultures. The genotype and type of explants strongly influenced the occurrence of somaclonal variation in many species such as strawberry (Popescu *et al*., 1997) and banana (Israeli *et al*., 1991; Martin *et al.,* 2006).

In order to study the effects of hormonal regulation and duration of cultures on cellular behaviour of *Oryza sativa* L. cv. MRQ 74, the root tips of the cultivar from different hormonal treatments were used in the current work. The occurrence of somaclonal variation among the regenerants was determined by cellular behaviour analysis including mitotic index (MI), cell and nuclear areas, nuclear to cell area ratios, chromosome counts and nuclear DNA content, and were compared with root tips of *in vivo* grown *Oryza sativa* L. cv. MRQ 74.

9.2 MATERIALS AND METHODS

9.2.1 Seeds Sterilization and Germination

The dehusked seeds of *Oryza sativa* L. cv. MRQ 74 were surface sterilized as described in section 3.2.1. The sterilized seeds were cultured on MS basal media (without hormone) containing 30 g/L sucrose and 8 g/L agar. The cultures were maintained at 25 ± 1 ºC with 16 hours light and 8 hours dark for four weeks. The sterilized seeds started to germinate after 2 days. The seedlings were used as sources of explants.

9.2.2 The Effects of Plant Growth Regulators and Duration of Cultures on Cellular Behaviour

The stem explants were excised from 4-week-old aseptic seedlings and were cultured on MS media supplemented with 30 g/L sucrose, 8 g/L agar and 0.5 mg/L 2,4-D, MS media supplemented with 1.0 mg/L NAA in combination with 0.1 mg/L kinetin and MS media supplemented with 1.0 mg/L NAA in combination with 0.5 mg/L kinetin. The pH of the medium employed in this experiment was adjusted to 5.8 prior to autoclaving process at 121 ºC, 105 kPa for 21 minutes. The cultures were maintained in the culture room at 25 ± 1 ºC under 16 hours light and 8 hours dark with 1000 lux of light intensity. The stem explants were also cultured on MS basal media as control. The five-day-old primary roots with standard length of 8.09 ± 0.79 mm were harvested from each treatment at 4, 8 and 12 weeks of culture for permanent slide preparations.

9.2.3 Permanent Slide Preparations

The primary roots of 5-day-old seedlings with standard length of 8.09 ± 0.79 mm were chosen as mean root length of the sample, in order to determine the mitotic index, cell and nuclear areas, chromosome counts and nuclear DNA content. The methods illustrated by Conger and Fairchild (1953) were followed. At least eight primary roots

of *in vivo* and *in vitro* grown *Oryza sativa* L. cv. MRQ 74 were fixed in 3: 1 (v/v) absolute alcohol : glacial acetic acid for 24 hours. The roots were hydrolysed in 1N HCl at 63 ºC for 13 minutes and were stained in Feulgen reagent for 2 hours. The stained roots were then treated with pectinase for 40 minutes. The apical region of a root tips (1-2 mm) was placed on a clean slide in a small drop of 45 % acetic acid. After the cells of the root segment were separated using dissecting needle, a coverslip was gently placed onto the slide. The coverslip was tapped firmly to spread the cells out to give a monolayer of the cells. The cells were then squashed by applying thumb pressure onto the coverslip. The slides were placed on a block of dry ice (solid CO_2) until completely frozen. The cover slips were carefully flicked off with a scalpel blade before the slides were made permanent by dehydration in a series of alcohol. Finally, to give a permanent preparation, the slides were mounted with a new coverslip using DPX. The prepared slides were then viewed using Axioskop Zeiss (Germany) microscope attached to AxionCam MRC video camera. The mitotic index, cell and nuclear areas, chromosome counts and nuclear DNA content were analysed using AxioVision 4.7 software.

9.2.4 Mitotic Index Determination

Mitotic index is a measurement to determine the percentage of cells undergoing mitosis. The mitotic index was measured by scoring cells in the dividing stages (prophase, metaphase, anaphase, telophase) from 1000 cells in a series of random transect across each of the three slides which was made permanent previously.

9.2.5 Measurement of Cell and Nuclear Areas Using Non Squash Preparations

Five-day-old primary roots of standard length (8.09 ± 0.79 mm) from each treatment were used to prepare non squash permanent slides fixed in 3:1 (v/v) absolute alcohol : glacial acetic acid for 24 hours. The roots were hydrolysed in 1N HCl at 63 ºC for 13

minutes and were stained in Feulgen reagent for 2 hours. The stained roots were then treated with pectinase for 40 minutes. The apical region of a root tip (1-2 mm) was placed on a clean slide in a small drop of 45 % acetic acid. After the cells of the root segments were separated using dissecting needle, a coverslip was gently placed onto the slide. Without squashing, the slides were then counterstained in 0.2 % (w/v) light green in absolute ethanol for 10 minutes and returned to absolute ethanol for another 10 minutes. Finally the slides were cleared in xylene for 10 minutes and mounted with DPX.

9.2.6 Chromosome Counts

At least ten cells at metaphase with proper chromosome spreads were counted from three prepared permanent slides for each treatment. All the counting were done under oil immersion (100 x magnification).

9.2.7 Measurement of Nuclear DNA Content

The prepared permanent slides were analysed using light microscope (Zeiss Axioscope) attached to VIDAS 21 image analysis system by Kontron Electronic. This image analysis system uses a software package for DNA measurement.

9.2.8 Statistical Analysis

Data obtained were analysed using the IBM SPSS Statistics 20 software (International Business Machines Corp., Armonk, NY). Statistical variance analysis was conducted using ANOVA (Duncan's Multiple Range Test or DMRT) at 5 % significance level where thirty replicates were used for each treatment. The effect of different treatments was quantified as mean ± SE (standard error).

9.3 RESULTS

9.3.1 Mitosis in Root Tip Meristem Cells of *Oryza sativa* L. cv. MRQ 74

All phases of mitosis (prophase, metaphase, anaphase and telophase) were clearly observed from the root tip meristem cells of *in vivo* grown *Oryza sativa* L. cv. MRQ 74, even though the cells were small in size. Cells at late prophase have shorten and thicken chromosomes as a result of chromatin condensation. With the aid of dye (Feulgen reagent) the chromosomes become visible as dark, rod-shape bodies (Figure 9.1). Following prophase is metaphase, the stage where the chromosomes line up along the equatorial plane of the cell (Figure 9.2). Spindle fibres attached to the centromeres of the chromosomes. Therefore, chromosomes can be seen more clearly at this stage than at any other phases. Hence, the chromosome number of a species can be determined at this stage.

During anaphase, the sister chromatids are pulled towards opposite poles by spindle microtubules. Each chromatid is now an independent chromosome. Figure 9.3 shows early anaphase observed in root tip meristem cells of *in vivo* grown *Oryza sativa* L. cv. MRQ 74. The final stage in mitosis is telophase, the stage where the chromosomes elongate by uncoiling and becoming chromatin threads. Two genetically identical daughter cells are produced after mitotic cell division had been completed. Figure 9.4 shows cell at telophase observed from root tip meristem cells of *in vivo* grown *Oryza sativa* L. cv. MRQ 74. The four stages of mitoses were also observed from root tip meristem cells of *in vitro* grown *Oryza sativa* L. cv. MRQ 74 (hormonal treatments), as shown in Figures 9.5, 9.6 and 9.7. The cells with bigger in size than normal was observed from the treatment containing 1.0 mg/L NAA in combination with 0.1 mg/L kinetin (Figure 9.8).

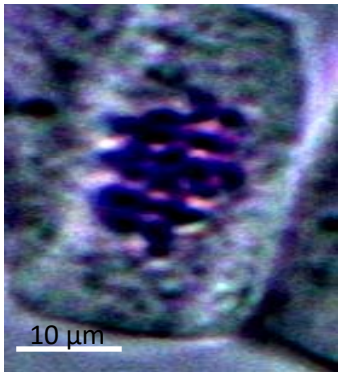


Figure 9.1 : Cell at prophase observed from squashed preparation of root tip meristem cell of *in vivo* grown *Oryza sativa* L. cv. MRQ 74.

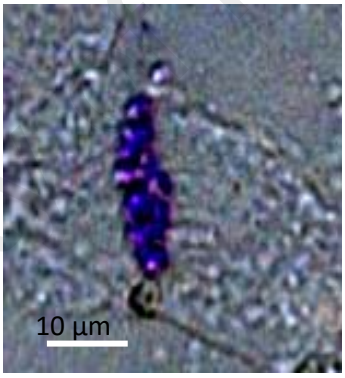


Figure 9.2: Cell at metaphase observed from squashed preparation of root tip meristem cell of *in vivo* grown *Oryza sativa* L. cv. MRQ 74.

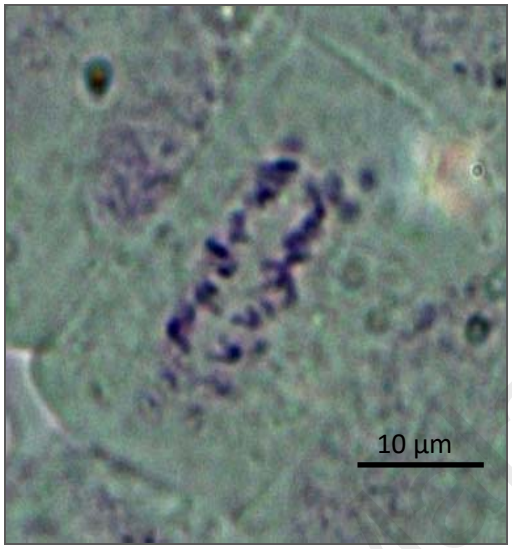


Figure 9.3: Cell at early anaphase observed from squashed preparation of root tip meristem cell of *in vivo* grown *Oryza sativa* L. cv. MRQ 74.

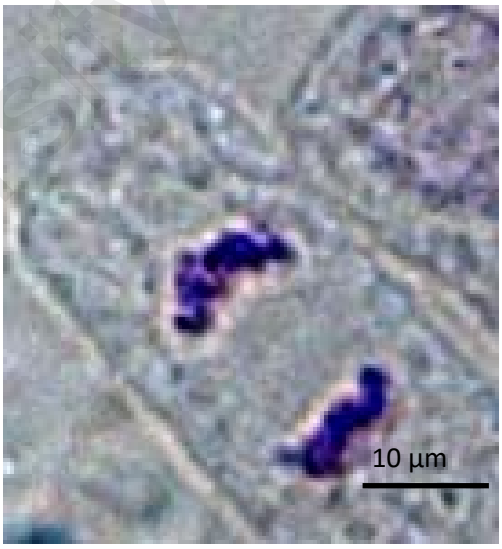


Figure 9.4: Cell at telophase observed from squashed preparation of root tip meristem cell of *in vivo* grown *Oryza sativa* L. cv. MRQ 74.

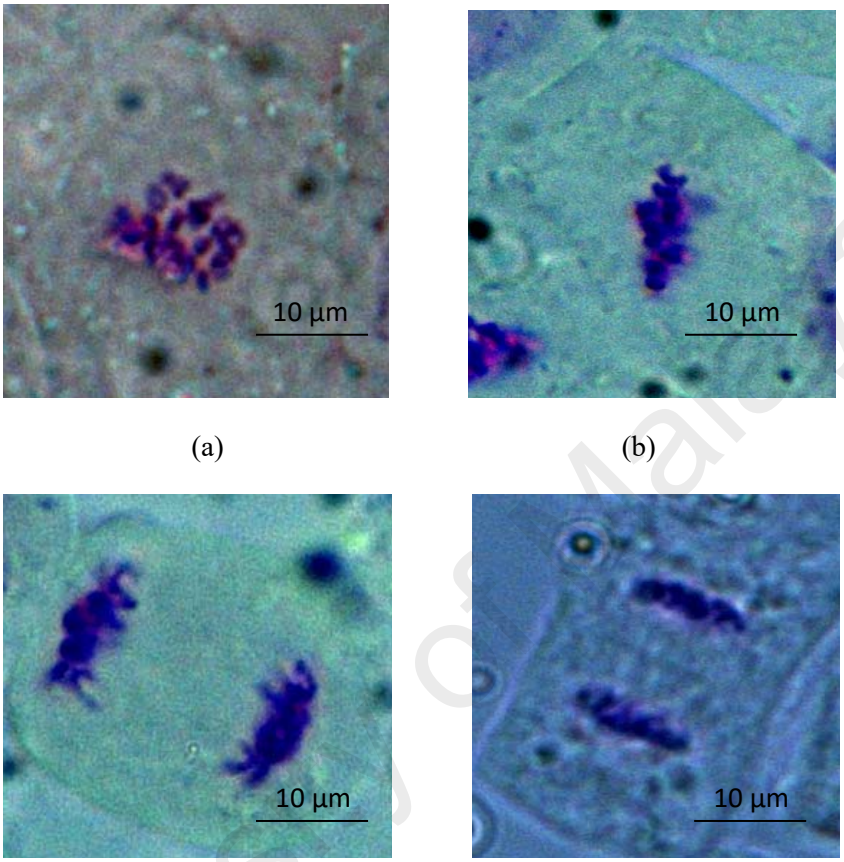


Figure 9.5: Stages of mitosis in root tip meristem cells of *Oryza sativa* L. cv. MRQ 74 grown *in vitro*. (a) and (b) Metaphase, (c) Anaphase, (d) Telophase.

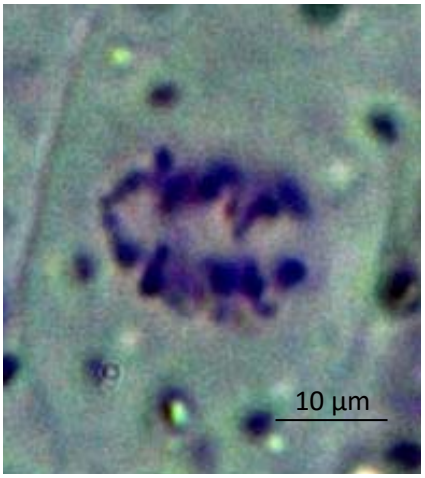


Figure 9.6: Early anaphase observed from root tip meristem cell of *Oryza sativa* L. cv. MRQ 74 grown *in vitro*.

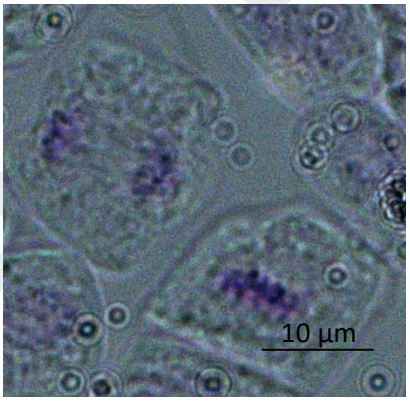


Figure 9.7: Metaphse (right) and late anaphase (left) observed from root tip meristem cells of *Oryza sativa* L. cv. MRQ 74 grown *in vitro*.

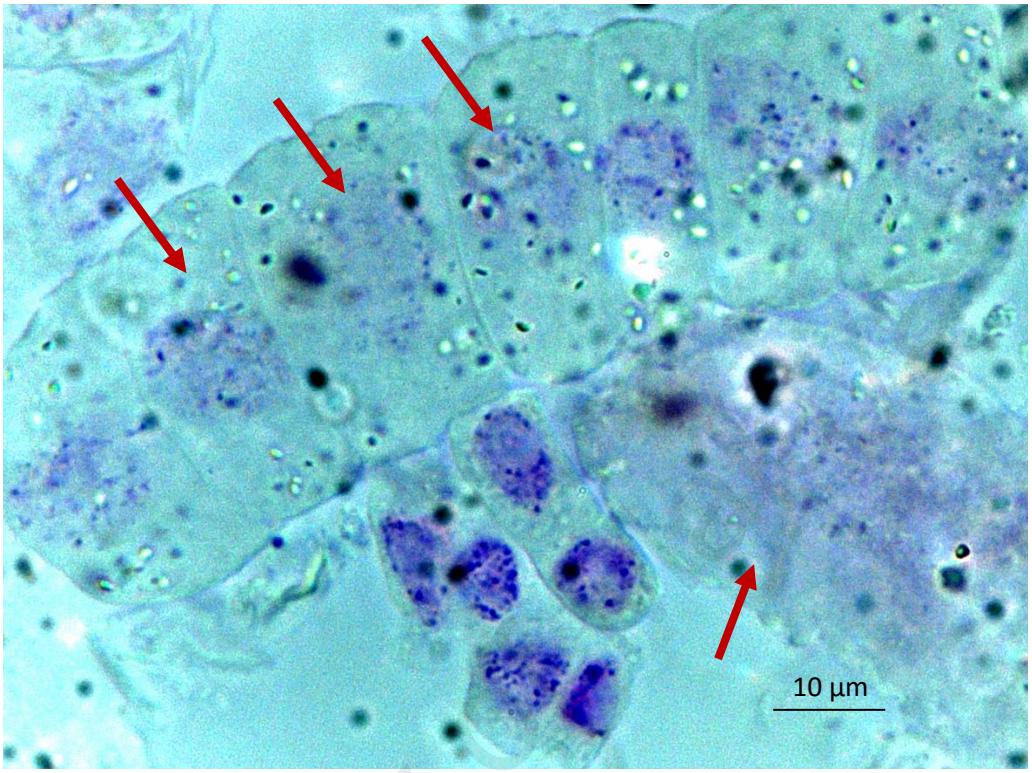


Figure 9.8: Bigger cells (red arrows) were observed from root tip meristem cells derived from MS medium containing 1.0 mg/L NAA in combination with 0.1 mg/L kinetin.

9.3.2 Mitotic Index (MI)

The mitotic index (MI) value was obtained by dividing the number of cells undergoing cell division with the total number of cells observed including interphase cells. The value is expressed as percentage. The MI was determined from a total of 500 cells in random transect across each of the three slides. Based on the calculation, the MI value for primary root of *Oryza sativa* L. cv. MRQ 74 grown *in vivo* was 10.45 ± 0.31 %. The mitotic activity of the cells was seriously influenced by the presence of plant growth regulators and their concentrations in the culture media and the duration of the cultures. The mitotic index (MI) values of rice root tip meristem cells were significantly

lower in MS media supplemented with NAA in combinations with kinetin and MS media supplemented with 2,4-D as compared with MS basal media (Table 9.1). The lowest MI value (2.67 ± 0.38 %) was observed in MS media supplemented with 0.5 mg/L 2,4-D after 12 weeks of culture. In fact, this hormone showed significantly lower MI values in weeks 4 and 8 as compared with all other treatments, 3.33 ± 0.38 % and 3.78 ± 0.22 %, respectively.

The results also showed that the mitoses of regenerants from MS basal media were most frequent at about 8 weeks of culture and dropped off markedly at week 12. However, the frequency remained the same for both weeks (8 and 12) for regenerants from MS media supplemented with 1.0 mg/L NAA in combination with 0.1 mg/L kinetin. On the other hand, at a slightly higher concentration of kinetin (0.5 mg/L), the frequency of mitoses was unstable and fluctuated on weeks 8 and 12 between 4.44 ± 0.55 % to 7.77 ± 0.80 %, respectively.

Table 9.1: The mitotic index (MI) values of root tip meristem cells of *Oryza sativa* L. cv. MRQ 74 grown *in vivo* and *in vitro*.

MS + Hormones (mg/L)	Period in culture (week)	Mitotic Index (MI) (%)
In vivo	-	$10.45 \pm 0.31_{f}$
MS basal	4	$8.00 \pm 0.39_{de}$
	8	$10.22 \pm 0.59_{f}$
	12	$9.11 \pm 0.22_{ef}$
1.0 NAA + 0.1 kinetin	4	$3.87 \pm 0.37_{ab}$
	8	$6.72 \pm 0.75_{cd}$
	12	$6.55 \pm 0.59_{cd}$
1.0 NAA + 0.5 kinetin	4	$6.00 \pm 0.67_{c}$
	8	$4.44 \pm 0.59_{b}$
	12	$7.77 \pm 0.80_{de}$
0.5 2,4-D	4	$3.33 \pm 0.38_{ab}$
	8	$3.78 \pm 0.22_{ab}$
	12	$2.67 \pm 0.38_{a}$

Mean values with same letters within a column are not significantly different at $p < 0.05$.

9.3.3 Mean Cell and Nuclear Areas, and Their Ratios

In general, the mean cell areas fluctuated from week 4 to week 12 in all treatments (Table 9.2). The highest mean cell area (237.93 ± 19.71 μm^2) was observed on week 8 in MS media supplemented with 0.5 2,4-D mg/L. Almost the same values were recorded on MS media supplemented with 1.0 mg/L NAA in combination with 0.1 mg/L kinetin and 1.0 mg/L NAA in combination with 0.5 mg/L kinetin at week 12, 236 ± 7.99 μm^2 and 235 ± 93 μm^2, respectively. It is interesting to note that the mean cell areas for these two treatments were not significantly different from week 4 to week 12. However, the mean cell areas for the MS media supplemented with 0.5 mg/L 2,4-D showed significantly different within these period.

