HISTOLOGY (SECTIONING AND MICROSCOPY) OF PUTATIVE SOMATIC EMBRYOS FROM DIRECT SOMATIC EMBRYOGENESIS OF SUNDEW

LIM CHEE SIEAN

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

FACULTY OF SCIENCE, TECHNOLOGY,
ENGINEERING AND MATHEMATICS
INTI INTERNATIONAL UNIVERSITY
PUTRA NILAI, MALAYSIA

2015

NON-PLAGIARISM DECLARATION

By this letter I declare that I have written this dissertation completely by myself, and that I have used no other sources or resources than the ones mentioned.

I have indicated all quotes and citations that were literally taken from publications, or that were in close accordance with the meaning of those publications, as such. All sources and other resources used are stated in the references.

Moreover I have not handed in a dissertation similar in contents elsewhere.

In case of proof that the dissertation has not been constructed in accordance with this declaration, the School of Applied Science has the right to consider the research dissertation as a deliberate act that has been aimed at making correct judgment of the candidate's expertise, insights and skills impossible.

I acknowledge that the assessor of this item may, for the purpose of assessing this item,

- reproduce this assessment item and provide a copy to another member of the University; and/or,
- communicate a copy of this assessment item to a plagiarism checking service (which may then retain a copy of the assessment item on its database for the purpose of future plagiarism checking).

In case of plagiarism the examiner has the right to fail me and take action as prescribed by the rules regarding Academic Misconduct practiced by INTI International University.

Lim Chee Siean	
Name	Signature
I12001819	8.5.2015
I.D.Number	Date

ACKNOWLEDGEMENT

First of all, I would like to express my highest gratitude to Dr. Choong Chieh Wean, the supervisor of the present project, for his dedication in guiding and providing support and constructive comments to me throughout the project. Deep appreciation is also devoted to him for his enthusiasms and willingness in volunteering himself to capture all the photographs in the present study professionally. I would also like to thank the co-supervisor of the present project, Mr. Meganathan Perumal for willing to share his experiences in histology as well as providing me necessary technical supports.

Faculty of Science, Technology, Engineering and Mathematics (FOSTEM) and Faculty of Health Science (FHS) are acknowledged for allowing me to utilize the chemicals and equipments in both Molecular Biosciences Laboratory and Multi-Disciplinary Laboratory. Accessibility to the chemicals, instruments and equipments in laboratories has enabled me to complete the present project smoothly. Thanks to INTI International University for providing the excellent facilities in laboratories. In addition to that, I am also grateful to the Head of Programme, Mr. Lim Chia Hong for his continuous support and excellent coordination in ensuring the smooth progression of this project.

The present study was carried out at the Molecular Biosciences Laboratory and Multi-Disciplinary Laboratory, INTI International University. I am grateful to Ms. Quah Hui Hsien, Mr. Ng Peng Wah and Ms. Nasrah Ab. Ghani, the laboratory technicians for providing their highest commitment throughout the project. I would also like to express heartfelt thanks to those wonderful friends who assisted me at different stages of the project: Farrah Wahidah, Jeremiah Sia, Heng Wei Seng, Vishnu, Madhavi Rajakumar and Kovienaah. Last but not least, special thanks are dedicated to my parents, for their financial, moral and spiritual support throughout this project.

ABSTRACT

Sundew has been a medicinally important carnivorous plant and also a favourable ornamental plant for centuries. Owing to this, sundew is being over-exploited in certain countries, causing their populations to reduce to a critical level. To address these problems, *in vitro* micropropagation of sundew serves as an ideal way to regenerate and propagate these plants in large scale. Being one of the plant regeneration pathways, somatic embryogenesis possesses several advantages over other methods, rendering it to be more preferred. Previously, nodular structures were induced on *Drosera* × *tokaiensis* leaf segments on Murashige & Skoog (MS) medium supplemented with thidiazuron (TDZ) plant growth regulator. In order to confirm that these observed structures were somatic embryos, histology study was conducted. *D.* × *tokaiensis* leaves were cultured on MS medium supplemented with 1 mg/L TDZ hormone, and were then sampled and fixed in FAA fixative followed by dehydration in ethanol series. The dehydrated leaf samples were then cleared in xylene and infiltrated with paraffin wax prior to sectioning. Sections were dried, dewaxed and stained either using toluidine blue or safranin-aniline blue prior to microscopy. Microscopy observation identified competent-like cells, and embryogenic cell at one-, two-, four-, eight-cell stages and globular somatic embryos. Identification of these embryo developmental stages confirmed the somatic embryogenesis pathway of the species. However, identity of competent-like cells could not be confirmed as the morphology of competent cells is highly variable across different species. The identity could be confirmed by molecular detection of early-embryogenesis gene marker in future.

