CHEMICAL AND PHYSICAL TREATMENT EFFECTS ON INDUCTION OF SOMATIC EMBRYOGENESIS OF EXPLANTS CULTURED ON MS MEDIUM CONTAINING THIDIAZURON.

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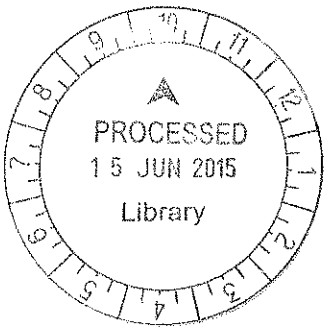
DISSERTATION SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF BACHELOR OF BIOTECHNOLOGY (HONOURS)



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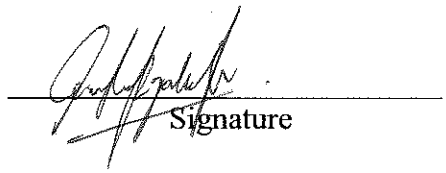
Madhavi Rajakumar	
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ABSTRACT

This project seeked to optimise the chemical and physical factors as parameters that induce somatic embryogenesis (SE) of *D.* × *tokaiensis* leaf explants grown on Murashige and Skoog (MS) medium supplemented with 1.0 mg/L thidiazuron (TDZ). The chemical parameters utilised were pH of value of 3, 4, 5, 6 and 7, while the physical parameters are varying light intensity, dark, dim, diffused and direct lighting as well as varying sub-culturing frequencies, at a period of 2 weeks, 4 weeks and 8 weeks. Observation of the cultures was performed at day 15 and 30. The cultures were photographed at day 30 and the induction of somatic embryogenesis (SE) was examined via the counting of number of embryos formed. The results obtained were then tested for significance using Analysis of Variance (ANOVA) and Fisher's Least Significant Difference (LSD) test with a 95% confidence level. The resultant somatic embryos were then transferred on to MS medium without TDZ and the various stages of somatic embryos, globular, heart, torpedo, cotyledonary and germinating plantlets were photographed. The explants sub-cultured at a 2-week interval, exposed to direct lighting and grown on pH 4 resulted in the induction of the highest number of somatic embryos. Experimental methodology of the exposure of explants to the varying light intensity could be modified by utilising specific wavelengths of light instead of white light. In addition to that, histological and molecular studies could be carried out, to further determine the effect of the chemical and physical factors at a cellular and molecular level.

TABLE OF CONTENT

	PAGE
DECLARATION	**ii**
ACKNOWLEDGEMENT	**iii**
ABSTRACT	**iv**
TABLE OF CONTENT	**v**
LIST OF TABLES	**vii**
LIST OF FIGURES	**viii**
LIST OF ABBREVIATIONS	**ix**
CHAPTER	
1. INTRODUCTION	**1**
2. LITERATURE REVIEW	**3**
2.1 Plant Tissue Culture Technology	3
2.1.1 History of Plant Tissue Culture	3
2.1.2 Micropropagation Technique	4
2.2 Somatic Embryogenesis (SE)	5
2.2.1 An Introduction to SE	5
2.2.2 SE Is Favoured Over Organogenesis	5
2.3 Genetic Factor and SE	6
2.3.1 Molecular Mechanism of SE	6
2.4 Effects of Physical Factors on SE	7
2.4.1 Sub-culturing Frequency	7
2.4.2 Light Intensity	7
2.5 Effect of Chemical Factors on SE	8
2.5.1 Potential Hydrogen as a Chemical Factor	8
2.6 Thidiazuron (TDZ)	9
2.6.1 A Brief Introduction to TDZ	9
2.6.2 The Mechanism of TDZ	9
2.6.3 TDZ in Stimulation of SE	10
2.7 Statistical Analysis	10
2.7.1 Analysis of Variance (ANOVA)	10
2.7.2 Fisher's Least Signifance Difference (LSD)	11
3. MATERIALS AND METHODS	**12**
3.1 Preparation of Murashige and Skoog (MS) with TDZ	12
3.1.1 MS Medium Containing TDZ Without pH Adjustment	12
3.1.2 MS Medium Containing TDZ With pH Adjustment	12
3.2 Source of Plant material	13