The same pattern of fluctuation was observed in mean nuclear areas for all treatments. The highest mean nuclear area (76.26 ± 2.76 μm^2) was recorded on week 12 in MS media supplemented with 1.0 mg/L NAA in combination with 0.1 mg/L kinetin. This treatment also showed higher mean nuclear areas ranging from 52.08 ± 1.91 μm^2 to 76.26 ± 2.76 μm^2 compared to 53.33 ± 1.25 μm^2 to 74.85 ± 2.44 μm^2 and 46.80 ± 1.33 μm^2 to 64.67 ± 2.51 μm^2 on MS media supplemented with 1.0 mg/L NAA in combination with 0.5 mg/L kinetin and 0.5 mg/L 2,4-D, respectively. In terms of the ratio of nuclear to cell areas, *Oryza sativa* L. cv. MRQ 74 grown on MS basal media showed significantly lower (0.27 ± 0.01 μm^2) nuclear to cell areas ratio at week 4 and had significantly increased on weeks 8 and 12 of culture as compared to *in vivo*. In contrast results were observed on MS media supplemented with 0.5 mg/L 2,4-D, whereby the nuclear area to cell area ratio had significantly decreased from 0.34 ± 0.03 μm^2 to 0.29 ± 0.01 μm^2 and 0.31 ± 0.02 μm^2 on weeks 4, 8 and 12, respectively. On the other hand, MS media supplemented with NAA in combinations with kinetin showed the slight differences throughout the culture period. The ratio was proven not significant by statistical analysis.

Table 9.2: The mean cell and nuclear areas, nuclear to cell area ratios of root tip meristem cells of *Oryza sativa* L. cv. MRQ 74 grown *in vivo* and *in vitro*.

MS + Hormones (mg/L)	Duration (week)	Mean Area (μm^2) Cell (C)	Nuclear (N)	Nuclear : Cell Area (μm^2)
In vivo	-	$191.00 \pm 4.95_{c}$	$62.20 \pm 1.96_{e}$	$0.33 \pm 0.03_{de}$
MS basal	4	$164.37 \pm 3.85_{ab}$	$44.18 \pm 1.16_{a}$	$0.27 \pm 0.01_{a}$
	8	$145.40 \pm 6.37_{a}$	$48.92 \pm 2.03_{abc}$	$0.34 \pm 0.03_{e}$
	12	$190.21 \pm 5.51_{c}$	$55.33 \pm 1.30_{d}$	$0.29 \quad 0.01_{b}$
1.0 NAA + 0.1 kinetin	4	$220.20 \pm 7.89_{c}$	$71.92 \pm 3.12_{f}$	$0.33 \pm 0.03_{de}$
	8	$168.12 \pm 5.40_{bc}$	$52.08 \pm 1.91_{bcd}$	$0.31 \pm 0.02_{cd}$
	12	$236.25 \pm 7.99_{c}$	$76.26 \pm 2.76_{f}$	$0.33 \quad 0.03_{de}$
1.0 NAA + 0.5 kinetin	4	$190.26 \pm 5.05_{c}$	$56.35 \pm 1.59_{d}$	$0.30 \pm 0.02_{bc}$
	8	$169.91 \pm 3.88_{bc}$	$53.33 \pm 1.25_{cd}$	$0.32 \pm 0.02_{d}$
	12	$235.93 \pm 7.30_{c}$	$74.85 \pm 2.44_{f}$	$0.32 \pm 0.2_{d}$
0.5 2,4-D	4	$143.36 \pm 5.41_{a}$	$48.41 \pm 2.04_{abc}$	$0.34 \pm 0.03_{e}$
	8	$237.93 \pm 19.71_{c}$	$64.67 \pm 2.51_{e}$	$0.29 \pm 0.01_{b}$
	12	$151.82 \pm 4.33_{ab}$	$46.80 \pm 1.33_{ab}$	$0.31 \pm 0.02_{cd}$

Mean values with same letters within a column are not significantly different at $p< 0.05$.

9.3.4 Chromosome Counts

Determination of chromosome counts for *Oryza sativa* L. cv. MRQ 74 was very hard due to the cells size was very small. In addition, most of the chromosomes were not lying entirely in a single plane. The mean number of chromosomes of *in vivo* and *in vitro* grown *Oryza sativa* L. cv. MRQ 74 is shown in Table 9.3. Interestingly, the same mean number of chromosomes (24.00 ± 1.15) was recorded from *in vivo*, MS basal and MS media supplemented with 1.0 mg/L NAA in combination with 0.1 mg/L kinetin throughout the culture period. However, the mean number of chromosomes was significantly different (18.00 ± 1.15) at week 12 of culture in the treatment with 1.0 mg/L NAA in combination with 0.1 mg/L kinetin. Although the supplement of 0.5

mg/L kinetin to MS media containing 1.0 mg/L NAA promotes the chromosome number to increase (26.00 ± 1.15) as compared to other treatments, this number was not significantly difference with the exception of week 12 for MS media supplemented with 1.0 mg/L NAA in combination with 0.1 mg/L kinetin. As observed in Table 9.3, the period of cultures had no effect on chromosome number of *in vitro* grown *Oryza sativa* L. cv. MRQ 74.

Table 9.3: Chromosome counts in root tip meristem cells of *Oryza sativa* L. cv. MRQ 74 grown *in vivo* and *in vitro*.

MS + Hormones (mg/L)	Period in culture (week)	Mean number of chromosome
In vivo	-	$24.00 \pm 1.15_{b}$
MS basal	4	$23.00 \pm 1.15_{ab}$
	8	$24.00 \pm 2.65_{b}$
	12	$24.00 \pm 2.13_{b}$
1.0 NAA + 0.1 kinetin	4	$24.00 \pm 0.58_{b}$
	8	$24.00 \pm 1.15_{b}$
	12	$18.00 \pm 1.15_{a}$
1.0 NAA + 0.5 kinetin	4	$25.00 \pm 0.58_{b}$
	8	$24.00 \pm 2.52_{b}$
	12	$26.00 \pm 1.15_{b}$
0.5 2,4-D	4	$24.00 \pm 1.15_{b}$
	8	$22.00 \pm 2.00_{ab}$
	12	$23.00 \pm 2.00_{ab}$

Mean values with same letters within a column are not significantly different at $p< 0.05$.

9.3.5 Nuclear DNA Content and Ploidy Level

A total of 150 interphase cells were observed in determining the nuclear DNA content of primary roots of *Oryza sativa* L. cv. MRQ 74. The percentage frequency of cells at G1, S, G2 phases and ploidy levels were determined (Table 9.4). According to Evans and Van't Hof (1974), cell nuclei with nuclear DNA content of 0.0 – 2.2C are considered as G1, 2.2 – 3.6C as S, 3.6 – 4.8C as G2 and > 4.8 as polyploid. It was

found that most of the cell nuclei of *Oryza sativa* L. cv. MRQ 74 grown *in vivo* were at G1 phase (62.75 %), followed by S phase (24.16 %) and G2 phase (12.75 %). A small amount of cells (1.34 %) had nuclear DNA content > 4.8. This indicates that a small percentage of cells were polyploid.

It was also observed that most of the cells which had been cultured on MS basal media were in G1 and S phases of the cell cycle. The percentage frequency of the nuclei in G1 decreased from 38.67 % at week 4 to 35.33 % at weeks 8 and 12. Meanwhile, the percentage of the nuclei in S phase ranged from 33.33 % to 36.00 %. The nuclei with DNA amount higher than 4.8C decreased from 8.66 % to 4.67 % after 12 weeks of culture. On the other hand, most of the cells from MS media supplemented with 1.0 mg/L NAA in combination with 0.1 mg/L kinetin were in S phase. The percentage of the cells in this phase fluctuated from 32.67 % to 54.67 % and 40.00 % at weeks 4, 8 and 12, respectively. The highest frequency (10.00 %) of the cells with DNA amount higher than 4.8C or polyploid cells was recorded on week 8. The addition of kinetin at a slightly higher concentration (0.5 mg/L) did not affect DNA amount of the cells, thus producing the same pattern of nuclear DNA content.

However, an obvious effect of plant growth hormone was observed on MS media supplemented with 0.5 mg/L 2,4-D. The percentage frequency of nuclei in G1 phase increased while the polyploidy nuclei fluctuated. The percentage frequency were 7.33 % in G1, 46.67 % in S, 26.00 % in G2 and 22.00 % in polyploidy stage at week 4 of culture. The percentage frequency of G1 increased to 11.33 % and 12.67 % at weeks 8 and 12, respectively. In contrast, the percentage frequency of nuclei in S, G2 and polyploidy stages fluctuated. In general, the addition of 0.5 mg/L 2,4-D on MS media had caused significant effect on cellular activity of the cells. The highest frequency (22.00 %) of polyploid cells was recorded on MS media supplemented with 0.5 mg/L 2,4-D on week 4 of culture. The frequency decreased to 13.37 % on week 8 and 18.00

% on week 12. Nevertheless, the highest ploidy level reached was 6.6C and it was recorded from MS media supplemented with 1.0 mg/L NAA in combination with 0.5 mg/L kinetin at week 4 of culture. The duration of culture did not influence the ploidy level since the frequency of the cells with the nuclear DNA amount higher than 4.8C fluctuated from week 4 to week 12 in all cultures with different types of hormones treatments. Meanwhile, for the hormone free treatment (MS basal media), the percentages decreased from 8.66 % to 5.34 % and 4.67 % at weeks 4, 8 and 12, respectively.

Figures 9.9 to 9.21 show the distribution of cell nuclei in root tip meristem cells of *in vivo* and *in vitro* grown *Oryza sativa* L. cv. MRQ 74. Based on Figure 9.9, most of the primary root tip meristem cells of *in vivo* grown *Oryza sativa* L. cv. MRQ 74 had nuclear DNA content between 1.0 - 2.2C (G1). Therefore, the cells were observed to be diploid. However, the cells with nuclear DNA content >4.8 was observed as early as four weeks of culture in root tip meristem cells of *in vitro* all hormonal treatments.

Table 9.4 : Percentage of nuclei in cell cycle phases of root tip meristem cells of *Oryza sativa* L. cv. MRQ 74 grown *in vivo* and *in vitro*.

MS + hormones mg/L	Duration (week)	Phase (%) G1	S	G2	Polyploidy (%)
In vivo	-	62.75	24.16	12.75	1.34
MS basal	4	38.67	34.00	18.67	8.66
	8	35.33	33.33	26.00	5.34
	12	35.33	36.00	24.00	4.67
1.0 NAA+ 0.1 kinetin	4	26.67	32.67	31.33	9.33
	8	21.33	54.67	14.00	10.00
	12	44.00	40.00	11.33	4.67
1.0 NAA+ 0.5 kinetin	4	33.33	46.67	16.00	4.00
	8	28.00	46.00	18.00	8.00
	12	22.00	58.00	15.30	4.70
0.5 2,4-D	4	7.33	46.67	26.00	22.00
	8	11.33	51.33	24.00	13.37
	12	12.67	49.33	20.00	18.00

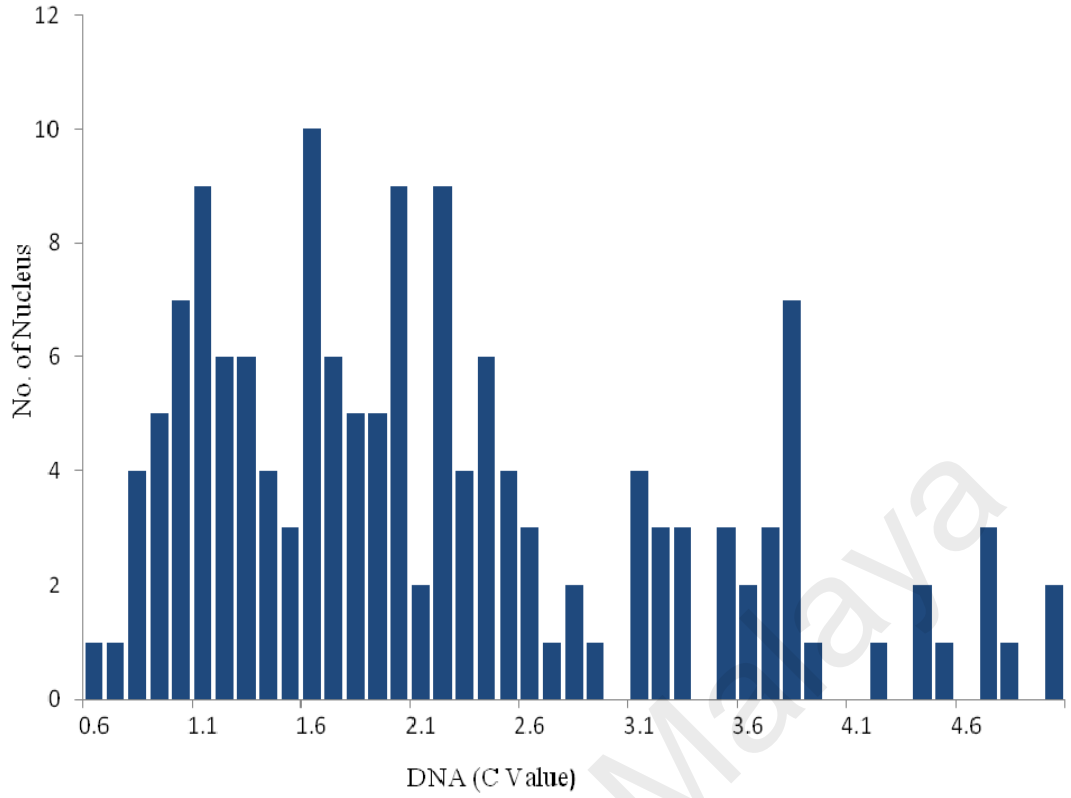


Figure 9.9: The distribution of DNA C values of interphase cells from root tip meristem of *Oryza sativa* L. cv. MRQ 74 grown *in vivo*.

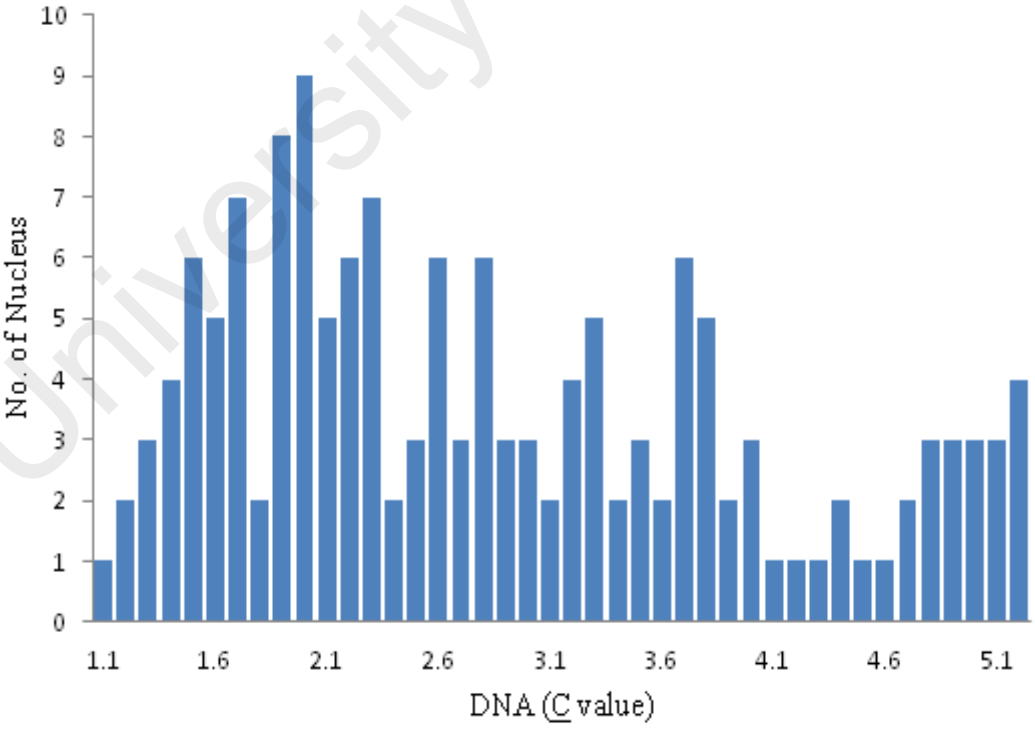


Figure 9.10: The distribution of DNA C values of interphase cells from root tip meristem of *Oryza sativa* L. cv. MRQ 74 grown *in vitro* on MS basal media after 4 weeks of culture.

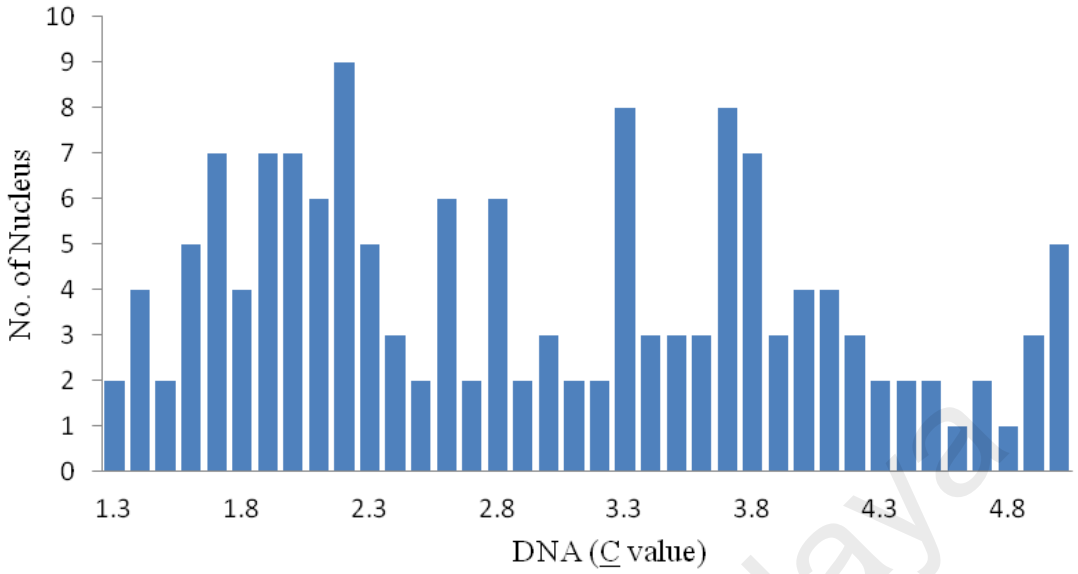


Figure 9.11: The distribution of DNA C values of interphase cells from root tip meristem of *Oryza sativa* L. cv. MRQ 74 grown *in vitro* on MS basal media after 8 weeks of culture.

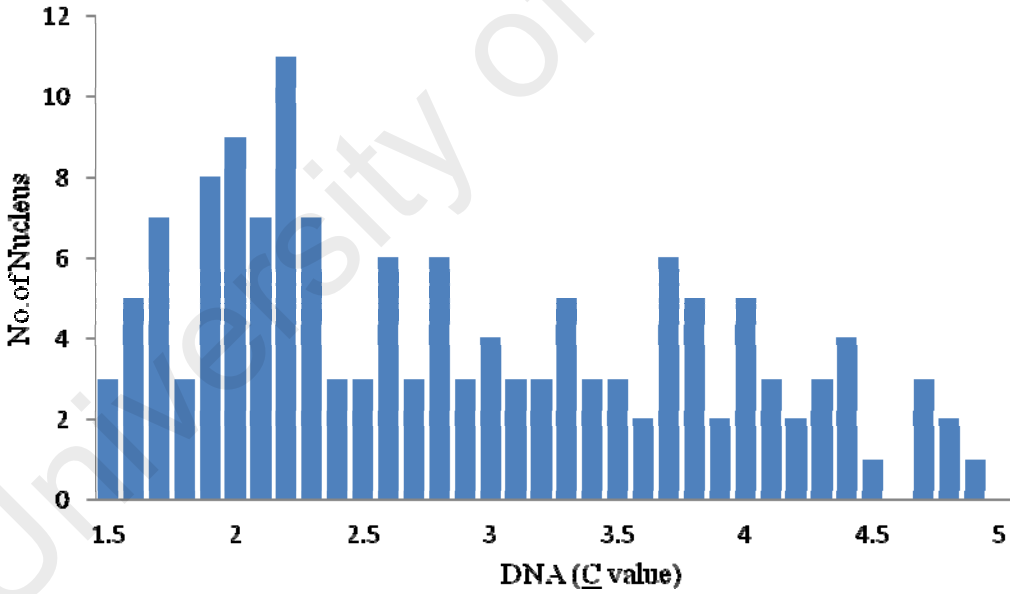


Figure 9.12: The distribution of DNA C values of interphase cells from root tip meristem of *Oryza sativa* L. cv. MRQ 74 grown *in vitro* on MS basal media after 12 weeks of culture.