TABLE OF CONTENT

LISTS OF TABLES

LISTS OF FIGURES

LIST OF ABBREVIATIONS

ac	Apical cell
atc	Anticlinal
BBM	*BABY BOOM*
bc	Basal cell
β-D-glucans	Beta-D-glucans
$CaCl_2 \cdot 2H_2O$	Calcium chloride dihydrate
cl	Competent-like
CLV	*CLAVATA*
$CoCl_2 \cdot 6H_2O$	Cobalt (II) chloride hexahydrate
$CuSO_4 \cdot 5H_2O$	Copper (II) sulfate pentahydrate
°C	Degree Celsius
°	Degree angle
D. carota	*Daucus carota*
DNA	Deoxyribonucleic acid
D. rotundifolia	*Drosera rotundifolia*
D. spathulata	*Drosera spathulata*
D. × tokaiensis	*Drosera × tokaiensis*
DWS	Dishwashing soap
ECM	Extracellular matrix
ep	Embryo proper
FAA	Formalin-Acetic acid-Alcohol
$FeSO_4 \cdot 7H_2O$	Iron (II) sulfate heptahydrate
g	Gram
g/L	Gram per litre

glo	Globular somatic embryo
HMC	Human mast cell
H_3BO_3	Boric acid
ic	Internal cell
in	Inch
kg/cm^2	Kilogram per square centimetre
KH_2PO_4	Monopotassium phosphate
KI	Potassium iodide
KNO_3	Potassium nitrate
L	Litre
mc	Meristematic cell
$MgSO_4 \cdot 7H_2O$	Magnesium sulfate heptahydrate
µL	Microlitre
µm	Micrometre
mg	Milligram
mg/L	Milligram per litre
mg/mL	Milligram per millilitre
mm	Millimetre
$MnSO_4 \cdot 4H_2O$	Manganese(II) sulfate tetrahydrate
mL	Millilitre
min	Minute
MS	Murashige & Skoog (1962)
$Na_2EDTA \cdot 2H_2O$	Ethylenediaminetetraacetic acid, disodium dihydrate
$Na_2MoO_4 \cdot 2H_2O$	Sodium molybdate dihydrate
ne	Non-embryogenic

NH_4NO_3	Ammonium nitrate
nm	Nanometre
olc	Outer layer cell
%	Percent
PC	Pheochromocytoma
pd	Protoderm
prc	Periclinal
Pyridoxine-HCl	Pyridoxine hydrochloride
s	Second
sc	Suspensor cell
SEM	Scanning electron microscopy
SERK	Somatic Embryogenesis Receptor-like Kinase
sg	Smaller globular somatic embryo
TDZ	Thidiazuron
TEM	Transmission electron microscope
Thiamine-HCl	Thiamine hydrochloride
UV	Ultraviolet
v	Vacuole
WUS	*WUSCHEL*
w/v	Weight per volume
×	Times
$ZnSO_4 \cdot 7H_2O$	Zinc sulfate heptahydrate

1.0 CHAPTER 1:

INTRODUCTION

Sundew, being one of the largest varieties of carnivorous plants, having at least 194 species, is a group of herbaceous plants that feeds by using its stalked mucilaginous glands covering its leaf surfaces to lure, trap and digest small creatures namely insects. Sundew is usually found in wet habitats where the acidic conditions minimise the amount of nutrients it can absorb from the soil. Thus, its alternative way of obtaining sufficient amount of nutrients to sustain life is to lure, capture and digest insects using its leaves covered with numerous 'tentacles', within each contains a nectar gland. The nectar gland produces digestive enzymes which help to digest the trapped prey.