3.3	Explants Collection	14
	3.3.1 Sub-culturing Frequency Treatments	14
	3.3.2 Light Intensity Treatments	14
	3.3.3 Potential Hydrogen (pH) Treatments	15
	3.3.4 Observation and Statistical Analysis	15
3.4	Morphology Study of Somatic Embryos Stages and Fixation	16
	3.4.1 Sampling of Explants	16
	3.4.2 Fixation of Explants	17
	3.4.3 Dehydration and Storage	17
4.	**RESULTS**	**18**
4.1	Preliminary Somatic Embryo Observation	18
	4.1.1 Explants Cultured With Varying Sub-Culturing Frequencies	18 18
	4.1.2 Explants Cultured With Varying Light Intensities	18
	4.1.3 Explants Cultured With Varying pH levels of Medium	18
4.2	Somatic Embryo Counting	19
	4.2.1 Statistical Analysis of Day 30 Observation	19
4.3	Morphological Study of Germinating Somatic Embryos	24
5.	**DISCUSSION**	**27**
5.1	Sub-culturing Frequency Effect on SE Induction	27
5.2	Light Intensity Effect on SE Induction	30
5.3	Potential Hydrogen Effect on SE Induction	31
5.4	Molecular Studies of SE Induction	34
5.5	Confirmation of the Occurence of SE Induction	34
6.	**CONCLUSION AND RECOMMENDATIONS**	**37**
6.1	Conclusion	37
6.2	Recommendations	37
	REFERENCES	**38**
	APPENDIX	**44**

LIST OF TABLES

Table		Page
1	Concentration of chemical composition of the prepared MS medium supplemented with TDZ, 20 g/L sucrose and 2.2 g/L Gelrite™.	13
2	ANOVA on the number of somatic embryo parameter of *D.×tokaiensis* explants treated with sub-culturing frequency, light intensity and pH parameters.	20
3	Morphological description of the various stage structures of somatic embryos, globular, heart, torpedo, cotyledonary and germinating plantlets, arising from *D.* × *tokaiensis* explants sub-cultured on MS medium supplemented with 1.0 mg/L TDZ.	25

LIST OF FIGURES

Figure		Page
1	The mean values of the number of somatic embryos from *D.* × *tokaiensis* explants subjected to various sub-culturing frequencies grown on MS medium supplemented with 1.0 mg/L TDZ.	22
2	The mean values of the number of somatic embryos from *D.* × *tokaiensis* explants subjected to various light intensities grown on MS medium supplemented with 1.0 mg/L TDZ.	22
3	The mean values of the number of somatic embryos from *D.* × *tokaiensis* explants subjected to varying pH level grown on MS medium supplemented with 1.0 mg/L TDZ.	23
4	Somatic embryo morphology from different germination stages of *D.* × *tokaiensis*. (**A**) Globular structure with no distinct cell organisation as revealed by histology study (C.S. Lim, personal communication, March 19, 2015). (**B**) Heart structure with shoot apical meristem (**sam**), root apical meristem (**ram**) and cotyledon primordia (**cp**). (**C**) Torpedo structure. (**D**) Cotyledonary structure with leaf primordia (**lp**) (**E**) Germinating plantlets with cotyledon residue (**cr**). Bar = 1 mm.	26
5	The mechanism by which auxin and cytokinin enable the transition of the cell cycle from the G1 phase to the S phase. CycD2 is the abbrevation for cyclinD2, CycD3 is the abbreviation for cyclin D3, Cdk stands for cyclin-dependent protein kinases, Rb stands for retinoblastoma protein, E2F represent a class of conserved transcription factors (Extracted from Gahan, 2007).	28
6	Gelrite™ and agar reduced in solidification as pH is reduced. Both Gelrite™ and tissue culture (TC) agar have no solidification capability at a pH level of pH 3.	32

LIST OF ABBREVIATIONS

ANOVA	Analysis of Variance
AP2/EREBP	APETALA2/ Ethylene-responsive element-binding protein
◦C	Degree celsius
$CaCl_2 \cdot 2H_2O$	Calcium chloride dihydrate
$CoCl_2 \cdot 6H_2O$	Cobalt (II) chloride hexahydrate
cp	Cotyledon primordia
cr	Cotyledon residue
$CuSO_4 \cdot 5H_2O$	Copper (II) sulfate pentahydrate
D. × tokaiensis	*Drosera × tokaiensis*
D. carota	*Daucus carota*
df	Degree of freedom
$FeSO_4 \cdot 7H_2O$	Iron (II) sulfate heptahydrate
ft	feet
F-value	ratio of two mean square values
G0	Gap 0
G1	Gap 1
G2	Gap 2
g/L	Gram per liter
H_3BO_3	Boric acid
HCl	Hydrochloric acid
hr	hour
IAA	Indole-3-acetic acid
kg/cm^2	Kilogram per centimeter square
KH_2PO_4	Monopotassium phosphate
KI	Potassium iodide
KNO_3	Potassium nitrate
KOH	Potassium hydroxide
L	Litre
lp	Leaf primordia
LSD	Fisher's Least Significant Difference