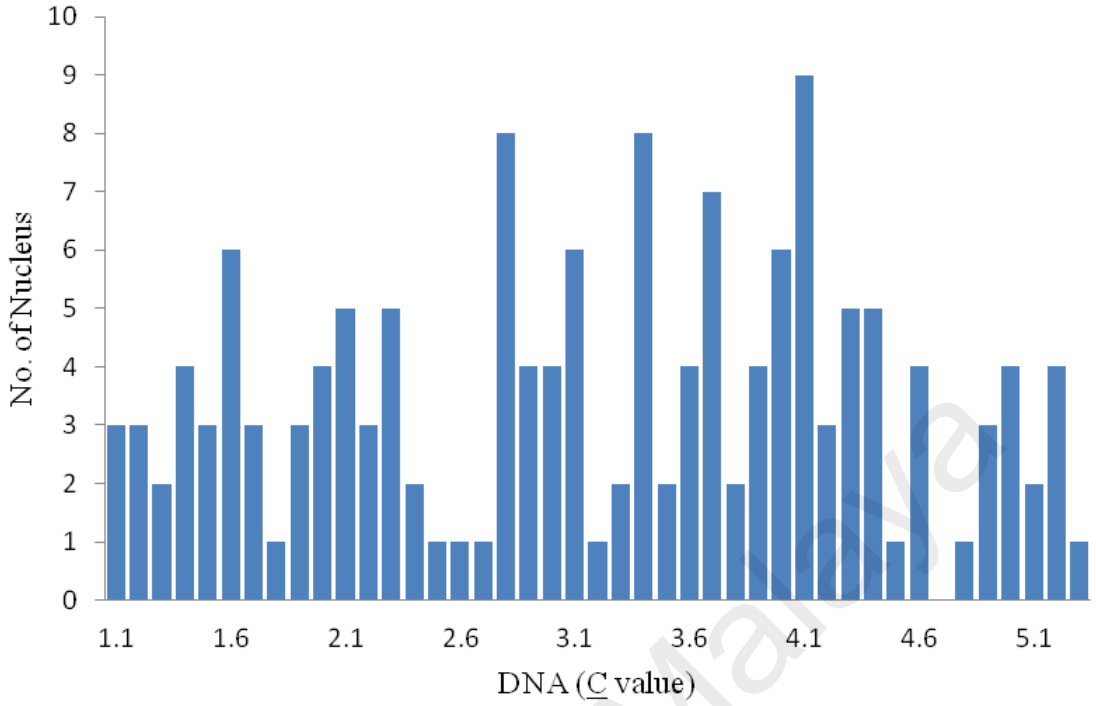


Figure 9.13: The distribution of DNA C values of interphase cells from root tip meristem of *Oryza sativa* L. cv. MRQ 74 grown on MS media supplemented with 1.0 mg/L NAA in combination with 0.1 mg/L kinetin after 4 weeks of culture.

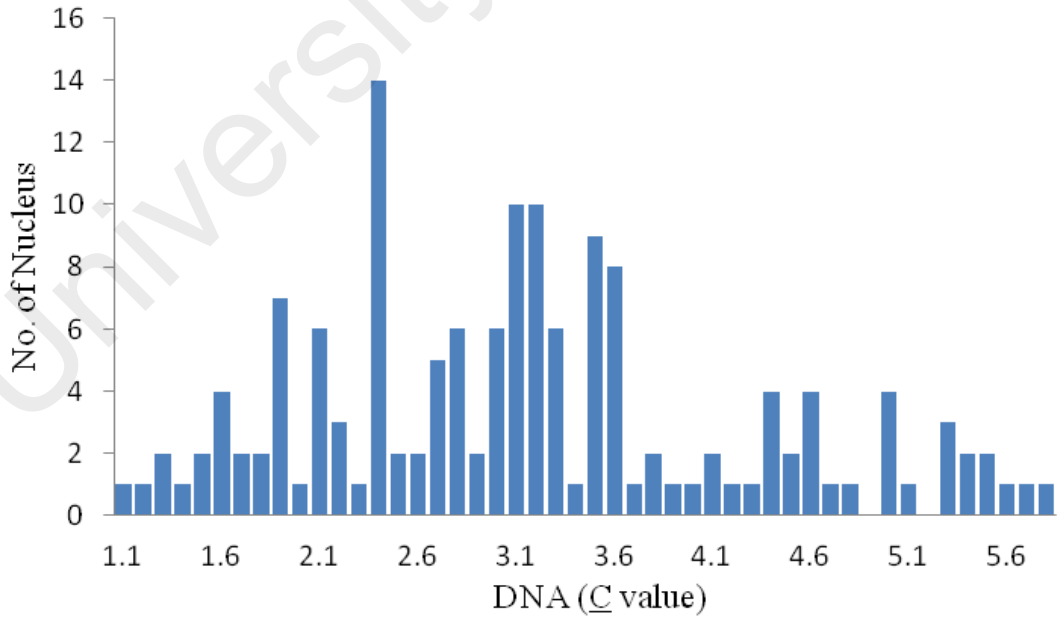


Figure 9.14: The distribution of DNA C values of interphase cells from root tip meristem of *Oryza sativa* L. cv. MRQ 74 cultured on MS media supplemented with 1.0 mg/L NAA in combination with 0.1 mg/L kinetin after 8 weeks of culture.

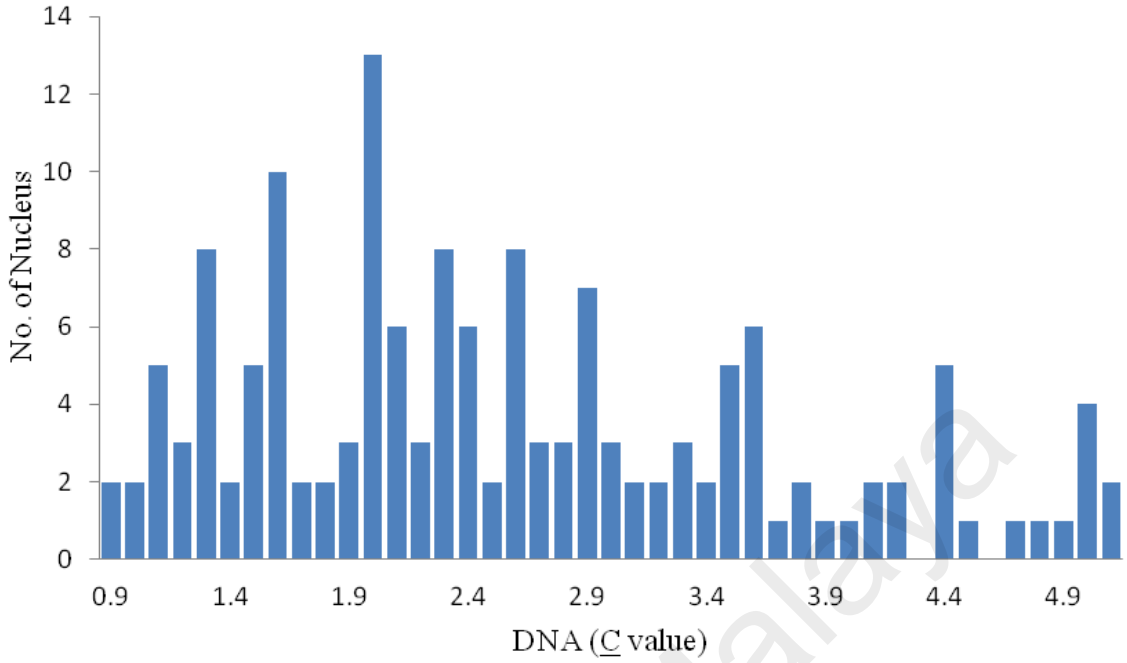


Figure 9.15: The distribution of DNA C values of interphase cells from root tip meristem of *Oryza sativa* L. cv. MRQ 74 cultured on MS media supplemented with 1.0 mg/L NAA in combination with 0.1 mg/L kinetin after 12 weeks of culture.

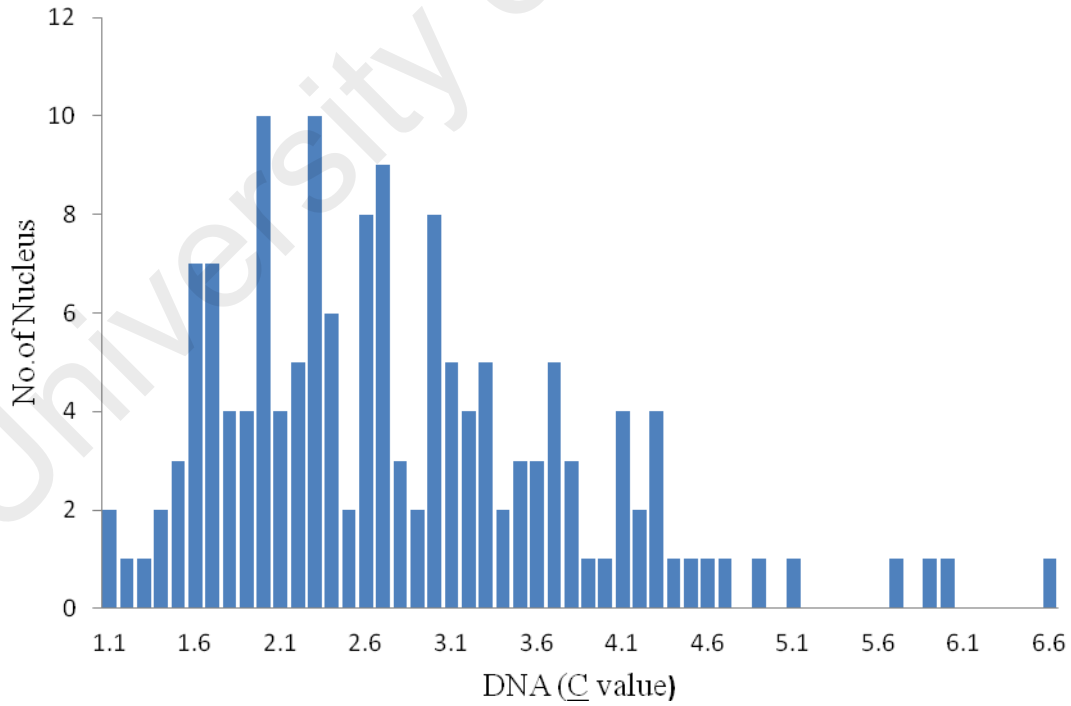


Figure 9.16: The distribution of DNA C values of interphase cells from root tip meristem of *Oryza sativa* L. cv. MRQ 74 cultured on MS media supplemented with 1.0 mg/L NAA in combination with 0.5 mg/L kinetin after 4 weeks of culture.

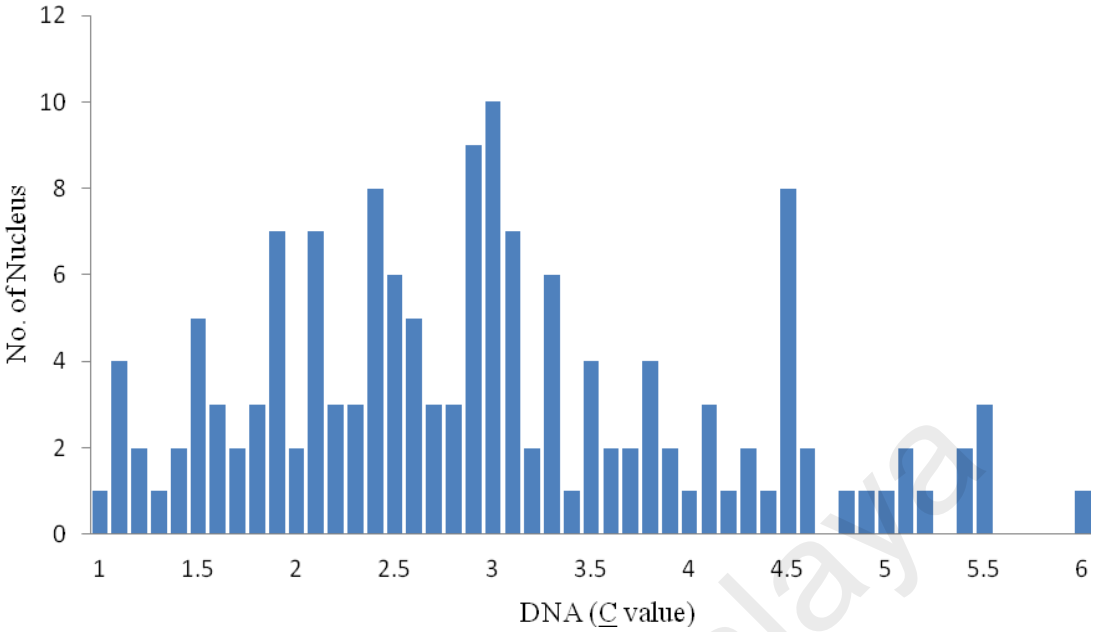


Figure 9.17: The distribution of DNA C values of interphase cells from root tip meristem of *Oryza sativa* L. cv. MRQ 74 cultured on MS media supplemented with 1.0 mg/L NAA in combination with 0.5 mg/L kinetin after 8 weeks of culture.

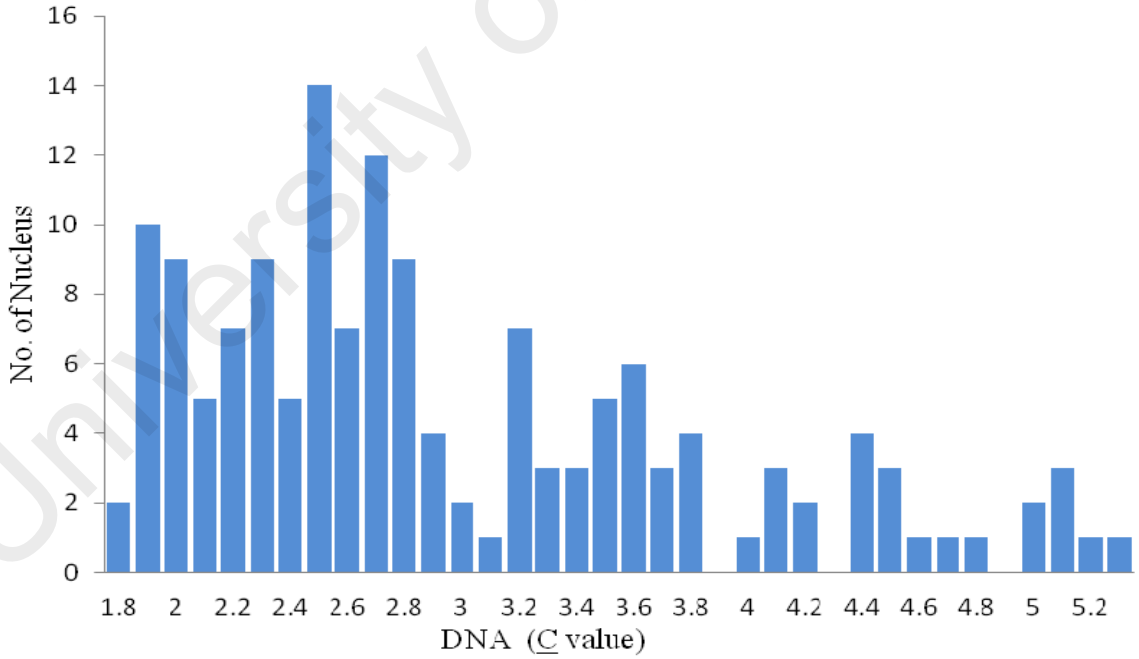


Figure 9.18: The distribution of DNA C values of interphase cells from root tip meristem of *Oryza sativa* L. cv. MRQ 74 cultured on MS media supplemented with 1.0 mg/L NAA in combination with 0.5 mg/L kinetin after 12 weeks of culture.

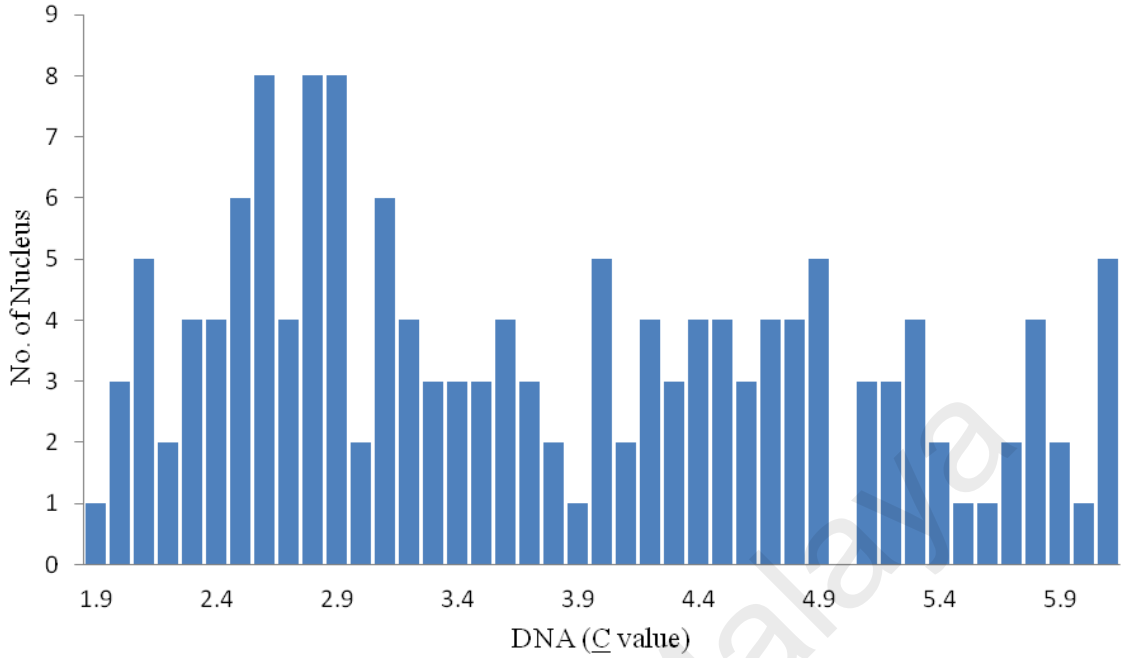


Figure 9.19: The distribution of DNA C values of interphase cells from root tip meristem of *Oryza sativa* L. cv. MRQ 74 cultured on MS media supplemented with 0.5 mg/L 2,4-D after 4 weeks of culture.

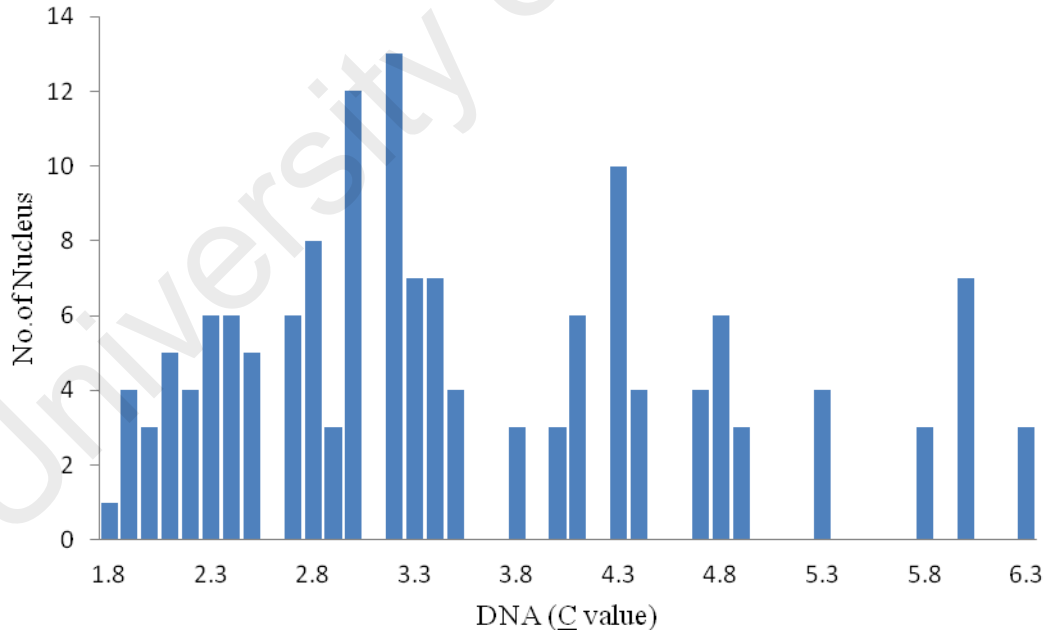


Figure 9.20: The distribution of DNA C values of interphase cells from root tip meristem of *Oryza sativa* L. cv. MRQ 74 cultured on MS media supplemented with 0.5 mg/L 2,4-D after 8 weeks of culture.

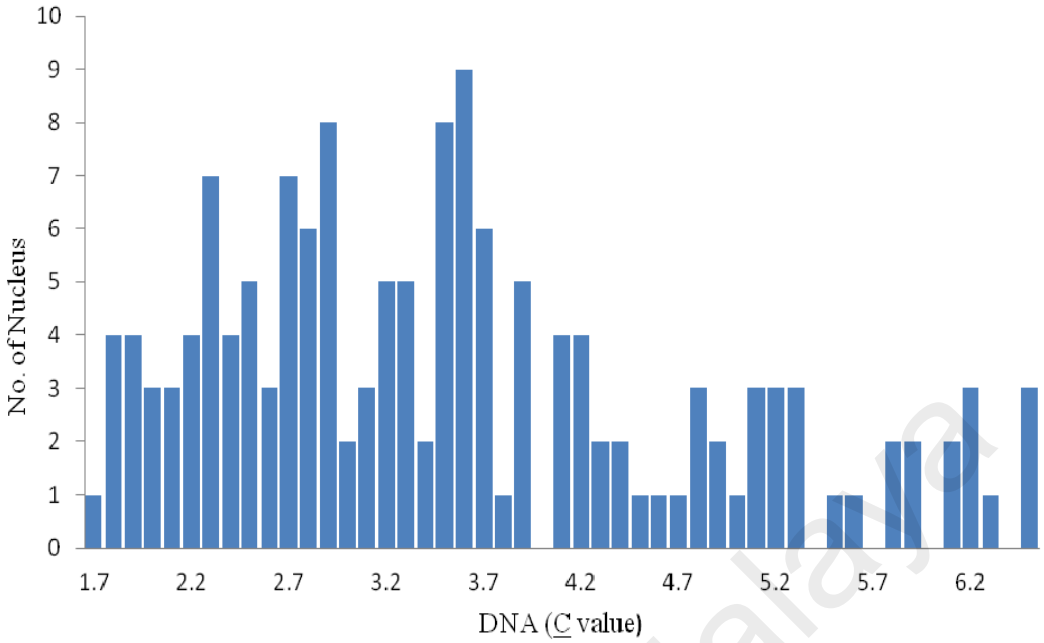


Figure 9.21: The distribution of DNA C values of interphase cells from root tip meristem of *Oryza sativa* L. cv. MRQ 74 cultured on MS media supplemented with 0.5 mg/L 2,4-D after 12 weeks of culture.

9.4 SUMMARY OF RESULTS

1. The mitotic index (MI) of primary roots of *in vivo* grown *Oryza sativa* L. cv. MRQ 74 was 10.45 ± 0.31 %.
2. The mean number of chromosome of *in vivo* grown *Oryza sativa* L. cv. MRQ 74 was 24.00 ± 1.15, while the reported value is 24.
3. The mean cell area of *in vivo* grown *Oryza sativa* L. cv. MRQ 74 was 191.00 ± 4.95 μm^2 and the mean nuclear area was 62.20 ± 1.96 μm^2, while the mean nuclear to cell area ratio was 0.33 ± 0.01 μm^2.
4. Most of the nuclei observed from the root tip meristem cells of *in vivo* grown *Oryza sativa* L. cv. MRQ 74 were in G1 phase (62.75 %), however, a small percentage (1.34 %) of the nuclei were polyploidy.
5. The MI values of *in vitro* grown *Oryza sativa* L. cv. MRQ 74 were higher (8.00 ± 0.39 %, 10.22 ± 0.59 % and 9.11 ± 0.22 %) in hormone free treatment (MS basal media). The lowest MI value was observed in MS media supplemented with 0.5 mg/L 2,4-D (2.67 ± 0.38 %).
6. The mean cell and nuclear areas were lower (with mean cell area ranging from 145.40 ± 6.37 μm^2 to 190.21 ± 5.51 μm^2 and nuclear area from 44.18 ± 1.16 μm^2 to 55.33 ± 1.30 μm^2) in MS basal media than in MS media supplemented with 1.0 mg/L NAA in combination with 0.1 mg/L kinetin and MS media supplemented with 1.0 mg/L NAA in combination with 0.5 mg/L kinetin. The lowest mean cell and nuclear areas was recorded on MS media supplemented with 0.5 mg/L 2,4-D, except for the week 8 of culture.
7. The number of chromosomes of *in vivo* grown *Oryza sativa* L. cv. MRQ 74 remained stable (24.00 ± 1.15) and ranging from 18.00 ± 1.15 to 26.00 ± 1.15 for *in vitro* grown *Oryza sativa* L. cv. MRQ 74.

8. The percentage of polyploid cells fluctuated with increasing period of culture. The presence of NAA and kinetin in MS media did not give remarkable effect on the percentage of ploidy level of cells.

9. The addition of 2,4-D in MS media at the concentration of 0.5 mg/L had increased the percentage frequency of polyploidy cells, ranging from 13.37 % to 22.00 %.

10. The highest ploidy level (6.6C) was recorded from MS media supplemented with 1.0 mg/L NAA in combination with 0.5 mg/L kinetin.

CHAPTER 10

DISCUSSION

Vegetative propagation through tissue culture system has become possible for many agricultural and horticultural crops. However, there are still many species, varieties or cultivars which remained non responsive even though major efforts have been done. For example, monocot plants such as rice are difficult to propagate. According to Khanna and Raina (1998), indica rice was less responsive than japonica rice in callus induction. In fact, significant variation response was observed within *in vitro* culture of indica rice subspecies. This indicates that the developed protocols are not universal and suitable for all cultivars, varieties and subspecies due to a few reasons such as genotype dependent. Thus, there is an urgent need to develop an efficient micropropagation protocol for an economically important species such as aromatic rice (*Oryza sativa* L. cv. MRQ 74). In the present study, plant tissue culture of *Oryza sativa* L. cv. MRQ 74 was successfully carried out which involved callus induction, somatic embryogenesis, *in vitro* regeneration, production of synthetic seeds, acclimatization and finally *in vivo* and *in vitro* cellular behaviour studies to detect early occurrence of somaclonal variation at cellular level.

Prior to this, the standard growth of roots was measured to determine the mean root length of the population samples. The standard growth of roots was obtained by germinating 100 of rice matured seeds on moist cotton wool in Petri dishes and were maintained for two weeks at 25 ± 1 ºC under 16 hours light and 8 hours dark with 1000 lux of light intensity. The primary root lengths were measured daily until the secondary roots appeared. The mean primary root length of the sample was used as standard root length for the subsequent experiments. However, other explants (stem and leaf) were tried and tested when root segments failed to regenerate. From the current work, the

mean of primary root length was 8.09 ± 0.79 mm. The growth rate of the primary root was 1.68 mm per day.

A lot of work had been done to study the effects of plant growth regulators and types of explants on callus induction in rice (Hoque and Mansfield, 2004; Bano *et al*., 2005; Joyia and Khan, 2012). Efforts have been made for callus induction and subsequent regeneration from green explants in many cereals such as maize (Ahmadabadi *et al*., 2007), Oat (Chen *et al*., 1995; Gless *et al*., 1998), wheat (Haliloglu, 2006), sugarcane (Franklin *et al.,* 2006) and rice (Ramesh *et al*., 2009). In order to examine the effect of plant growth regulators and types of explants on callus induction of *Oryza sativa* L. cv. MRQ 74, 2,4-D was applied singly and in combinations with BAP at various concentrations (0.5 to 2.5 mg/L). The mean callus dry weight and morphogenic responses varied depending on the types and concentrations of growth regulators. The mean callus dry weight varied from 3.50 mg to 71.60 mg in stem explants and 0.20 mg to 66.70 mg in root explants (Tables 4.1 and 4.2). The application of 2,4-D singly at 0.5 mg/L resulted in the highest callus dry weight (71.60 ± 6.40 mg) from stem explants. Joyia and Khan (2012) reported similar findings, whereby maximum callus of rice cultivar Super Basmati (*Oryza sativa* L. ssp indica) was induced at 1.0 mg/L 2,4-D, while minimum at 5.0 mg/L 2,4-D. Similar result was also reported by Lee *et al*., (2012) on callus induction of *Elymus dahuricus* L. The application of 2,4-D singly was found to increase the callus induction of this species. Studies have shown that among all auxins, 2,4-D has been used widely for callus induction of the Gramineae family (Sirkka and Immonen, 1993; Bai and Qu, 2000; Chaudhury and Qu, 2000). It was also observed that plantlets were regenerated from stem derived callus but not from root derived callus. However, Mandal *et al*., (2003) reported that embryogenic callus was produced from roots of eight-day-old seedlings of indica rice varieties viz. Quing Livan 1 and IET 13856. In contrast, results which were observed in this study suggested that

this behaviour could be species-dependent. Furthermore, different types of plant growth hormones were used in the present work.

Based on the results obtained from callus induction experiment, the most suitable type and concentration of hormones were chosen for somatic embryogenesis induction in the following experiment. Even though the highest mean callus dry weight was observed on MS media supplemented with 0.5 mg/L 2, 4-D from stem explants, however, the best quality of callus (creamy white, globular, friable) was observed on MS media supplemented with 2,4-D in combination with BAP. According to Fauziah *et al*., (2013), embryogenic callus of indica rice is characterized as pale-yellow white in color with a loose friable texture in appearance, while non embryogenic callus shows a white color and rough texture. Therefore, MS media supplemented with 2,4-D and BAP at the concentrations of 2.0 mg/L and 1.0 mg/L respectively, was used as embryogenic callus induction medium in the current work. Furthermore, some of the calli produced from the culture media regenerated into green plantlets. This indicates that the combination of these two hormones at appropriate concentrations can stimulate indirect somatic embryogenesis. It has been shown that 2,4-D played an important role in cell division and differentiation in rice somatic embryogenesis (Meneses *et al*. 2005).

The induction of somatic embryos is generally controlled by regulating the composition of plant growth hormones in the culture medium. Among these hormones, ABA is often used to promote somatic embryogenesis and enhance somatic embryo quality by increasing desiccation tolerance and preventing precocious germination. According to Vahdati *et al*., (2008), somatic embryos seem to become less responsive to ABA during maturation but show better respond at globular stage. Besides ABA, kinetin has been used to promote somatic embryogenesis in many plant species such as *Elaeis guineensis* Jacq. (Sumaryono *et al*., 2008) and *Saccharum officinalis* (Aamir *et al.,* 2007). In this study, an effort was made to examine the effect of these hormones

on somatic embryogenesis of *Oryza sativa* L. cv. MRQ 74. The results obtained indicate that the addition of kinetin at the concentration of 1.0 mg/L significantly increased the mean number of somatic embryos regardless of the concentrations of ABA supplemented in the culture media. The best culture media for somatic embryogenesis induction was MS fortified with 1.5 mg/L ABA in combination with 1.0 mg/L kinetin with 15.47 ± 0.24 mean number of somatic embryos. The callus differentiation pathway involves somatic embryogenesis and organogenesis (Jiménez, 2005). Both pathways generate entire plantlets from callus in rice, but mainly through organogenesis (Jiang *et al*., 2006; Huang *et al*., 2012). Indirect organogenesis of *in vitro* grown *Oryza sativa* L. cv. MRQ 74 was also observed in the current work, whereby organs such as root was observed to be growing from embryogenic callus. This can be seen from Figure 5.7, a root was produced when embryogenic calli were subcultured on MS media supplemented with 1.0 mg/L kinetin in combination with 2.0 mg/L ABA.

Supplementation of amino acids in culture media has been reported to enhance somatic embryogenesis in a number of monocots (Claparols *et al*., 1993; Wang *et al*., 2002; Kopertekh and Stribnaya, 2003; Abdullah *et al*., 2005). Amino acids are organic form of nitrogen (reduced state), which are readily metabolized by plant cells, stimulating faster cell growth and development (Gamborg 1970; Grimes and Hodges 1990). However, the optimal composition of plants growth hormones and requirements of other supplements varies with plant species. In order to study the effect of amino acids on somatic embryogenesis of *Oryza sativa* L. cv. MRQ 74, L-Proline was added in the somatic embryogenesis induction medium at the concentrations of 100, 200, 300, 400 and 500 mg/L. The results revealed that exogenous application of L-Proline had significant effect on somatic embryogenesis of *Oryza sativa* L. cv. MRQ 74. The highest mean number of somatic embryos (17.37 ± 0.66) was observed on MS media supplemented with 400 mg/L L-Proline. Amali *et al*., (2014) reported that the highest

number of 33.3 somatic embryos was obtained when 500 mg/L L-Proline was added onto MS medium supplemented with 2.5 mg/L 2,4-D, 0.25 mg/L kinetin and 500 mg/L casein hydrolysate.

The success of micropropagation of any species relies on several factors such as genotype, the type and age of explants, the type and concentration of plant growth regulators and organic components in the culture medium. Plant growth regulators are the most important factors for successful plant regeneration. *In vitro* shoot proliferation and multiplication are largely based on media formulations containing cytokinins as a major plant growth regulators (Mamidala and Nanna, 2009; Hoque, 2010).

In the present study, an attempt had been made in order to determine the most favourable plant growth hormones in terms of type and concentration for optimum shoots multiplication of rice (*Oryza sativa* L. cv. MRQ 74) from leaf, stem and root explants. Three types of cytokinins namely BAP, kinetin and TDZ were used in the present study to induce shoot multiplication. Besides these hormones, NAA and IBA were supplemented in the culture media in order to stimulate roots formation. MS medium containing 3 % (30 g/L) sucrose was employed throughout the experiments. Even though the most commonly used concentrations of sucrose were 2 to 5 %, however, the ideal concentration was 3%. According to Summart *et al*., (2008), the highest growth rate of Thai Aromatic Rice callus was found on the N6 medium fortified with 4 % sucrose. However, the high quality of callus was obtained from MS medium supplemented with 3 % sucrose. Therefore, the latest case was implemented in this study.

The root, leaf and stem explants were excised from healthy juvenile **(**one-month-old aseptic seedlings). The explants were cultured vertically on MS media supplemented with BAP, kinetin, TDZ, NAA and IBA at various concentrations either applied singly

or in combinations. The results revealed that the most responsive explant was the stem, the basal segment, 1 to 10 mm in length. The leaf and root explants were found unsuitable for *in vitro* regeneration of this cultivar, since these explants did not response to all types of hormones supplemented in shoot induction media. In fact, the explants became brownish after two weeks in the culture media and died after the forth weeks. According to Joyia and Khan (2012), no systems are available to regenerate plants from leaf explants in rice. Habibi *et al*., (2009) reported that monocots have always offered challenges in tissue culture system due to lack of meristematic areas in them. A poor regeneration response from root explants was also reported in other plant species such as in *Prunus microcarpa* (Nas *et al*., 2010). Until today, very few literatures had been published on the success of *in vitro* regeneration, callus and somatic embryos induction of rice from stem explants. On the other hand, dehusked rice seeds are the most preferred explants due to its distinct advantages of easy access and storage.

The results obtained from *in vitro* regeneration of *Oryza sativa* L. cv. MRQ 74 showed that there were differences in the effects of BAP, TDZ and kinetin either applied singly or in combinations with NAA and IBA. The highest mean number of shoots (4.03 ± 0.31) was obtained at the concentration of 1.5 mg/L BAP (Table 6.1). This was significantly higher compared to control (2.30 ± 0.16). The results indicate that BAP alone was sufficient for formation of multiple shoots. Similar to these findings, many researchers reported that cytokinins induced multiple shoot formation and shoot length (Van Staden, 2008; Jafari and Hamidoghli, 2010; Tornero, 2009; Gomes *et al.,* 2010; Ritu *et al.*, 2013; Hashem and Kaviani, 2010). Saini and Jawal (2005) reported that the efficiency of BAP for shoot regeneration in *V. Mungo* was significantly decreased when it was combined with IAA, NAA or IBA. Similar findings were observed in this rice cultivar. The combinations of BAP and NAA resulted in lower mean number of shoots per explant (Table 6.1).

Combinations of kinetin with NAA for adventitious shoots development were reported in many plant species (Lu *et al*., 1995; Gonzalez *et al*., 1999). Borthakur *et al*., (2012) stated that kinetin was antagonistic to shoot proliferation for leguminous tree *Albizzia chinensis* (Osbeck) Merr. The inefficiency of kinetin for shoot proliferation rate was also observed in *Petroselinum crispum* (Vandermoortele *et al*., 1996), banana (Shirani *et al*., 2010) and *Aloe vera* (Kumar *et al*., 2011), which are in agreement with the findings in the present study. Similar results were reported in *Salix* (Khan *et al*., 2011) and in *Bacopa chamaedryoides* (Sk Moquammel and Biswajit, 2013) whereby BAP was found to be more effective than kinetin. In contrast, TDZ was highly effective in promoting shoot formation (8.23 ± 1.09) at a low concentration (0.1 mg/L). However, high concentration of TDZ (0.5 mg/L) drastically inhibited shoot formation (4.20 ± 0.33). These results were in agreement with the findings of Mithila *et al*., (2003). They reported that a low concentration of TDZ induced shoot organogenesis in African violet explants, whereas at higher doses (5 to 10 μM) somatic embryos were formed. According to Shirani *et al*., (2010), higher concentrations of TDZ hindered further growth and development of the regenerants. Huetteman and Preace (1993) stated that at maximum concentration of TDZ, shoots might be prone to maximum hidricity and fasciation. Such a harmful effect may be due to hindering consequences of TDZ on endogenous auxin (IAA) and particularly cytokinin (BA) concentration (Zhang *et al*., 2005). Similar results were observed in this study. The plantlets produced at the concentrations of 0.3 to 0.5 mg/L TDZ showed abnormal growth (Figure 6.1D). TDZ has been reported to be more effective than BAP (Espinosa *et al*., 2006; Canli and Tian, 2008), while other reports stated that BAP had been found to be more effective than TDZ (Tang *et al*., 2002; Ruzic and Vujovic, 2008).

In the present study, the combinations of BAP, NAA and TDZ yielded no significant difference in mean number of shoots produced per explant. On the other hand, Mutasim and Kazumi (1999) found that explants obtained from 7-day-old seedlings cultured on MS medium containing BAP in combination with TDZ (2 mg/L each) produced higher number of shoots per explant than that of BAP applied alone. These contradictory results could be attributed to the use of different species, explant types and also possible effects of genotypes. According to Jordan *et al.,* (1996), TDZ induced shoot organogenesis when other cytokoninins failed. Studies on *Matthiola incana* showed that the effect of kinetin was significant on the number and length of root (Afshin *et al*., 2011).

Nowadays, the plant growth regulators have been used widely in modern agriculture and in tissue culture systems to promote shoots and roots formation. However, there is still lack of information on *in vitro* rooting of rice. An interesting result was observed in rice cultivar MRQ 74. In the present study, the cultivar did not require any exogenous plant growth regulators for root induction. The mean number of roots per explant (5.27 ± 0.76) was observed in the control treatment (hormone free medium). The presence of IBA (1.0 mg/L) in combination with a low concentration of BAP (0.1 mg/L) promoted better root formation, which was 11.07 ± 0.78 (Table 6.8). A similar observation was reported by Ling *et al*., (2013) whereby the highest number of roots per explant was 4.3 at 1 mg/L IBA. They also stated that the higher concentrations of IBA (3 to 7 mg/L) significantly reduced the number of roots per explant. In the present study, the highest mean number of roots (25.33 ± 1.89) was found on the MS media supplemented with 0.1 mg/L BAP in combination with 0.1 mg/L NAA, followed by 24.37 ± 1.91 at the concentration of 0.1 mg/L NAA. These findings suggest that NAA was the best rooting hormone for *Oryza sativa* L. cv. MRQ 74. The results were in line with the previous study on *Aloe vera* (Lee *et al*., 2011). On the other hand, IBA was found to be the most

efficient plant growth regulator for root induction in *Labisia pumila* var. alata (Ling *et al.,* 2013). According to Nandagopal and Kumari (2007), the effect of auxin on adventitious roots induction and elongation is highly species dependent.

Root length growth was significantly influenced by different levels of growth regulators. Among the treatments, the highest root length was obtained on MS media supplemented with 0.1 mg/L IBA, followed by 0.5 mg/L kinetin and 0.1 mg/L BAP in combination with 0.1 mg/L NAA that ranged from 0.3 - 15.0 cm, 0.7 - 9.0 cm and 0.5 - 7.0 cm, respectively (Tables 6.2, 6.4 and 6.8).

Somatic embryos have been used widely as favoured micropropagules for synthetic seed production in many plants species such as *Vitis vinifera*, *Solanum melongena*, *Psidium guajava*, *Daucus carota* (Ara *et al*., 2000) and *Oryza sativa* L. Hybrid CORH-2 (Kumar *et al*., 2005). According to Gray (1987), synthetic seed technology has been developed based on the use of somatic embryos as functional seeds. However, success of the synthetic seed technology is constrained due to scarcity and undesirable qualities of somatic embryos making it difficult for their development into complete plants. Besides somatic embryos, various types of micropropagules like axillary shoot buds, apical shoot tips and protocom like bodies were also used in this technology. To date, only a few studies have used microshoots for the production of synthetic seeds (Soneji *et al*., 2002). Therefore, in the current work, microshoots of *Oryza sativa* L. cv. MRQ 74 (3-5 mm) were chosen as micropropagules.