lx	lux
$MgSO_4 \cdot 7H_2O$	Magnesium sulfate heptahydrate
mg	Milligram
mg/L	Milligram per litre
mL	Millilitre
$MnSO_4 \cdot 4H_2O$	Manganese sulfate tetrahydrate
MS	Mean square
MS medium	Murashige and Skoog (1962) medium
N	Normality
$Na_2EDTA \cdot 2H_2O$	Ethylenediaminetetraacetic acid, disodium dihydrate
NAC	N (derived from no apical meristem) A (derived from *Arabidopsis* transcription activation factor) C (derived from cup-shaped cotyledon)
NaOH	Sodium hydroxide
$Na_2MoO_4 \cdot 2H_2O$	Sodium molybdate
NH_4NO_3	Ammonium nitrate
pH	Potential hydrogen
Pyridoxine-HCl	Pyridoxine hydrochloride
P- value	Probability value
PIN proteins	Auxin efflux carrier plasma proteins
ram	Root apical meristem
S	Synthesis
SS	Sun of squares
sam	Shoot apical meristem
SE	Somatic embryogenesis
TC	Tissue culture
TDZ	Thidiazuron
TF	Transcription factor
Thiamine-HCl	Thiamine hydrochloride
µM	Micromolar
v/v	Volume/volume
$ZnSO_4 \cdot 7H_2O$	Zinc sulfate heptahydrate
%	Percentage

1.0 CHAPTER 1:

INTRODUCTION

Somatic embryogenesis (SE) is not foreign to tissue culture; it is in fact on of the most preferred methods of plant regeneration. Placing SE into definition, embryogenesis is derived from either undifferentiated callus cell(s) or embryogenic cell(s) which develops to form an embryo, while SE constitute of embryos obtained from somatic cells. SE was first examined in carrot but is now known to be fundamental to higher plants due to cellular totipotency (Karami, Aghavaisi & Pour, 2004). Commercial crops, plants of medicinal value and those that are endangered have been propagated using SE. Aside that, SE also plays a role in many cellular and development studies.

SE is known to be affected by an array of factors ranging from chemical to genetic and even physical factors (Karami et al., 2004). While both chemical and genetic factors have been the focus of many researches, physical factors are rarely highlighted. Chemical and physical factors that potentially influence SE includes pH, temperature, light intensity and sub-culturing frequency. With dependence on species, SE can be chemically induced on a broad range of pH, such as the induction of SE in soybean which has been found to occur between a range of pH 5.7 to 7.0 (Santarem, Pelissier & Finer, 1996). However, the optimum induction of SE is upon a narrow margin of pH, which in the case of soyabean has been found to be pH 7.0 (Bonacin, Di Mauro, de Oliveira & Perecin, 2000), while carrot at pH 4 (George & Debergh, 2008).

SE induction across varying light intensities was first tested in carrot. Light intensities utilised can either be as simple as providing light directly, indirectly or not providing light. Soybean was found that in both darkness (completely no light exposure) and direct lighting resulted in SE induction (Lazzeri, Hildebrand & Collins, 1987). However, not all species of plants, such as herbs or orchids have been tested along the lines of varying light intensities.

Sub-culturing has been known to increase the rate of SE induction especially when carried out at an interval of 2 weeks (Ismail, Rani & Batra, 2011). This is because the amount of plant growth regulators readily available in the medium diminishes within the same interval (Jimenez & Thomas, 2005). In terms of herbs and orchids, very little emphasis have been given to understand how these physical factors would result in an optimum induction of SE.

Previously, *Drosera* × *tokaiensis* explants grown on Murashige & Skoog (MS; 1962) medium supplemented with thidiazuron (TDZ) were found to form somatic embryo structures. It is known that continuous exposure to TDZ at concentration of 0.5-1.0 μM (1-2 mg/L) is vital to assist the induction of SE (Chhabra, Chaudhary, Varma, Sainger & Jaiwal, 2008) in *Lens culinaris* Medik species. Many sources correlated TDZ with the induction of SE but not the correlation of chemical and physical factors with the induction of SE. Hence it is vital to study this area as well as to provide answers to the appropriate pH range, light intensity and sub-culturing frequency. A comprehensive understanding would ultimately result in a potential optimum combination of chemical, genetic and physical factors to optimally induce SE. This would enable for an increase in mass propagation of this and other plant species with high medicinal or commercial value as well as to ensure the sustenance of endangered plant species.

As such the objectives of this research were to determine the optimum pH range, light intensity and sub-culturing frequency for SE induction of *D.* × *tokaiensis* explants on MS medium supplemented with 1.0 mg/L TDZ as well as to determine and differentiate the various stages of somatic embryo development , globular, heart, torpedo, cotyledonary and germinating plantlets.