Various factors were examined in line with the effort of producing synthetic seeds. The factors including the presence of plant growth hormones in encapsulation matrices, type of sowing substrates, storage period and storage temperature were taken into consideration. Even though a number of substances such as potassium alginate, carrageenan, agar, gelrite and sodium pectate have been tested as hydrogels, however, sodium alginate is the most popular. Sodium alginate was widely used due to its

solubility at room temperature and ability to form completely permeable gel with calcium chloride, low toxicity and has bio compatibility characteristics (Saiprasad, 2001). The quality in terms of the texture and shape of the synthetic seeds produced are dependent on sodium alginate and calcium chloride concentrations and the duration of exposure to complexing agent. According to Asmah *et al*., (2011), 3 % sodium alginate produced uniform and sufficiently firm beads of *Acacia* hybrid. Higher sodium alginate concentration (4 to 5 %) produced hard texture beads and this reduced the ability of shoots and roots emergence. Therefore, 3 % sodium alginate with 30 minutes exposure to 0.2 M calcium chloride was applied in this study.

Based on the results obtained, the highest germination rate (100 %) was found in all types of encapsulation matrices including control (nonencapsulated microshoots). For production of synthetic seeds from apical shoot tips and axillary shoot buds, these organs are usually first treated with auxins for root induction. In this case, 0.1 mg/L BAP in combination with 0.1 mg/L NAA were added in encapsulation matrix to stimulate shoots and roots formation. However, Bapat and Rao (1990) reported that mulberry plantlets were obtained from alginate encapsulated shoot buds without any specific root induction treatment. Taha *et al*., (2009) found that MS basal media supplemented with 30 g/L sucrose was the best substrate for germination of the synthetic seeds of *Saintpaulia ionantha* Wendl. The same finding was reported on banana (Ganapathi *et al*., 1992). Roy and Mandal (2008) also stated that embryos and pro-embryos of elite indica rice (*Oryza sativa* L. Var IR 72) developed into plantlets on MS basal medium without any growth hormones. These results were in line with the findings of the present study. The well rooted plantlets were observed from all the germinated synthetic seeds.

The presence of sucrose in encapsulation matrix of the synthetic seeds gave significant effect on plantlets survival rate. The lowest survival rate (90.00 ± 0.55 %) was recorded on control treatment (non-encapsulated) followed by Ca-free MS + distilled water (93.33 ± 0.48 %) as compared to Ca-free MS + 30 g/L sucrose and Ca-free MS + 30 g/L sucrose + 0.1 mg/L BAP + 0.1 mg/L NAA which were 100.00 ± 0.00 % and 96.67 ± 0.48 %, respectively (Table 7.1). These results were supported by those findings obtained by Thobualuepop *et al*. (2009). They found that the inoculated somatic embryos with various concentrations of sucrose (0, 30 and 60 mg/L) gave synthetic seeds germination rates of 43 %, 57 % and 46 %, respectively. Similar findings were reported on other species such as *Psidium guajava* L. The percentage of germination of encapsulated somatic embryos of *Psidium guajava* L. decreased significantly with increasing concentration of sucrose (3-9%) in medium. Other studies have shown that low germination and conversion capacity of synthetic seeds was due to absence of nutritive tissues like the endosperms of the natural seeds (Sanada *et al*., 1993). These results indicate that coating material and the concentration of the coating material are important limiting factors for the synthetic seed technology. On the other hand, Sandova-Yugar *et al*.,(2009) reported that the higher germination rate (83.3 %) was found on sucrose-free capsule than sucrose-enriched capsule (56.7 %) of *Musa* sp. cultivar 'Grand Naine'.

An interesting feature of the synthetic seeds was their ability to retain viability in terms of germination potential even after a considerable period of storage. A low temperature (4 ^{o}C) was reported as good storage temperature for synthetic seeds of many plants species such as mulberry (Patnaik *et al*., 1995), strawberry and raspberry (Lisek and Orlikowska, 2004). However, in the present study, the viability of the synthetic seeds had fallen drastically from 93.33 % to 3.33 % after one month of storage at 4 ^{o}C. Therefore, further research for the development of a better technique is

required. For example, increasing the storage temperature may increase regeneration levels. Simultaneously, Elvax 4260 (ethylene vinyl acetate acrylic acid terpolymer, Du Pont, USA) could be used for coating the capsules to avoid rapid water loss when calcium alginate capsules are exposed to the ambient atmosphere (Ara *et al.,* 2000). Storage of synthetic seeds using an alginate encapsulation protocol has been attempted in a few species with minimal success (Redenbaugh *et al*., 1987). The synthetic seeds of sweet corn germinated successfully up to 43 % and 55 % after 2 weeks of storage under 15 ± 2 ºC and 25 ± 2 ºC, respectively (Thobunluepop *et al*., 2009). The decline in the regeneration frequency among the stored encapsulated propagules may be due to the inhibited respiration of plant tissue by the alginate and accumulation of metabolic waste in the alginate capsules during the long storage.

An addition of plant growth regulators in the culture medium not only stimulated better growth, but sometimes it caused morphological changes especially on leaves structure. In order to identify whether this phenomena had occurred, the microscopic studies of the structure of *in vitro* leaves from synthetic seeds grown plantlets and *in vitro* leaves from plantlets cultured on MS media supplemented with 0.1 mg/L BAP in combination with 0.1 mg/L NAA were compared with intact (*in vivo*) leaves. The findings revealed that no morphological changes in both *in vivo* and *in vitro* leaves. There was no significant differences in terms of stomata number on adaxial and abaxial leaf surfaces of *in vitro* and *in vivo* leaves. Apart from that, silica bodies were also observed on *in vitro* and *in vivo* leaf surfaces. In rice, silica body content may be correlated with resistance to fungal diseases, example brown spot and blast, and insect pests (Kim *et al*., 2002).

Micropropagation allows rapid production of high quality, disease free and uniform planting materials irrespective of the season and weather. However, a major limitation in large scale application of this technology is high mortality experienced by

micropropagated plants during or following laboratory to land transfer. Microshoots on being transferred to *ex vitro* conditions are exposed to abiotic and biotic stress conditions, so need acclimatization process for successful establishment and survival of plantlets (Deb and Imchen, 2010).

The ultimate success of micropropagation of a species is the successful growing and survival of the micropropagated plantlets in the field. The micropropagated plantlets must undergo a period of acclimatization, more specifically, a period of transitional development in which both anatomical characters and physiological performance overcome the influence of *in vitro* culture conditions (Donnelly *et al.*, 1986). The successful *ex vitro* acclimatization of micropropagated plants determine the quality of the end-product and, in commercial production, economic viability of the enterprise (Conner and Thomas, 1982). Therefore, research on acclimatization of many plants species have received a lot of attention from biotechnologists.

In the present study, an efficient protocol for *ex vitro* hardening of rice cultivar MRQ 74 was successfully established. In this experiment, acclimatized plantlets were obtained from two different regeneration media. From the previous experiment (Chapter 6), MS media supplemented with 0.1 mg/L BAP in combination with 0.1 mg/L NAA was found to be the best regeneration media. Hence, plantlets were regenerated on MS media containing these two plant growth hormones from stem explants of 6-week-old aseptic seedlings. Besides that, plantlets were also regenerated on MS media fortified with 2,4-D at the concentration of 0.5 mg/L. The two-month old *in vitro* raised plantlets with five to ten healthy roots were acclimatized on three different types of soil namely black soil, red soil and mixture of black and red soil in 1: 1 ratio. In order to avoid direct exposure to sun light, the acclimatized plantlets were covered by plastic and were kept in the culture room for two months. After the first month, the plastic covers were removed. Thus, the plantlets experienced a lower air humidity compared in cultured

containers and covered plastics. The acclimatized plantlets were irrigated every alternate day before they were transferred to a green house. A few morphological parameters such as plant height, leaf length, number of seeds per stalk and number of leaf were measured.

The results showed that the mixture of red and black soil (1:1 ratio) gave the highest plantlets' survival rate (Tables 8.1 and 8.2), regardless of the types of regeneration induction media (MS media containing 0.1 mg/L BAP + 0.1 mg/L NAA and MS media containing 0.5 mg/L 2, 4-D). The least preferred substrate was the red soil. The different survival rate might be due to the different types and amount of nutrients content in each soil. In general, black or dark brown soil usually indicates the presence of decaying organic matter, therefore it is generally fertile. Meanwhile, red soil typically indicates extensive weathering and good drainage, but often needs nutrients and organic matter. Soil nutrient analysis is important in determining the soil fertility levels. In the present study, the soil samples were analysed using XRF (X-Ray Fluorescence) method. This method can be used for *in-situ* screening and *ex-situ* analysis. Based on the results obtained from XRF soil analysis, the black soil contains higher values of the most important nutrients such as P, K, Ca, Mg and S than that of the red soil (Tables 8.4 and 8.5). These macronutrients are required for plants growth and development. For example, phosphorus is a component of the complex nucleic acid structure of plants, which regulates protein synthesis. Therefore, it is important in cell division and development of new tissues. Phosphorus also plays a role in photosynthesis, respiration, energy storage and transfer, and several other processes in plants. Calcium is required for various structural roles in cell wall and membranes (White and Broadley, 2003).

As presented in Tables 8.4 and 8.5, the survival rate of the plantlets decreased eventually. The ability of the plantlets to survive was influenced by the regeneration pathway. It was observed that the plantlets produced on MS media containing 0.5 mg/L

2,4-D had bigger size of roots. Therefore, the plantlets showed better survival rate than that of MS media supplemented with 0.1 mg/L BAP in combination with 0.1 mg/L NAA. The mixture of black and red soil gave the highest survival rate which was 90.00 ± 1.53 % and had decreased to 83.33 ± 1.20 %, while none of the plantlets survived after two months on the red soil.

An abnormal morphology of leaf was observed among the regenerants where the leaf blade became wavy (Figure 8.2b). According to Abe *et al*., (2010), the growth of the central region of the leaf would fail to synchronize with the lateral region. In order to validate this phenomena, histological studies was carried out to compare between *in vivo*, *in vitro* and acclimatized plants. The cross section of the *in vitro* leaf revealed that cell enlargement and proliferation was imperfect in the wavy leaf. Abnormal differentiation in the bundle sheath was also observed in which cells were increased in number and arranged randomly (Figure 8.4). Generally, *in vitro* conditions cause rapid growth and shoot proliferation, resulted in abnormal histological features of regenerated plantlets such as altered leaf morphology, absence of thick cuticle and poor water transport (Pati *et al*., 2013). Lack of prominent cuticle is a regular feature of *in vitro* plants. The absence of leaf cuticle was observed in the present study of *Oryza sativa* L. cv. MRQ 74. The presence of cuticle is crucial in preventing excessive water loss upon transpiration and one of the factors that determine the survival rate of the plants. A similar observation was reported in *Uniola paniculata* (Carmen *et al*., 2008). The low survival rate of acclimatized plants was due to an abnormal tissue organization, stomata aperture blockage and thylakoid membrane disruption.

A wide variation in plant morphology such as the number of seeds per stalk, plant height, leaf length and number of leaf were also observed. Even though the plants managed to produce seeds after two months in the green house, however, the number of seeds per stalk was much lesser compared to *in vivo* or intact plants. The plant height,

leaf length and the number of leaf were also significantly lower than that of *in vivo* plants (Table 8.3). These results were in agreement with those findings obtained by Karim *et al*., (2015). A wide range of variations was also observed on quantitative and qualitative characters of the *in vitro* callus derived from strawberry plants (*Fragaria x ananassa* Dutch.).

The poor plant growth and development of acclimatized plants is due to different environmental conditions such as light intensity, temperature and humidity. *In vitro* growing plantlets are under low light intensity, temperature and hight humidity. Hence, direct transfer to sunlight and high temperature might cause flaccid of plantlets. The retardation in development of cuticle, epicuticular waxes and functional stomatal apparatus during *in vitro* culture cause high stomatal and cuticular transpiration rates of leaves in plantlets when taken out of the culture vessels. Thus, in order to avoid this, the plantlets should be slowly transferred from high humidity to low humidity conditions.

It is well known that plant growth regulators play a crucial role in plant growth and development. In tissue culture system, the presence of auxins and cytokinins are very important for proper growth and maintenance of culture. However, the exogenous application of these hormones in the culture medium causes chromosomal abnormalities. Nicuţă *et al*., (2012) stated that the frequency of chromosomal abnormalities was significantly influenced by the presence of growth regulators in the culture medium. Since a few types of plant growth hormones such as kinetin, 2,4-D and NAA were used in this study, the effects of these hormones on cell activities of *in vivo* and *in vitro* grown *Oryza sativa* L. cv. MRQ 74 were investigated.

In order to examine the effect of plant growth hormones on cellular behaviour such as mitotic index, cell areas, nuclear areas, chromosome counts and nuclear DNA content of *Oryza sativa* L. cv. MRQ 74, the stem explants from aseptic seedlings as described in section 4.2.1 were cultured on MS media containing 1.0 mg/L NAA in combination

with 0.1 mg/L kinetin, 1.0 mg/L NAA in combination with 0.5 mg/L kinetin and 0.5 mg/L 2,4-D. The stem explants were also cultured on MS basal media as a control treatment. As for the *in vivo* grown *Oryza sativa* L. cv. MRQ 74 roots, the sterilized seeds were cultured on moist cotton wool as described in section 3.2.1. The cellular parameters measured were compared between roots of *in vivo* and *in vitro* grown *Oryza sativa* L. cv. MRQ 74 to detect cellular changes when the plant was grown *in vitro*. Based on the standard growth of primary roots (Chapter 3), the roots with the mean root length of 8.09 ± 0.79 mm and standard age of five-day-old were used in preparing the permanent slides for all treatments. Since the rice root tips were small and thick, the root tips were hydrolyzed using 5M HCl at 63 ºC for 13 minutes. The hydrolyzed root tips were then submerged in 4 % pectinase to break down pectin, a compound found in cell wall. Pectinase therefore helps to break down cell wall and thus allowing better staining. The mean mitotic index (MI) value of the *in vivo* grown *Oryza sativa* L. cv. MRQ 74 was 10.45 ± 0.31 %. The mean chromosome number was 24 ± 1.15 while the mean cell and nuclear areas were 191.00 ± 4.95 μm^2 and 62.20 ±1.96 μm^2, respectively. The ratio of nuclear to cell areas was 0.33 ± 0.01 μm^2.

The effects of plant growth hormones on cellular behaviours of plants species have been reported by many researchers (Selma and Signem, 2012; Yaacob *et al*., 2013). Ud-Deen and Kabir (2009) reported that the mitotic index and cell size of onion cells were decreased with increasing concentration of plant growth regulators such as GA_3. The results obtained from the current work were in line with the findings of Ud-Deen and Kabir (2009). The mitotic index (MI) values were higher in MS basal media than MS media supplemented with various plant growth hormones. Most of the MI values were higher at week 8 of culture, except for MS media fortified with 1.0 mg/L NAA in combination with 0.1 mg/L kinetin. However, the statistical analysis revealed that there were no significant differences of MI values at weeks 8 and 12 for roots grown on MS

media supplemented with 1.0 mg/L NAA in combination with 0.1 mg/L kinetin and at weeks 4 and 8 for roots cultured on MS media supplemented with 0.5 mg/L 2,4-D. The lowest MI value was observed on MS media supplemented with 0.5 mg/L 2,4-D, suggesting that the presence of this hormone in the culture medium gave significant reducing effect on MI of the cells. It has been reported that 2,4-D caused changes in mitotic activity, chromosome and chromatin structure as well as changes during the cell cycle (Pavlica *et al*., 1991). Mishiba *et al*., (2001) reported that the exogenous application of 2,4-D arrested the cell cycle at G2 phase in *Doritaenopsis* cells. On the other hand, a slightly higher concentration of kinetin (0.5 mg/L) produced significantly higher MI values, ranged from 4.44 ± 0.59 % to 7.77 ± 0.80 % compared to 3.87 ± 0.37 % to 6.72 ± 0.75 % at the concentration of 0.1 mg/L. It has been well documented that auxin and cytokinins participated in regulation of the cell cycle by controlling the activity of cyclin-dependent kinases.

In general, the addition of NAA, kinetin and 2,4-D in the culture media of *in vitro* grown *Oryza sativa* L. cv. MRQ 74 had no significant influenced on the chromosome number of this species. As presented in Table 9.3, the mean number of chromosomes slightly different within and between treatments. The results obtained from the present study revealed that the chromosome numbers of *in vivo* and *in vitro* grown *Oryza sativa* L. cv. MRQ 74 was 24.00 ± 1.15. It has been reported that the diploid rice (*Oryza sativa* L) has 24 chromosome number (2n = 24)(Cheng *et al.*, 2001).

Besides MI and chromosome counts, cell areas, nuclear areas, nuclear to cell area ratios of *in vivo* and *in vitro* grown *Oryza sativa* L. cv. MRQ 74 were also investigated. The mean cell areas of 4, 8 and 12 weeks of *in vitro* grown *Oryza sativa* L. cv. MRQ 74 on MS media supplemented with 1.0 mg/L NAA in combination with 0.1 mg/L kinetin were 220.20 ± 7.89 μm^2, 168.12 ± 5.40 μm^2 and 236.25 ± 7.99 μm^2, respectively (Table 9.2). The same pattern of fluctuation was observed for mean cell areas derived from

MS media supplemented with 1.0 mg/L NAA in combination with 0.5 mg/L kinetin. The mean cell areas at week 4 of culture was 190.26 ± 5.05 μm^2, followed by 169.91 ± 3.88 μm^2 and 235.93 ± 7.30 μm^2 at weeks 8 and 12, respectively. Even though the mean cell areas of these treatments were significantly higher than MS basal medium, however, it showed no significant difference as compared with the mean cell areas of *in vivo* grown *Oryza sativa* L. cv. MRQ 74, which was 191.00 ± 4.96 μm^2.

The mean nuclear areas of all treatments were also fluctuated with different culture period. The lowest mean nuclear areas was observed from MS basal media (44.18 ± 1.16 μm^2), followed by MS media supplemented with 0.5 mg/L 2,4-D which were 48.41 ± 2.04 μm^2 and 46.80 ± 1.33 μm^2 at weeks 4 and 12 of culture, respectively. On the other hand, MS media supplemented with 1.0 mg/L NAA in combination with 0.1 mg/L kinetin produced significantly higher nuclear areas at weeks 4 (71. 92 ± 3.12 μm^2) and 12 (76.26 ± 2.76 μm^2). It was also noted that the mean nuclear areas of *Oryza sativa* L. cv. MRQ 74 grown on MS media supplemented with 1.0 mg/L NAA in combination with 0.5 mg/L kinetin was significantly higher (74.85 ± 2.44 μm^2) at week 12 as compared with weeks 4 and 5 of culture, which were 56.35 ± 1.59 μm^2 and 53.33 ± 1.25 μm^2, respectively. Since the results showed the same pattern of fluctuation for cell and nuclear areas, this indicates a positive correlation between these two cytological parameters of *in vitro* grown *Oryza sativa* L. cv. MRQ 74. These results was supported by the results obtained by Jovtchev *et al*., (2006) whom reported a significant positive correlation between nuclear volume and cell volume of *Allium thaliana* and *Allium cepa*.

The ratio of mean nuclear to cell areas was also investigated in this study. In general, the ratio of mean nuclear to cell areas of *in vitro Oryza sativa* L. cv. MRQ 74 grown on MS media supplemented with 1.0 mg/L NAA in combination with 0.1 mg/L kinetin and MS media supplemented with 1.0 mg/L NAA in combination with 0.5 mg/L kinetin

ranged from 0.30 ± 0.01 μm^2 to 0.33 ± 0.01 μm^2. The highest mean nuclear to cell area ratio (0.34 ± 0.01 μm^2) was observed on MS media supplemented with 0.5 mg/L 2,4-D. Nuclear size is influenced by DNA content and it varies widely within a single plant due to DNA replication without intervening mitosis, or endoreduplication (Galbraith *et al*., 1991). Jovtcher *et al*., (2006) reported a linear relationship between DNA content (ploidy level) and nuclear volume. This indicates that there is a fixed amount of DNA that can be packaged per unit volume in the nucleus.

In the present study, no chromosomal aberrations was observed in all treatments. This might be due to low concentration of hormone presence in culture medium. Bairu *et al*., (2011) stated that media with low concentrations or totally absent of plant growth regulators are often give an advantage to cells of normal ploidy. On the other hand, the presence of high levels of BA (30 mg/L) greatly increased the genetic variability of rice callus cultures (Oono, 1985).

Mitotic cell division in multicellular organisms is important for growth and cell repair. The daughter cells produced in this process must undergo cell cycle i.e the period from the beginning of one division to the beginning of the next division. G_1 phase is the first of four phases of the cell cycle that takes place in mitotic cell division of eukaryotic. During this phase, the cell grows in size and synthesizes mRNA and proteins in preparation for subsequent steps. G_1 phase ends when the cell moves into the S phase of interphase. This phase begins with the replication of deoxyribonucleic acid (DNA), resulted in double DNA content. The cell enters the last phase in interphase (G2), after it completes the S phase. During G2 phase, protein synthesis increases in preparation for mitosis and cytokinesis. To ensure the proper division of the cell, there are control mechanisms known as cell cycle checkpoints. The first checkpoint is between G1 and S phases, also known as restriction point. The cell will pass this checkpoint if the cell size, quantity of energy, the presence of nucleotides

and growth factor, nutrient level and environment are in favourable condition. The second checkpoint (between G2 and mitosis) ensures that all DNA is replicated. Therefore, cells with damaged DNA are restricted from undergoing cell division until the damaged DNA is repaired. The third checkpoint (before anaphase) checks whether the chromosomes are paired properly, aligned at spindle fibres before anaphase begins. This mechanism closely controls the frequency of mitotic cell division.

In the present study, most of the root meristem cell nuclei of *Oryza sativa* L. cv. MRQ 74 grown *in vivo* were at G1 phase (62.75%), followed by S phase (24.16%) and G2 phase (12.75%). The results also showed that a small portion (1.34%) of the cell nuclei were polyploid (Table 8.4). On the other hand, the meristematic cell nuclei of *in vitro* grown *Oryza sativa* L. cv. MRQ 74 showed higher percentage of the cell with nuclear DNA content > 4.8 C value. The ploidy levels of root meristematic cell nuclei derived from MS basal media decreased with culture period. The highest percentage (8.66 %) was recorded on week 4 of culture, followed by 5.34 % and 4.67 % on weeks 8 and 12, respectively. In general, most of the nuclei were at G1 and S phase. In contrast, the ploidy levels of root meristematic cell nuclei of *Oryza sativa* L. cv. MRQ 74 grown on MS media supplemented with 1.0 mg/L NAA in combination with 0.1 mg/L kinetin and MS media supplemented with 1.0 mg/L NAA in combination with 0.5 mg/L kinetin fluctuated with the culture period. The highest percentage of ploidy level was observed on week 8 for both treatments (MS media supplemented with 1.0 mg/L NAA in combination with 0.1 mg/L kinetin and MS media supplemented with 1.0 mg/L NAA in combination with 0.5 mg/L kinetin). The results also indicate that 46.67 %, 46.00 % and 58.00 % of the meristematic cells derived from MS media supplemented with 1.0 mg/L NAA in combination with 0.5 mg/L kinetin were in S phase at weeks 4, 8 and 12, respectively.

Based on the results obtained, it can be said that the ploidy levels of *Oryza sativa* L. cv. MRQ 74 was significantly influenced by the presence of 0.5 mg/L 2,4-D in the culture media as compared to kinetin. There were no nuclei with DNA amount lower than 1.9C, whereas 22.0 % of the nuclei had DNA content higher than 4.8C, indicating high frequency of polyploid at week 4 of culture. However, the percentage of polyploid decreased after 8 weeks of culture (13.00%) and slightly higher after 12 weeks (18.00%). Similarly, most of the cell nuclei were at S phase at weeks 4, 8 and 12 of culture, which were 46.67 %, 51.33 % and 49.33 %, respectively. Polyploidy can naturally arise in a number of different ways. For example, chromosome doubling in meristematic cells can occur due to a disorder in mitosis. According to Ramsey and Schemske (1998), about 47-70 % of flowering plants are polyploidy in origin.

It has been well documented that phenotypic variation occurs in plant regeneration from cultured cells. In rice, phenotypically variant originating from seed-derived callus was first reported by Nishi *et. al*., (1968). Variation in number of tillers per plant, plant height, panicle length, fertility and the number of seeds produced are commonly observed in tissue –culture-derived plants (Jelodar *et al*., 2002; Nwauzoma and Jaja, 2013). This variation was observed in the present study. The agronomic parameters such as plant height, leaf length, the number of seeds per stalk of acclimatized plants (Chapter 8) were significantly lower as compared to *in vivo* plants. Besides variation in these parameters, cytological studies (Chapter 9) revealed that the percentage of polyploid cells in root tip meristem of *Oryza sativa* L. cv. MRQ 74 grown on MS media supplemented with 0.5 mg/L 2,4-D was also significantly higher (13.37 % to 22.00 %) as compared to *in vivo* (1.34 %).

The findings of the current study would be useful for plant breeders and molecular biologists in producing better quality aromatic rice since genetic transformation has been employed in creating of novel rice varieties. However, the success of this

technique could not be achieved without an efficient protocol on production of embryogenic calli and its subsequent regeneration into complete plantlets. Hence, the developed protocols for callus and somatic embryos induction in the present work could be used as a reference in producing better quality of aromatic rice.

CHAPTER 11

CONCLUSIONS

Tissue culture and cellular behaviour studies of *Oryza sativa* L. cv. MRQ 74 was successfully carried out in the present work. Even though, rice is one of the recalcitrant cereal crops with only a limited number of tissues suitable for *in vitro* culture and plant regeneration, an efficient protocol was completely developed for callus induction, somatic embryogenesis, *in vitro* regeneration, synthetic seeds production and acclimatization via stem explants. In order to get better understanding of this study, cellular behaviour of *in vivo* and *in vitro* grown *Oryza sativa* L. cv. MRQ 74 was also investigated and compared. From the current work, stem was found to be the most responsive explant. The stem explants responded well in callus induction and *in vitro* regeneration media, whilst, root explants only responded in callus induction medium. On the other hand, leaf explants did not give any response in both culture media.

It was observed that the quality and mean callus dry weight ultimately depend on the type and concentration of plant growth regulator supplemented to the culture medium. Even though the highest mean callus dry weight was observed on MS media supplemented with 0.5 mg/L 2,4-D from stem explants, the best quality of callus (creamy white, globular, friable) was recorded on MS media supplemented with 2,4-D and BAP. The stem explants produced significantly higher mean callus dry weight than that of root explants. It was also observed that the green plantlets regenerated from stem explants derived calluses, suggesting that the produced callus were embryogenic. This was proven by double staining method.

The addition of L-Proline at a concentration of 400 mg/L gave significantly positive effect on the formation of somatic embryos. The mean number of somatic embryos produced was 17.37 ± 0.66. All the developmental stages of somatic embryos (globular,

scutellar and coleoptilar) were clearly observed. Subsequently, microshoots development occurred and elongated to form complete plantlets.

The results obtained from *in vitro* regeneration protocol showed that the optimum regeneration media for this species was MS supplemented with 0.1 mg/L BAP in combination with 0.1 mg/L NAA with regard to the number of shoots (2.80 ± 0.23) and roots (25.33 ± 1.89) per explant. Thus, it can be suggested that the most effective hormones for *in vitro* regeneration of *Oryza sativa* L. cv. MRQ 74 were BAP in combination with NAA compared to kinetin in combination with NAA and BAP in combination with IBA. Direct *in vitro* regeneration was observed in all treatments for all types and concentrations of hormones either applied singly or in combinations and including hormone free treatment (control). All the cultures produced shoots regardless of the types and concentrations of hormones utilised.

Synthetic seeds production of *Oryza sativa* L cv. MRQ 74 was successfully achieved using microshoots. The presence of 30 g/L sucrose in encapsulation matrix gave the maximum survival rate whereby 100 % of the plantlets survived after 30 days of germination. It is interesting to note that the addition of 0.1 mg/L BAP and 0.1 mg/L NAA in encapsulation matrix did not influence the germination rate of the synthetic seeds. MS basal and MS media supplemented with 0.1 mg/L BAP were the most preferred sowing substrates for the synthetic seeds of this species. However, the viability of the synthetic seeds decreased significantly after two weeks and 100 % of the seeds failed to germinate after 45 days of storage at 4 ºC. Scanning electron microscopic (SEM) studies revealed that the plantlet leaves derived from stem explants cultured on MS media supplemented with 0.1 mg/L BAP in combination with 0.1 mg/L NAA had higher number of stomata on both surfaces. However, there was no significant difference between *in vivo* and *in vitro* grown plants in terms of stomata number on both

adaxial and abaxial leaf surfaces, indicating that no morphological changes had occurred throughout this study.

The mixture of black and red soil (1:1 ratio) was found to be the best growing substrate since 83.3 ± 0.07 % plantlets had survived after 8 weeks being acclimatized in the culture room. The X-Ray Fluorescence (XRF) analysis revealed that the black soil contains higher value of macronutrients (P, K, Ca, Mg and S) than that of the red soil. The survived plantlets managed to reach fruiting stage after 8 weeks being transferred to the green house. However, the plants had significantly lower number of leaves, leaf length, plant height and number of seeds per stalk as compared to *in vivo* grown plants. Histological studies of leaf and root of *in vivo, in vitro* and acclimatized plants showed normal leaf and root anatomy, except some abnormal cell division had occurred in the bundle sheath of *in vitro* leaf.

Cellular behaviour studies of *in vivo* and *in vitro* grown *Oryza sativa* L. cv. MRQ 74 were successfully investigated. Even though the presence of kinetin and 2,4-D in the culture media had significantly decreased the MI of root tip meristem cells of *Oryza sativa* L. cv. MRQ 74, these hormones had no significant influenced on the changes of chromosome number. As compared with kinetin, 2,4-D had significant effect on the ploidy levels of the cell nuclei (producing higher ploidy level).

For future work, aspects in physiological processes such as photosynthesis, stomata conductance and transpiration rate as well as protein synthesis should be determined to figure out the correlation between these processes and plant growth as a whole. Since rice production is strongly affected by climate change, some molecular work such as gene transformation should be carried out to produce better quality or rice and increase rice production. Furthermore, many rice cultivars are prone to drought and high temperature. Therefore, rice cultivar with drought tolerance is needed to cope with global warming.

REFERENCES

Aamir, A., Shagufta, N. and Javed, I. (2007). Effect of different explants and media composition for efficient somatic embryogenesis in sugarcane (*Saccharum officinalis*). *Pakistan Journal of Botany, 39*(6), 1877-1961.

Abdullah, M. Z., Mohamad, O., Azlan, S., Habibuddin, H., Saad, A., Hadzim, K. and Senawi, M. T. (2003). Rice breeding and germplasm exchange: The Malaysian perspective. Modern Rice Farming. *Proceeding of an International Rice Conference* 2003, 13- 16 October 2003, Alor Setar, Kedah, Malaysia.

Abdullah, R., Zainal, A., Heng, W. Y., Li, L. C., Beng, Y. C., Phing, L. M., Sirajuddin, S. A., Ping, W. Y. S. and Joseph, J. L. (2005). Immature embryo. A useful tool for oil palm (*Elaeis guineensis* Jacq.) genetic transformation studies. *Electronic Journal of Biotechnology*. *8*(1), 24-34.

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

Abe, M., Yoshikawa, T., Nosaka, M., Sakakibara, H., Sato, Y., Nagato, Y. and Itoh, J. (2010). WAVY LEAF1, an ortholog of Arabidopsis HEN1, regulates shoot development by maintaining microRNA and trans-acting siRNA accumulation in rice. *Plant Physiology*, *154*(3), 1335-1346.

Abolade, S., Yebanji, O. 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 Nerica cv. Faro 55. *African Journal of Plant Science*, *2*, 109-112.

Afshin, A. H., Kavini, B., Tarang, A. and Zanjani, S. B. (2011). Effect of different concentrations of kinetin on regeneration of (*'Matthiola incana'*). *Plant Omics*, *4*(5), 236-238.

Ahmed, E., U., Hayash,i T., and Yazawa, S. (2004). Auxins increase the occurrence of leaf-colour variants in *Caladium* regenerated from leaf explants. *Scientia Horticulturae*, *100*, 153-159.

Ahmad, S. and Mohd Razi, I. (2009). Deterministic model approaches in identifying and quantifying technological challenges in rice production and research and in predicting population, rice production and consumption in Malaysia. *Pertanika Journal of Tropical Agricultural Science, 32*(2), 267-291.

Ahmadabadi, M., Ruf, S., and Bock, R. (2007). A leaf-based regeneration and transformation system for maize (*Zea mays* L.). *Transgenic Research*, *16*, 437-448.

Ali, S., and Mizra, B. (2006). Micropropagation of rough lemon (*Citrus jambhiri* Lush.); Effect of explant type and hormone concentration. *Acta Botanica Croatica*, *65*(2), 137-146.

Amali P., Kingsley, S. J. and Ignacimuthu, S. (2014). Enhanced plant regeneration involving somatic embryogenesis from shoot tip explants of *Sorghum bicolour* (L. Moench). *Asian Journal of Plant Science and Research*, *4*(3), 26-34.

Amemiya, A., Akemine, H., and Toriy, K. (1956). Culture condition and growth of immature embryo in rice plant (studies on the embryo culture in rice plant. *Bulletin of the National Institute of Agricultural Science, 6*, 1-40.

Andarwulan, N., and Shetty, K. (1999). Phenolic content in differentiated tissue cultures of untransformed and agrobacterium-transformed roots of Anise (*Pimpinella anisum* L.). *Journal of Agricultural and Food Chemistry*, *47* (4), 1776-1780.

Ara, H., Jaiswal, U., and Jaiswal, V. S. (2000). Synthetic seed: Prospects and limitations. *Current Science*, *78* (12), 1438-1444.

Ashok, K. J. and Bashir, M. (2011). An efficient regeneration and plantlet development protocol from somatic tissues of *Plumbago zeylanica* L. *Journal of Pharmacy Research*, *4*(8), 2860-2863.

Asmah, H. N, Hasnida, H. N., Zaimah, N. A. N., Noraliz,a A. and Salmi, N. N. (2011). Synthetic seed technology for encapsulation and regrowth of *in vitro* derived *Acacia* hybrid shoot and axillary buds. *African Journal of Biotechnology*, *10*, 7820-7824

Bai, Y. and Qu, R. (2000). An evaluation on callus induction and plant regeneration of 25 turf-type tall fescue (*Festuca arundinacea* Schreb.) cultivars. *Grass Forage Science*, *55*, 326-330.

Bairu, M., W., Fennell, C., W., Van Staden, J. (2006). The effect of plant growth regulators on somaclonal variation in Cavendish banana (*Musa* AAA cv. 'Zelig'). *Scientia Horticulturae*, *108*, 347-351.

Bairu, M. W., Aremu, A. O. and Staden, J. V. (2011). Somaclonal variation in plants: causes and detection methods. *Plant Growth Regulators*, *63*, 147-173

Bano, S., Jabeen, M., Rahim, F., I and llahi, I. (2005). Callus induction and regeneration in seed explants of Rice (*Oryza sativa* cv. Swat-ll). *Pakistan Journal of Botany*, *37*, 829-836.

Barik, D. P., Naik, S. K., Mudgal, A., and Chand, P. K. (2007). Rapid plant regeneration through *in vitro* axillary shoot proliferation of butterfly pea (*Clitoria ternatea* L.) – a twinning legume. *In Vitro Cellular and Developmental Biology – Plant*, *43*, 144-148.

Bapat, V. A., and Rao, P. S. (1990). *In vivo* growth of encapsulated axillary buds of mulberry (*Morus indica* L.). *Plant Cell Tissue and Organ Culture*, *20*(1), 69-70.

Baskaran, S., and Smith, R. H. (1990). Regeneration in cereal tissue culture: A review. *Crop Science*, *30*, 1328-1336.

Beena, M. R., Winter, S., and Makeshkumar, T. (2014). Influence of age of explants and genotype on somatic embryogenesis in African and Indian Cassava cultivars. *Journal of Root Crops, 40*(2), 21-27.

Bhojwani, S. S., and Rajdan, M. K., (2004). Plant tissue culture: theory and practice, a revised Edition, Elsevier Publication, Netherland, 47- 48.

Blinstrubiene, A., Sliesaravicius, A., and Burbulis, N. (2004). Factors affecting morphogenesis in tissue culture of linseed flax (*Linum isitatissimum* L.). *Acta Universitatis Latviensis, Biol*ogy, *676*, 149-152.

Boissot, N., Valdez, M., and Guiderdoni, E. (1990). Plant regeneration from leaf and seed-derived calli and suspension cultures of the African perennial wild rice, *Oryza longistaminata*. *Plant Cell Reports*, *9*, 447-450.

Borthakur, A., Das, S. C., Kalita, M. C., and Sen, P. (2012). An *in vitro* plant regeneration system for conservation of the leguminous tree *Albizzia chinensis* (Osbeck) Merr. *Advances in Applied Science Research*, *3*(3), 1727-1732.

Bouman, B. A. M., Yang, X. G., Wang, H. G., Wang, Z. M., Zhao, J. F. and Chen, B. (2006). Performance of temperate rice varieties under irrigated aerobic conditions in North China. *Field Crops Research*, *98*, 53-65.

Bradley, D. E., Bruneace, A. H., and Qu, R. (2001). Effect of cultivar, explants treatment and medium supplements on callus induction and plantlets regeneration in perennial rye grass. *International Turf Grass Society Research Journal, 9*, 152-156.

Canli, F.A., and Tian, L., (2008). *In vitro* shoot regeneration from stored mature cotyledons of sweet cherry (*Prunus avium* L.) cultivars. *Scientia Horticulturae*, *116*, 34–40.

Capuano, G., Piccioni, E., and Standardi, A. (1998). Effect of different treatments on the conversion of M.26 apple rootstock synthetic seeds obtained from encapsulated apical and axillary micropropagated buds. *Journal of Horticultural Science and Biotechnology*, *73*, 299–305.

Carle, S. A., Bates, G.W. and T.A. Shannon, T. A. (1998). Hormonal control of gene expression during reactivation of the cell cycle in Tabacco mesophyll protoplasts. *Journal of Plant Growth Regulators*, *17,* 221-230.

Carmen, V. A., Michael E. K, Sandra, B. W., and Nancy L. P., (2008). Comparative growth, morphology, and anatomy of easy- and difficult-to-acclimatize Sea Oats (*Uniola paniculata*) genotypes during *in vitro* culture and *ex vitro* acclimatization. *Journal of American Society of Horticultural Science, 133*(6), 830–843.

Chakravarthi Dhavala V. N., Tejeswara, R. D., Yechuri, V. R., and Prabavathi, K. (2009). Effect of explant age, hormones on somatic embryogenesis and production of multiple shoot from cotyledonary leaf explants of *Solanum trilobatum* L. *African Journal of Biotechnology*, *8*(4), 630-634.

Chamhuri, S., Nor Diana, M, I., Muhammad, Y., and Golam, M. (2014). Issues and challenges facing rice production and food security in the granary areas in the East Coast Economic Region (ECER), Malaysia. *Research Journal of Applied Sciences, Engineering and Technology, 7*(4), 711-722.

Chan, C. S., Zainudin, H., Saad, A. and Azmi, M. (2012). Productive water use in aerobic rice cultivation. *Journal of Tropical Agriculture and Food Science*. *40*(1), 117-126.

Chand, S., and Sahrawat, A. K. (1997). Somatic embryogenesis and plant regeneration from coleoptiles tissues of indica rice. *Rice Biotechnology*, *32*, 19-20.

Charriere, F., Sotta, B., and Migniac, E. (1999). Induction of adventitious shoots or somatic embryos on *in vitro* culture. *Plant Physiology and Biochemistry, 37*(10), 752-757.

Chaudhury, A. and Qu, R. (2000). Somatic embryogenesis and plant regeneration of turf type bermuda grass: Effect of 6-benzyladenine in callus induction medium. *Plant Cell Tissue and Organ Culture, 60*, 113-120.

Chen, Z., Zhuge, Q,. and Sundqvist C. (1995). Oat leaf base: tissue with an efficient regeneration capacity. *Plant Cell Reports*, *14*, 354-358.

Cheng, Z. K., Yan, H., Yu, H., Tang, S., Jiang, J., Gu, M. H., and Zhu, L. (2001). Development and applications of a complete set of rice telotrisomics. *Genetics*, *157*, 361–368.

Cheong, E., J, and Pooler, M., R. (2004). Factors affecting somatic embryogenesis in *Prunus incisa* cv. February Pink. *Plant Cell Reports*, *22*, 810-815.

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, Q. R., Cao, H. X. and Linscombe, S. D. (1997). A novel medium for induction of embryogenic callus in rice anther culture of Southern US. Crosses. *Rice Biotechnology, 32*, 1-19

Claparols, I., Santos, M. A. and Torne, J. M. (1993). Influence of some exogenous amino acids on the production of maize embryogenic callus and on endogenous amino acid content. *Plant Cell Tissue and Organ Culture*, *34*, 1–11.

Cocu, S., Uranbey, S., Ipek, A., Khawar, K. M., Sarihan, E. O., Kaya, M. D., Parmaksiz, I., and Ozcan, S. (2004). Adventitious shoot regeneration and micropropagation in *Calendula officinalis* L. *Biologia Plantarum*, *48*, 449-451.