2.0 CHAPTER 2:

LITERATURE REVIEW

2.1 PLANT TISSUE CULTURE TECHNOLOGY

One of the most prominent branches in biotechnology is plant biotechnology. The basis of plant biotechnology is plant tissue culture which permits modern genetic engineering and large scale cultivation in bioreactors. Plant tissue culture can be summed as the *in vitro* growth of plants provided with sufficient environmental, physical and chemical factors that enable for the maximum exploitation of their growth (Hussain, Qarshi, Nazir & Ullah, 2012).

2.1.1 History of Plant Tissue Culture

The concept of *in vitro* plant tissue culture was conceived by Gottlieb Haberlandt in 1902 (Hussain et al., 2012). The concept was in pursuit of Schleiden and Schwann's hypothesis who in 1839, proposed that cell is the basic unit of organisms. Their theory was that each individual cell was capable of autonomy and hence had the capability to regenerate into a whole plant under certain conditions (Hussain et al., 2012).

Haberlandt worked with plant species such as *Laminum purpurem* and *Eicchornia crassiples* by isolating single fully developed individual plant cells (Bhojwani & Dantu, 2013). Haberlandt was also the first to culture the isolated plant cells on a medium known as Knop's salt solution which was enriched with glucose. However, Haberlandt's research was unfruitful as the plants he had cultured failed to grow. Despite his failure, Haberlandt did contribute to the mechanism of totipotency (Bhojwani & Dantu, 2013).

The year 1904 saw Hanning trying to improvise on Haberlandt's failure; instead of culturing fully developed individual cells, Hanning chose to culture

embryogenic tissue. Hanning was able to successfully grow the cells on a mineral salt and sugar solution (Hussain et al., 2012).

It was in the 1930s, when progress in plant tissue culture accelerated rapidly owing to an important discovery that vitamin B and natural auxins were necessary for the growth of isolated tissues containing meristems. This breakthrough came from White (in the year 1934) who reported that not only could cultured tomato root tips grow but they could be repeatedly subcultured. The discovery of plant growth regulators such as indole-3-acetic acid further enhanced the field of plant tissue culture (Bhojwani & Dantu, 2013).

The most commendable impact in the plant tissue culture discipline would be the development of the Murashige & Skoog (MS) medium in 1962, commonly referred to as the MS medium. Murashige & Skoog (1962) prepared a medium that had twenty-five times more salts concentration over Knops medium. The MS medium was found to enhance the growth of tobacco callus by five -fold (Murashige & Skoog, 1962).

2.1.2 Micropropagation Technique

Plant tissue culture is also referred to as micropropagation, which is defined as the propagation of plants under a sterile and controlled environment. This process produces plants that are known as microplants (George & Debergh, 2008). Micropropagation is a vital tool utilised by the agricultural industry to ensure that plants with superior and desired traits can be continuously utilised, while in biodiversity conservation, micropropagation is vital to ensure the survival of endangered plant species as well as in research to test parameters that affect the growth of plants. Aside that micropropagation lowers crops and plants cost while saving on propagation space (George & Debergh, 2008).

2.2 SOMATIC EMBRYOGENESIS (SE)

2.2.1 An Introduction to SE

The first incidence of SE was observed in carrots, which thereafter resulted in the fundamental knowledge that somatic cells of plants result in SE (Karami et al., 2004). Commercial crops, plants of medicinal value and those that are endangered have been known to be propagated using SE. Aside that, SE also plays a role in many cellular and plant developmental studies (Karami et al., 2004).

The induction of SE is considered a primary stage in the study of SE. In order to further understand the SE induction, morphological study of SE has to be conducted; this would enable the induced SE to be characterised in one of the following stages (in sequence) globular, heart, torpedo or cotyledonary, which eventually lead to germinated plantlets (Aslam, Mujib & Sharma, 2014). Morphological study of SE has determined the extent of the model plant's SE capabilities and whether SE is the best method of propagation of a particular plant (Aslam et al., 2014).

2.2.2 SE Is Favoured Over Organogenesis

When compared to organogenesis, it was found that SE produced a higher number of regenerates (Normah, Rohani & Mohamed-Hussein, 2013). The induction of SE is relatively simple requiring just a single cell derived from an embryogenic callus, while organogenesis requires differentiated cells to form organs such as the stems, leaves or roots (Normah et al., 2013).

Jiménez (2001) and Mukherj & Bandyoph (2014) shared the same opinion that the mode of culture of SE permits easy scale-up transfers with low labour inputs, can be synchronised and purified as the origins are from single cells, and somatic embryos that are cultivated into plants are less variable.