Conger, A. D., and Fairchild, L. M. (1953). A quick freeze method for making smear slide permanent. *Biologia Plantarum*, *28*, 281-283.

Conner, A.J. and Thomas, M.B. (1982). Re-establishing plantlets from tissue culture: a review. *International Plant Propagation Society*, *31*, 342-357.

Cunha, A., and Fernandes-Ferreira, M. (1999). Influence of medium parameters on somatic embryogenesis from hypocotyls explants of flax (*Linum usitatissinum* L.). *Journal of Plant Physiology*, *155*, 591-597.

Daniela, G. S., Lucimeire, S. M. L., Antonio, D. S. S and Fernada, V. D. S. (2013). Somatic embryogenesis of *Neoglaziovia variegata* (Arruda) Mez, an important source of fiber from Native Brazilian Bromeliads. *Brazilian Archives of Biology and Technology*, *56*(4), 547-555.

Datta, S. K., Datta, K., Soltinafar, N., Donn, G. and Potrykus, I. (1992). Herbicide resistant indica rice plants from IRRI breeding line IRRI after PEGmediated transformation of protoplast. *Plant Molecular Biology*, *20*, 619-629.

Deb, C. R. and Imchen, T. (2010). An efficient *in vitro* hardening technique of tissue culture raised plants. *Biotechnology*, *9*(1), 79-83.

Delporte, F., Pretova, A., Jardin, D. U., and Watillon, B. (2014). Morpho-histology and genotype dependence of *in vitro* morphogenesis in mature embryo cultures of wheat. *Protoplasma*, *251*(6), 1455–1470.

Deverno, L. L. 1995. An evaluation of somaclonal variation during somatic embryogenesis. In S. Jain, P. Gupta, and R. Newton (Eds.). *Somatic embryogenesis in woody plants*. (pp. 361-377). Dordrecht: Kluwer Academic Publishers.

Dogara, A. M., and Jumare, A. I. (2014). Origin, distribution and heading date in cultivated rice. *International Journal of Plant Biology and Research*, *2*(1), 1008.

Donnelly, D.J., Skelton, F.E. and Daubeny, H.A. (1986). External leaf features of tissue cultured 'Silvan' blackberry. *Horticultural Science, 21*, 306-308.

Duncan, R.R., (1997). Tissue culture-induced variation and crop improvement. *Advances in Agronomy*, *58*, 201-204.

Espinosa, A. C., Pijut, P. M., and Michler, C. H., (2006). Adventitious shoot regeneration and rooting of *Prunus serotina in vitro* cultures. *Hortscience, 41* (1), 193–201.

Faisal, M., Ahmad, N and Anis, M. F. (2005). Shoot multiplication in *Rauvolfia tetraphylla* L. using thidiazuron. *Plant Cell Tissue and Organ Culture, 80* (2), 87-190.

Faiz, A. J., and Muhammad, S. K. (2012). Reproducible and expedient rice regeneration system using *in vitro* grown plants. *African Journal of Biotechnology*, *11*(1), 138-144.

FAO. (2015). Rice Marker Monitor. *15*(2), 1-35.

Fauziah, I. A., Nur Shafiqoh, J. and Alina, W. (2013). Effect of 2,4-D on embryogenic callus induction of Malaysian indica rice (*Oryza sativa* L.) cultivars MR123 and MR127. *Jurnal Teknologi (Science & Engineering)*, *64*(2), 75-78.

Fitzgerald, M. A., Rahman, S., Resurreccion, A. P., Conception, J., Daygon, V. D., Dipti, S. S., Kabir, K. A., Klinger, B., Morell, M. K and Bird, A. R. (2011). Identification of a major genetic determinant of glycaemic index in rice. *Rice*, *4*(2), 66-74.

Franklin, G., Arvinth, S., Sheeba, C. J., Kanchana, M., and Subramonian, N. (2006). Auxin pretreatment promotes regeneration of sugarcane (*Saccharum* spp. hybrids) midrib segment explants. *Journal of Plant Growth Regulation, 50*, 111-119.

Fujiwara, A., and Ojima, K. (1955). Physiological studies of plant roots (part I).Influence of some environment conditions on growth of isolated roots of rice and wheat. *Journal of the Science of Soil and Manure, 28*, 9-12.

Gairi, A., and Rashid, A. (2004). Direct differentiation of somatic embryos on different regions of intact seedlings of *Azadirachta* in response to thidiazuron. *Journal of Plant Physiology*, *161*, 1073-1077.

Galbraith, D. W., Harkins, K. R. and Knapp, S. (1991). Systemic endopolyploidy in *Arabidopsis thaliana*. *Plant Physiology*, *96*, 985-989.

Gamborg, O. L.(1970). The effects of amino acids and ammonium on the growth of plant cells in suspension cultures. *Plant Physiology, 45*, 372-373.

Ganapathi, T. R., Suprasanna, P., Bapat, V. A., and Roa, P. S. (1992). Propagation of banana through encapsulated shoot tips. *Plant Cell Reports*, *11*(11), 571-575.

Genovesi, A. D., and Magill, C. W. (1982). Embryogenesis in callus derived from rice microspores. *Plant Cell Reports*, *6*, 257-260.

Gless, C., Lorz, H, and Jahne-Gartner, A. (1998). Establishment of a highly efficient regeneration system from leaf base segments of oat (*Avena sativa* L.). *Plant Cell Reports*, *17*, 441-445.

Golam, G. M. C., Ashrafuzzaman, M., Raihan, F., and Shamsul, H. P. (2012). Comparative study on regeneration potentiality of aromatic indica rice (*Oryza sativa* L.) of Bangaldesh. *Research Journal of Biology, 2*(3), 98-103.

Gomes, F., Simoes, M., Lopes, M. L. and Canhoto, M. (2010). Effect of plant growth regulators and genotype on the micropropagation of adult trees of *Arbutus unedo* L. (strawberry tree). *New Biotechnology*, *27*, 882-892.

Gonzalez, R. G., Sanchez, D. S., Campos, J. M., Vazquez, E. P., Guerra, Z., Quesada, A. Q., Valdivia, R. M., and Gonzalez, M. G. (1999). Plant regeneration from leaf and stem explants from two sweet potato (*Ipomoea batatas* L. Lam.) cultivars. *Biotechnologia Aplicada*, *16*, 11-14.

Gray, D. J. (1987). Synthetic seed technology for the mass cloning of crop plants: problems and prospects. *Horticultural Science*, *22*, 795-814.

Grimes, H. D. and Hodges, T. K. (1990). The inorganic NO_3 : NH_4 ratio influences 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.

Gubis, J., Lajchová, Z., Fragó, J., and Jureková, Z. (2003). Effect of explant type on shoot regeneration in tomato (*Lycopersicon esculentum* Mill.) *in vitro. Czech Journal of Genetics and Plant Breeding*, *39*(1), 9-14.

Habibi, N., Suthar, R. K. and Purohit, S. D. (2009). Role of PGRs and inhibitors in induction and control of somatic embryogenesis in *Themeda quadrivalvis*. *Indian Journal of Experimental Biology*, *47*, 198-203.

Haliloglu, K. (2006). Efficient regeneration system from wheat leaf base segments. *Biologia Planta*, *50*, 326-330.

Hashem, A. D. and Kaviani, B. (2010). *In vitro* proliferation of an important medicinal plant Aloe - a method for rapid production. *Australian Journal of Crop Science*, *4*(4), 216-222.

Hazarika, B. N. (2006). Morpho-physiological disorders in *in vitro* culture of plants. *Scientia Horticulturae*, *108*, 105-120.

Henry R. J. (1998). Molecular and biochemical characterization of somaclonal variation. In S. M. Jain, D. S. Brar, D. S. Ahloowalia (Eds.). *Somaclonal Variation and Induced Mutations in Crop Improvement*. (pp. 485-499). Dordrecht: Kluwer Academic Publisher.

Holme, I, B., Krogstrup, P., and Hansen, J. (1997). Embryogenic callus formation, growth and regeneration in callus and suspension cultures of *Miscanthus x ogiformis* Honda Giganteus as affected by proline. *Plant Cell Tissue and Organ Culture, 50*, 203-210.

Hoque, M. E. (2010). *In vitro* tuberization in potato (*Solanum tuberosum*) L.). *Plant Omics Journal*, *3*(1), 7-11.

Hoque, M. E., and Mansfield, J. W. (2004). Effect of genotype and explants age on callus induction and subsequent plant regeneration from root-derive callus of indica rice genotypes. *Plant Cell Tissue and Organ Culture*, *78*, 217-223.

Hronkova, M., Zahradnickova, H., and Simkova, M. (2003) The role of abscisic acid in acclimation of plants cultivated *in vitro* to ex vitro conditions. *Biologia Plantarum, 46*, 535-541.

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.

Huetteman, C. A., and Preece, J. E. (1993). Thidiazuron-A potent cytokinin for woody plant-tissue culture. *Plant Cell Tissue and Organ Culture*, *33*(2), 105-119.

Husen, A., and Pal, M. (2007). Metabolic changes during adventitious root primordium development in *Tectona grandis* Linn. f. (teak) cuttings as affected by age of donor plants and auxins (IBA and NAA) treatment. *New Forests*, *33*(3), 309-323.

Ismail, N., Uzma, R., Madan, M. S., and Amla, B. (2011). Influence of plant growth regulators on *in vitro* shoot regeneration via cotyledonary node in *Clitoria ternatea* L. *International Journal of Pharmaceutical Sciences and Research*. *2*(3), 552-557.

Israeli, Y., Reuveni, O. and Lahav, E. (1991). Qualitative aspects of somaclonal variations in banana propagated by *in vitro* techniques. *Scientia Horticulturae*, 48, 71-88.

Jafari, N. A. and Hamidoghli, Y. (2010). Micropropagation of thorn less trailing blackberry (*Rubus* sp.) by axillary bud explants. *Australian Journal of Crop Science*, *3*(4), 191-194.

Jelodar, N. B., Davey, M. R., and Cocking, E. C. (2002). Plant regeneration from cell suspension protoplasts of two Iranian Japonica rice cultivars and ploidy level of regenerated plants. *Journal of Agricultural Science and Technol*ogy, *4*, 141-149.

Jennings, P. R., Coffman, W. R. and Kauffman, H. E. (1979). Rice improvement. *International Rice Research Institute*, Los Banos, Philippines, 1-186.

Jiang, H., Chen, J., Gao, X. L., Wan, J., Wang, P. R., Wang, X. D., and Xu, Z. J. (2006). Effect of ABA on rice callus and development of somatic embryo and plant regeneration. *Acta Agronomica Sinica, 32*, 1379-1383.

Jiménez, V. (2001). Regulation of *in vitro* somatic embryogenesis with emphasis on the role of endogenous hormones. *Brazilian Journal of Plant Physiology, 13*, 196-223.

Jiménez, V. M. (2005). Involvement of plant hormone and plant growth regulators on *in vitro* somatic embryogenesis. *Plant Growth Regulation*, *47*, 91-110.

Jones, M. P. A, Yi, Z., Murch, S. J., and Saxena, P. K. (2007). Thidiazuron induced regeneration of *Echinacea purpurea* L. micropropagation in solid and liquid culture systems. *Plant Cell Report*s, *26*, 13-19.

Jordan, M., Velozo, J. and Sabja, A. M. (1996). Organogenesis *in vitro* of *Nothofagus alpina* (P.et E.) Oerst. Fagaceae. *Plant Cell Reports, 15*, 795-798.

Josefina, O. N., and Kazumi, H. (2010). Genotypic differences in morphology and ultrastructures of callus derived from selected rice varieties. *Phillippine Science Letters, 3*(1), 145-160.

Joyia, F. A., and Khan, M. S. (2012). Reproducible and expedient rice regeneration system using *in vitro* grown plants. *African Journal of Biotechnology*, *11*(1), 138-144.

Jovtchev, G., Schuber,t V., Meister, A., Barow, M. and Schubert, I. (2006). Nuclear DNA content and nuclear and cell volume are positively correlated in angiosperms. *Cytogenetic Genome Research*, *114*, 77-82.

Karim, R., Ahmed, U., Krishna Roy, U., Ara, T., Islam, R., and Hossain, M. (2015). Varietal improvement of strawberry (*Fragaria* x *ananassa* Dutch.) through

somaclonal variation using *in Vitro* techniques. *Journal of Agricultural Science and Technology, 17*, 977-986.

Khan, M. I., Ahmad, N., and Anis, M. (2011). The role of cytokinins on *in vitro* shoot production in *Salix tetrasperma* Roxb: a tree of ecological importance. *Trees, 25*, 577-584.

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

Kim, S. G., Kim, K. W., Park, E. W. and Choi, D. (2002). Silicon-induced cell wall fortification of rice leaves: A possible cellular mechanism of enhanced host resistance to blast. *Phytopathology*, *92*, 1095-1103.

Koetje, D. S., Grimes, H. D., Wang, Y. C., and Hodges, T. K., (1989). Regeneration of indica rice (*Oryza sativa* L.) from primary callus derived from immature embryos. *Journal of Plant Physiology, 35*, 184-190.

Konstantin, V. K., Iraida, N. T., Nataliay, V. O., Maria, E. P., Olga, V. K., and Eugene, A. V. (2014). Somaclonal variation of haploid *in vitro* tissue culture obtained from Siberian larch (*Larix sibirica* Ledeb.) megagametophytes for whole genome de novo sequencing. *In Vitro Cellular and Developmental Biology – Plant, 50*, 655-664.

Kopertekh, L. G. and Stribnaya, L. A. (2003). Plant regeneration from wheat leaf explants. *Russian Journal of Plant Physiology*, *50*(3), 365-368.

Kumar, M. B. A., Vakeswaran, V., and Krishnasamy, V. (2005). Enhancement of synthetic seed conversion to seedlings in hybrid rice. *Plant Cell Tissue and Organ Culture, 81*, 97-100.

Kumar, M., Singh, S., and Singh, S. (2011). *In vitro* morphogenesis of a medicinal plant – *Aloe vera* L. *Asian Journal of Plant Science and Research*, *1*(1), 31-40.

Lee, K., Jeon, H. and Kim, M. (2002). Optimization of 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.

Lee, K. W., Chinzorig, O., Choi, G. J., Kim, K. Y., Ji, H. C., Park, H. S., Kim, W. H. and Lee, S. H. (2012). Factors influencing callus induction and plant regeneration of Dahurian wildrye grass (*Elymus dahuricus* L.). *African Journal of Biotechnology*, *11*(4), 815-820.

Lee, Y. S., Yang, T. J., Park, S. U., Baek, J. H., Wu, S. Q., and Lim, K. B. (2011). Induction and proliferation of adventitious roots from *Aloe vera* leaf tissues for *in vitro* production of aloe-emodin. *Plant Omics Journal*, *4*(4), 190-194.

Lema-Ruminska, J., Goncerzewicz, K. and M. Gabriel, M. (2013). Influence of abscisic acid and sucrose on somatic embryogenesis in Cactus *Copiapoa tenuissima* Ritt. forma *mostruosa. The Scientific World Journal*, 1-6.

Li, R., Bruneau, A. H. B., and Qu, R. (2010). Tissue culture-induced morphological somaclonal variation in St. Augustine grass [*Stenotaphrum secundatum* (Walt.) Kuntze]. *Plant Breeding, 129*, 96-99.

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

Ling, A. P. K., Tan, K. and Sobri, H. (2013). Comparative effects of plant growth regulators on leaf and stem explants of *Labisia pumila* var. *Alata*. *Journal of Zhejiang University Science*, *14*(7), 621-631.

Lisek, A. and Orlikowska, T. (2004). *In vitro* storage of strawberry and raspberry in calcium-alginate beads at 4°C. *Plant Cell Tissue and Organ Culture*, *78*, 167-172.

Lu, C. H., Lee, P. D., and Su, J. C. (1995). Preparation of amyloplasts from sweet potato callus culture. *Botanical Bulletin Academia Sinica, 36*, 223-228.

Ludwig-Müller, J., Vertocnik, A., and Town, C. D. (2005). Analysis of indole-3-butyric acid-induced adventitious root formation on *Arabidopsis* stem segments. *Journal of Experimental Botany, 56*(418), 2095-2105.

Lulsdorf, M. M., Tautorus, T. E., Kikcio, S. I., Bethune, T. D., and Dunstan, D. I. (1993). Germination of encapsulated embryos of interior spruce (*Picea glauca engelmannii* complex) and black spruce (*Picea mariana* Mill.). *Plant Cell Reports*, *12*(7), 385-389.

Malgorzatta, D. G. (2004). Factors influencing somatic embryogenesis induction and plant regeneration with particular reference to *Arabidopsis thaliana* (L.) Heynh. *Plant Growth Regulation*, *43*, 27-47.

Malik, K. A., and Saxena, P. K. (1992). *In vitro* regeneration of plants: A novel approach. *Naturwissenschaften*, *79*, 136-137.

Mamidala, P. and Nanna, R. S. (2009). Efficient *in vitro* plant regeneration, flowering and fruiting of dwarf tomato cv. Micro-Msk. *Plant Omics Journal*, *2*(3), 98-102.

Mandal,A. B., Aparna, M. and Anusrita, B. (2003). Somatic embryogenesis in root derived callus of indica rice. *Plant Tissue Culture. 13*(2), 125-133.

Martin, K., Pachathundikandi, S., Zhang, C., Slater, A., and Madassery, J. (2006). RAPD analysis of a variant of banana (*Musa* sp.) cv. Grande naine and its

propagation via shoot tip culture. *In Vitro Cellular Developmental Biology-Plant*, *42*, 188-192.

Masuda, K., Kudo-Shiratori, A. and Inoue, M. (1989). Callus formation and plant regeneration from rice protoplasts purified by density gradient centrifugation. *Plant Science*, *62*, 237-246.

Mathur, J., Ahuja, P. S., Lal, N., and Mathur, A. K. (1989). Propagation of *Valeriana wallichii* DC using encapsulated apical and axial shoot buds. *Plant Science*, *60*(1), 111-116.

Meneses, A., D. Flores, M. Muñoz, G. A. and Espinoza, A. M. (2005). Effect of 2,4-D, hydric stress and light on indica rice (*Oryza sativa*) somatic embryogenesis. *Revista de Biologia Tropical, 53*, 361-368.

Mikami, T., and Kinoshita, T. (1988). Genotypic effects on the callus formation from different explants of rice, *Oryza sativa* L. *Plant Cell Tissue and Organ Culture*, *12*, 311-314.

Mishiba, K, I., Okamoto, T., and Mii, M**.** (2001). Increasing ploidy level in cell suspension cultures of *Doritaenopsis* by exogenous application of 2,4-dichlorophenoxyacetic acid. *Physiologia Plantarum*, *112*(1), 142-148.

Mithila, J., Hall, J. C., Victor, J. M. R., and Saxena, P. K. (2003). Thidiazuron induces shoot organogenesis at low concentrations and somatic embryogenesis at high concentrations on leaf and petiole explants of African violet (*Saintpaulia ionantha* Wendl.). *Plant Cell Reports, 2*(15), 408-414.

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*). *Euphytica, 112*(2), 151-156.

Mohanty, S., Panda, M., Subudhi, E., and Nayak, S. (2008). Plant regeneration from callus culture of *Curcuma aromatica* and *in vitro* detection of somaclonal variation through cytophotometric analysis. *Biologia Plantarum*, *52*, 783-786.

Mohebodini, M., Javaran, M. J., Freidoon Mahboudi, F., and Alizadeh, H. (2011). Effects of genotype, explant age and growth regulators on callus induction and direct shoot regeneration of Lettuce (*Lactuca sativa* L.). *Australian Journal of Crop Science*, *5*(1), 92-95.

Molina, D., Aponte, M., Cortina, H and Moreno. G. (2002). The effect of genotype and explant age on somatic embryogenesis of coffee. *Plant Cell Tissue and Organ Culture*, *71*, 117-123.

Morita, M., Xing, X. H., and Unno, H. (1999). Synchronized shoot regeneration of rice (*Oryza sativa* L.) calli on solid medium by adjustment of intracellular 2, 4 - dichlorophenoxyacetic acid concentration. *Plant Cell Reports*, *18*, 633-639.

Moura, D. S., Zapata-Arias, F. J., Ando, A. and Neto, A. T. (1997). Plant regeneration from protoplast isolated from primary calli using mature embryos of two Brazilian rice cultivars. *Euphytica*, *94*, 1-5.

Mroginski, E., Rey, H. Y., Gonzalez, A. M., and Mroginski, L. A. (2004). Thidiazuron promotes *in vitro* plant regeneration of *Arachis correntina* (Leguminosae) via organogenesis. *Plant Growth Regulation*, *23*, 129-134.

Murashige, T., and Skoog, F. (1962). A revised medium for rapid growth and bioassay with tobacco. *Physiologia Plantarum*, *15*, 473-497.

Murthy, B. N. S., Murch, S. J., and Saxena, P. K. (1998). Thidiazuron: A potent regulator of *in vitro* plant morphogenesis. *In Vitro Cellular and Developmental Biology – Plant*, *34*, 267-275.

Mutasim, M. K., and Kazumi, H. (1999). A combination of thidiazuron and benzyladenine promotes multiple shoot production from cotyledonary node explants of faba bean (*Vicia faba* L.). *Plant Growth Regulation*, *27*, 145-148.

Namasivayam, P. (2007). Acquisition of embryogenic competence during somatic embryogenesis. *Plant Cell Tissue and Organ Culture*, *90*, 1-8.

Nandagopal, S. and Ranjitha Kumari, B. D. (2007). Effectiveness of auxin induced *in vitro* root culture in chicory. *Journal of Central European Agriculture*, *8* (1), 73-80.

Napasintuwong, O. (2012). *Survey of Recent Innovations in Aromatic Rice*. Paper prepared for presentation at the 131st EAAE Seminar 'Innovation for Agricultural Competitiveness and Sustainability of Rural Areas', Prague, Czech Republic, 1-20.

Nas, M.. N., Bolek, Y. and Sevgin, N. (2010). The effects of explant and cytokinin type on regeneration of *Prunus microcarpa*. *Scientia Horticulturae*, *126*, 88-94.

Nwauzoma, A,. B. and Jaja, E., T. (2013). A review of somaclonal variation in plantain (Musa spp): mechanisms and applications. *Journal of Applied Biosciences, 67*, 5252-5260.

Nicuţă, D., , Ghiorghiţă, D. and Maftei, D. E. (2012). Cytogenetic observations on some *in vitro* regenerants provided by ovaries of *Brassica oleracea* L. Var. Capitata. *Biologie Vegetală*, *58*, 2, 31-46.

Nishi, T., Yamada, Y., and Takahashi, E. (1968) Organ redifferentiation and plant restoration in rice callus. *Nature*, *219*, 508-509

Nowak, B. K., Miczynski, K., and Hudy, L. (2004). Sugar uptake and utilization during adventitious bud differentiation on *in vitro* leaf explant of Wegierka

zwykla plum (*Prunus domestica*). *Plant Cell Tissue and Organ Culture, 76*, 255-260.

Oono, K. (1985). Putative homozygous mutations in regenerated plants of rice. *Molecular and General Genetics*, *198*, 377-384.

Osuga, K.; Masuda, H. and Komamine, A. (1999). Synchronization of somatic embryogenesis at high frequency using carrot suspension cultures: model systems and application in plant development. *Methods in Cell Science*, *21*, 129-140.

Pandeya, K., Tiwari, K. N., Singh, J., Verma, J. P. and Dubey, S. D. (2010). *In vitro* propagation of *Clitoria ternate* L: A rare medicinal plant. *Journal of Medicinal Plants Research. 4*(8), 664-668.

Pati, R., Mishra, M., Chandra, R., and Muthukumar, M. (2013). Histological and biochemical changes in *Aegle marmelos* Corr. before and after acclimatization. *Tree Genetics and Molecular Breeding*, *3*(3), 12-18.

Patnaik, S.K., Sahoo, Y and Chand, P. K. (1995). Efficient plant retrieval from alginate-encapsulated vegetative buds of mature mulberry trees. *Scientia Horticulturae*., 61: 227-239.

Paul Vincent, R. (2010). Prospects for rice production in Sarawak. *Proceedings of the National Rice Conference 2010*, Damai Laut, Perak, 213-241.

Pavlica, M., Papes, D., and Nagy, B. (1991). 2,4-Dichlorophenoxyacetic acid causes chromatin and chromosome abnormalities in plant cells and mutation in cultured mammalian cells. *Mutation Research*, *263*(2), 77-81.

Pescador, R., Kerbauy, G.B., Vivian, I. D., and Kraus, J. E. (2008). Anomalous somatic embryos in *Acca sellowiana* (O. Berg) Burret (Myrtaceae). *Revista Brasileira de Botania, 31*, 155-164.

Pilet, P. E. (1991). Root growth and gravireaction. In Y. Waisel, Eshel, A., and Kafkafi, U. (Ed.), *Plant roots, the hidden half* (pp. 179-204). New York: Marcel Dekker.

Pinto, G., Santos, C., Neves, L., and Araujo, C. (2002). Somatic embryogenesis and plant regeneration in *Eucalyptus. globules*. Labill. *Plant Cell Reports*, *21*, 208-213.

Popescu, A., N., Isac, V., S., and Coman, M., S. (1997). MSR Somaclonal variation in plants regenerated by organogenesis from callus cultures of strawberry (*Fragaria ananassa*). *Acta Horticulturae*, *439*, 89-96.

Pospíšilová, J., Synková, H., Haisel, D., and Semorádová, Š. (2007). Acclimation of plantlets to *ex vitro* conditions: Effects of air humidity, irradiance, CO_2 concentration and abscisic acid (a Review), *Acta Horticulturae*, *748*, 29-38.

Pospisilova, J., Haisel, D., Synkova, H., and Batkova-Spoustova, P. (2009) Improvement of *ex vitro* transfer of tobacco plantlets by addition of abscisic acid to the last subculture. *Biologia Plantarum, 53*, 617-624.

Puhan, P and Siddiq, E. A., (2013). Protocol optimization and evaluation of rice varieties response to *in vitro* regeneration. *Advances in Bioscience and Biotechnology, 4*, 647-653.

Qin, Y. H., Zhang, S. L, Zhang, L. X., Zhu, D. Y., and Syed, A. (2005). Response of *in vitro* strawberry to silver nitrate ($AgNO_3$). *Horticultural Science*, *40*(3), 747-751.

Quiroz-Figueroa, F.R., Fuentes-Cerda, C. F. J., Rojas-Herrera, R., and Loyola-Vargas, V. M. (2002). Histological studies on the developmental stages and differentiation of two different somatic embryogenesis systems of C*offea arabica*. *Plant Cell Reports, 20*, 1141-1149.

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

Rai, M. K., Jaiswal, V. S., and Jaiswal, U. (2008). Effect of ABA and sucrose on germination of encapsulated somatic embryos of guava (*Psidium guajava* L.). *Scientia Horticulturae*, *117*(3), 302-305.

Ramakrishnan, K., Gnanam, R., Sivakumar, P., and Manickam, A. (2005). *In vitro* somatic embryogenesis from cell suspension cultures of cowpea (*Vigna unguiculata* L. Walp). *Plant Cell Reports*, *24*(8), 449-461.

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*(1), 68-74.

Ramsey, J., and Schemske, D. W. (1998). Pathways, mechanisms, and rates of polyploid formation in flowering plants. *Annual Review of Ecology and Systematics, 29*, 467-501

Rashid, H., Toriyama, A., Qurashi, K., Hinta, and Malik, K. A. (2000). An improved method for shoot regeneration from calli of indica rice. *Pakistan Journal of Biological Sciences*, *3*(12), 2229-2231.

Rashidun, N. (2012). Callus induction ability and plant regeneration potentiality of local varieties of rice. *Genetics and Plant Breeding*, 1-57.

Rathore, J. S., Rathore, V., Shekhawat, N. S., Singh, R. P., Lilar, G., Phlwaria, M., and Dagla, H. R. (2004). *Plant Biotechnology and Molecular Markers.* (pp. 195-205). Netherlands: Springer.

Rauf, S., Rahman, H., and Khan, T. M. (2004). Effect of kinetin on multiple shoot induction in cotton (*Gossypium hirsutum* L.) cv. NIAB-999. *Iranian Journal of Biotechnology*. *2*(4), 279-282.

Redengbaugh, K., Paasch, B. D., Nichol, J. W., Kossler, M. E., Viss, P. R., and Walkee, K. A. (1986). Somatic seed: encapsulation of asexual plant embryos. *Biotechnology*, *4*, 797- 801.

Redenbaugh, K., Slade, D, Viss, P. R., and Fujii, J. A. (1987). Encapculation of somatic embryos in synthetic seed coats. *Hortscience*, *22*, 803-809.

Resurreccion, A. P., Juliano, B. O. and Tanaka, Y. (1979). Nutrient content and distribution in milling fractions of rice grain. *Journal of the Science of Food and Agriculture*, *30*, 475-481.

Ritu, M., Lubna, A. and Hejazy, K. (2013). Effect of growth regulators on *in vitro* cultures of two basmati rice genotypes: ranbir basmati and basmati 370. *International Journal of Pharmaceutical, Chemical and Biological Sciences, 3*(4), 1131-1138.

Rosniyana, A, Khairunizah, H. K., Hashifah, M. A, and Sharifah, N. S. A. (2010). Quality characteristics of organic and inorganic Maswangi rice variety. *Journal of Tropical Agriculture and Food Science*, *38*(1), 71-79.

Roy, B., and Mandal, A. B. (2008). Development of synthetic seeds involving androgenic and pro-embryos in elite indica rice. *Indian Journal of Biotechnology*, *7*(4), 515-519.

Ruzic, D.J., V. and Vujovic, T .I., (2008). The effects of cytokinin types and their concentration on *in vitro* multiplication of sweet cherry cv. Lapins (*Prunus avium* L.). *Hortscience, 3*, 12-21.

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.

Saini, R. and Jaiwal, P. K. (2005). Transformation of a recalcitrant grain legume, *Vigna mungo* L. Hepper, using *Agrobacterium tumefacians* mediated gene transfer to shoot apical meristem cultures. *Plant Cell Reports, 24*, 164-171.

Saiprasad, G.V.S. (2001). Artificial seeds and their applications. *Resonance*, *6*, 39-46.

Sanada, M., Sakamoto, Y.,Hayashi, M., Mashiko, T., Okamoto, A. and Onosh, N. (1993). Celery and lettuce. In K. Redenbaugh, (Ed.). *Synseeds*. (pp. 305-327). Boca Raton, USA: CRC Press.

Sandoval-Yugar E.W., Dal Vasco L.L., Steinmacher D.A., Stolf E.C., and Guerra M.P. (2009). Microshoots encapsulation and plant conversion of *Musa* sp. cv. 'Grand Naine'. *Ciencia Rural*, *39(*4), 998-1004.

Selma, T., and Signem, O. (2012). Comparison of cytogenetic antagonism between abscisic acid and plant growth regulators. *Pakistan Journal of Botany*, *44*(5), 1581-1586.

Shahnewaj, S., and Bari, M. A. (2004). Effect of concentration of sucrose on the frequency of callus induction and plant regeneration in anther culture of rice (*Oryza sativa* L.). *Plant Tissue Culture, 14*(1), 37-43.

Shah, M. I., Jabeen, M. and Ilahi, I. (2003). *In vitro* callus induction, its proliferation and regeneration in seed explants of wheat (*Triticum aestivum* L.) var. Lu-26S. *Pakistan Journal of Botany*, *35*(2), 209-217.

Shirani, S., Mahdavi, F., and Maziah, M. (2010). Morphological abnormality among regenerated shoots of banana and plantain (*Musa* spp) after *in vitro* multiplication with TDZ and BAP from excised shoot-tips. *African Journal of Biotechnology*, *8*(21), 5755-5761.

Sirka, A. and Immonen, T. (1993). Comparison of callus culture with embryo culture at different times of embryo rescue for primary triticale production. *Euphytica*, *70*, 185-190.

Siwach, P., Gill, A. R., and Kumari, K. (2011). Effect of season, explants, growth regulators and sugar level on induction and long term maintenance of callus cultures of *Ficus religiosa* L. *African Journal of Biotechnology*, *10*(24), 4879 -4886.

Sk Moquammel, H and Biswajit, G. (2013). Micropropagation, *in vitro* flowering and cytological studies of *Bacopa chamaedryoides*, an ethno-medicinal plant. *Environmental and Experimental Biology*, *11*, 59-68.

Soneji, J. R., Rao, P. S. and Mhatre, M. (2002). Germination of synthetic seeds of pineapple (*Ananas comosus* (L.) Merr. *Plant Cell Reports, 20*, 891-894.

Sumaryono, Riyadi, I., Pauline, D. K. and Gale, G. (2008). Growth and differentiation of embryogenic callus and somatic embryos of oil palm (*Elaeis guineensis* Jacq.) in temporary immersion system. *Indonesian Journal of Agriculture*, *1*(2), 109-114.

Summart, J., Panichajakul, S., Prathepa, 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*(2), 246-251.

Swartz, H J. (1991). Post culture behaviour, genetic and epigenetic effects and related problems. In: Debergh PC, Zimmerman RH (eds.) *Micropropagation: technology and application*. Dodrecht : Kluwer Academic Publishers, 95-122.

Syaiful Bahri, P., Siti Nor Akmar, A., Maheran, A., Sariah, M., and Othman, O. (2009). Somatic embryogenesis from scutellar embryo of *Oryza sativa* L.

var. MR219. *Pertanika Journal of Tropical Agricultural Science, 32*(2), 185 - 194.

Taha, R. M., Daud, N., Hasbullah, N. A., and Awal, A. (2009). Somatic embryogenesis and production of artificial seeds in *Saintpaulia ionantha* Wendl, in Proceedings of the 6th International Symposium on *In Vitro* Culture and Horticultural Breeding, R. J. Geijskes, P. Lakshmanan and A. Taji, Eds., of *Acta Horticulturae*, Brisbane, Australia, 331-336.

Taha, R. M., Saleh, A. Mahmad, N., Hasbullah, N. A. and Mohajer, S. (2012). Germination and plantlet regeneration of encapsulated microshoots of aromatic rice (*Oryza sativa* L. Cv. MRQ 74). *Scientific World Journal*, 1-6.

Tang, W. (2000). High-frequency plant regeneration via somatic embryogenesis and organogenesis and *in vitro* flowering of regenerated plantlets in *Panax ginseng*. *Plant Cell Reports*, *19*, 727-732.

Tang, H., Ren, Z., Reustle, G., and Krczal, G., (2002). Plant regeneration from leaves of sweet and sour cherry cultivars. *Scientia Horticulturae*. *93*, 235-244.

Tang, K., Suni, X., An, D., Power, J. B., Cocking, E.C., and Davey, M. R. (2001). A simple and rapid procedure to establish embryogenic cells suspension as a source of protoplasts for efficient plant regeneration from two Chinese commercial rice cultivars. *Plant Cell Tissue and Organ Culture, 66*, 149-153.

Thobunluepop, P., Pawelzik, E. and Vearasilp, S. (2009). Possibility of sweet corn synthetic seed production. *Pakistan Journal of Biological Sciences*, *12*(15), 1085-1089.

Tornero, L. (2009). Citrus lemon micropropagation: effect of different phytohormones on multiplication and rooting. *Acta Horticulturae*, *839*, 57-62.

Tremblay, L., Levasseur, C. and Tremblay, F. M. (1999). Frequency of somaclonal variation in plants of black spruce (*Picea mariana*, Pinaceae) and white spruce (*P. glauca*, Pinaceae) derived from somatic embryogenesis and identification of some factors involved in genetic instability. *American Journal of Botany*, *86*, 1373-1381.

Tsay, H. S., Lee, C. Y., Agrawal, D. C., and Basker, S. (2006). Influence of ventilation closure, gelling agent and explant type on shoot bud proliferation and hyperhydricity in *Scrophularia yoshmurae* – a medicinal plant. *Plant*, *42*(5), 445-449.

Tuong, T. P. and Bouman, B. A. M. (2003). Rice production in water scare environment. In J. W. Kijne and D. Molden (Eds.). *Water productivity in agriculture: Limits and opportunities for improvement*. (pp. 53-67). Wallingford (UK): CABI Publishing.

Ud-Deen, M. M. and Kabir, G. (2009). Effects of growth regulators on root tip cells of onion. *Bangladesh Journal of Botany*, *38*(1), 99-102.

Vahdati, K., Bayat, S., Ebrahimzadeh, H., Jariteh and Mirmasoumi, M. (2008). Effect of exogenous ABA on somatic embryo maturation and germination in Persian walnut (*Juglans regia* L). *Plant Cell Tissue and Organ Culture, 93*, 163-171.

Vandemoortele, J. L., Billard, J. P., Boucaud, J., and Gaspar, T. (1996). Micropropagation of parsley through axillary shoot proliferation. *Plant Cell Tissue and Organ Culture*, *44*(1), 25-30.

Van Staden, D. (2008). Plant growth regulators, II: cytokinins, their analogues and inhibitors. In E. F. George, M. A. Hall and G. J. Klerk (Eds.). *Plant Propagation by Tissue Culture*. (pp. 205-226). Dordrecht: Springer.

Vasil, I. K. (1983). Isolation and culture of protoplasts of grasses. *International Review of Cytology Supplement, 16*, 79-88.

Victor, J. M. R., Murch, S. J., Krishnaraj, S., and Saxena, P. K. (1999). Somatic embryogenesis and organogenesis in peanut: The role of thidiazuron and N-6-benzylaminopurine in the induction of plant morphogenesis. *Plant Growth Regulation, 28*(1), 9-15.

Vietez, A. M., and San Josè, M. C. (1996). Adventitious shoot regeneration from *Fagus sylvatica* leafex plants *in vitro*. *In vitro Cellular & Development Biology, 32*(3), 140-147.

Wang, Y., Ruemmele, B., Chandlee, J., Sullivan, M., Knapp, J., and Kausch, A. (2002). Embryogenic callus induction and plant regeneration media for bentgrasses and annual bluegrass. *In Vitro Biology, 38*, 460-467.

White, P. J, and Broadley, M.R. (2003) Calcium in plants. *Annals of Botany, 9*, 487-511.

Yaacob, J. S., Taha, R. M., and Esmaeili, A. K. (2013). Comparative studies on cellular behaviour of Carnation (*Dianthus caryophyllus* Linn. cv. Grenadin) grown *in vivo* and *in vitro* for early detection of somaclonal variation. *Scientific World Journal*, 1-6.

Youssef, M., James, A., Mayo-Mosqueda, A., Jose Roberto Ku-cauich, Rosa Grilajva-Arango and Rosa Maria Escobedo-GM (2010). Influence of genotype and age of explants source on the capacity for somatic embryogenesis of two Cavndish banana cultivars (*Musa acuminata* Colla, AAA). *African Journal of Biotechnology*, *9*(15), 2216-2223.

Zahida, Y. R., Md. Mahmudul, I., Md. Pallob, E. S., Md. Saiful Islam, A., Shah Md. S., Dipesh, D., Md. Mujjammil, E. H. and Md. Khalekuzzaman. (2014). *In vitro* callus induction and regeneration potentiality of aromatic rice (*Oryza*

sativa L.) cultivars in differential growth regulators. *International Journal of Applied Sciences and Biotechnology*, *2*(2), 160-167.

Zhang, Y 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*(6), 1641-1654.

Zhang, C. G., Li, W., Mao, Y. F., Zhao, D. L., Dong, W., and Guo, G. O. (2005). Endogenous hormonal levels in *Scutellaria baicalensis* calli induced by thidiazuron. *Russian Journal of Plant Physiology, 52*(3), 345-351.

Zheng, K. (2003). The application of biotechnology in the development of modern rice cultivars in China in Modern Rice Farming. *Proceedings of an International Rice Conference 2003*, Alor Setar, Kedah, Malaysia, 122-132.

Zuraida, A. R., Suri, R., and Wan Zaliha, W. S. (2010). Regeneration of Malaysian indica rice (*Oryza sativa*) Variety MR232 via optimised somatic embryogenesis system. *Journal of Phytology*, *2*(3), 30-38.

Zuraida, A. R., Zulkifli, A. S., Habibuddin, H. and Naziah, B. (2012). Regeneration of Malaysian rice variety MR219 via somatic embryogenesis. *Journal of Tropical Agriculture and Food Science*, *39*(2), 167-177.

LIST OF PUBLICATIONS AND PAPERS PRESENTED

1. Rosna Mat Taha, **Azani Saleh**, Noraini Mahmad, Nor Azlina Hasbulah and Sadegh Mohajer (2012). Germination and plantlet regeneration of encapsulated microshoots of aromatic rice (*Oryza sativa* L. Cv. MRQ 74). *The Scientific World Journal*, 1-6.

2. **Azani Saleh**, Rosna Mat Taha, Noraini Mahmad, Hashimah Elias and Hairul Amani Abdul Hamid (2017). Detection of somaclonal variation in micropropagated *Oryza sativa* L. cv. MRQ 74 from Stem Explants. *Planta Daninha*. (Accepted)

3. Paper entitled "The effects of growth regulators on callus induction and plant regeneration of aromatic rice (*Oryza sativa* L. Cv. MRQ 74)" was presented at The International Symposium on Medical and Aromatic Plants, Chiang Mai, Thailand, 15 – 18 November 2011.

4. Paper entitled "Germination and plantlet regeneration of encapsulated microshoots of aromatic rice (*Oryza sativa* L. Cv. MRQ 74)," was presented at 19th General Congress of UECARPIA, Budapest, Hungary, 21- 24 May 2012.