Sundew has been used as a medicinal plant as early as 12th century when an Italian doctor, Matthaeus Platearius reported that sundew as a remedy for coughs under the name *herba sole.* Herbalists also recommend sundew tea for various lung diseases such as bronchitis, asthma, dry coughs, whooping coughs, and ''bronchial cramps'' (Chakraborty & Bhattacharya, 2013). Besides that, sundew was also found to exhibit antimicrobial property. The phytochemical dubbed plumbagin produced by sundew was believed to be the substance responsible for the antimicrobial property. This made those sundew extracts to emerge as a potential treatment for various oral infectious diseases (Didry, Dubreuil, Trotin & Pinkas, 1998).

Sticky adhesive of sundew possesses potential for a variety of cutting-edge medical uses encompassing chronic wound healing, regenerative medicine and tissue engineering. Dried sundew's adhesive coated on a silicon wafer was found to comprise of a complex network of nanofibres which cross-linked to produce a porous scaffold useful for cell attachment in tissue engineering procedures (Zhang, Lenaghan, Xia, Dong, He, Henson & Fan, 2010). Besides possessing medical and therapeutic properties, sundew has also become a favourable ornamental plant owing to the beauty of its glistening traps. Some species of sundew can be used as dyeing agents in making yarn (Williams, 2011). Some tuberous sundews which are native to Australia produce corms which serve as a delicacy of the Australia Aborigines (Clarke, 2013).

Since sundew is attributed to various therapeutic properties by botanical treatises and pharmacopoeias, some sundew species have encountered over-exploitation in certain countries in Europe (Lange, 1998). Destruction of their natural habitats in peats or bogs and collection for research purposes further reduce their populations. Many studies including *in vitro* propagation have been conducted to propagate sundew for commercial, research and conservation purposes. There have been various methods to propagate sundew namely direct (not involving callus intermediate) and indirect somatic embryogenesis (involving callus intermediate) and organogenesis, propagation from pre-existing meristems (e.g. leaf segments), leaf cuttings, root cuttings and so on.

There has been a scenario that more plant cultivators are becoming more interested in sundew micropropagation rather than conventional propagation methods. Extended juvenility has made conventional propagation methods to be relatively more time consuming than somatic embryogenesis in mass production of plants. In addition, limited improvements have been made through conventional propagation methods due to inherent problems such as hybridization barriers, long term inbreeding depression and sterility. In contrast, somatic embryogenesis allows large-scale vegetative propagation which could be enhanced by using bioreactors to further scale up the propagation (De Klerk, Hall & George, 2007).

Somatic embryogenesis, being one of the plant micropropagation methods, possesses several advantages over other micropropagation methods and conventional propagation (Litz & Gray, 1992). The somatic embryos could act as a research model system in embryological studies besides serving as a target for gene transformation. Since somatic embryo cultures often originate from a single plant cell, the formation of chimeras could be minimised after gene transformation to ensure genetic uniformity among the plantlets. This distinguishes somatic embryogenesis from organogenesis which often produces chimeras (Kamle, Bajpai, Chandra, Kalim & Kumar, 2011).

Somatic embryogenesis in most cases also eliminates the need for a separate root induction which might be difficult in some species. This enables plantlets to be

propagated and acclimatised more rapidly compared to organogenesis that requires root induction (Kamle et al., 2011). In addition, somatic embryos could be cryopreserved, enabling the establishment of gene banks and thus serving conservation purposes.

In a previous study, leaf segments of a model sundew plant were used as explants for the induction of somatic embryogenesis using thidiazuron (TDZ) plant growth regulator (unpublished data). However, the preliminary result obtained from the induction study of sundew remains unconfirmed. Thus, in this study, histological analysis of the structure and ontogeny of the somatic embryos were studied to confirm the occurrence of somatic embryogenesis pathway on treated sundew explants. Another alternative to confirm the somatic embryogenesis pathway of sundew is to regenerate sundew plants from the somatic embryos. However, the latter requires longer time to achieve in many species.

Therefore the aim of this study is to confirm the occurrence of somatic embryogenesis pathway of a research model sundew, *D.* × *tokaiensis* (Komiya & Shibata) T. Nakam & K. Ueda (Apps.kew.org, n.d.), by performing histological analysis (sectioning and microscopy) of the putative somatic embryos induced by TDZ. In order to achieve the aim, cultured sundew leaf explants were sampled at particular days after induction by TDZ for histological analysis. The null hypothesis of this study is the nodular structures obtained from direct somatic embryogenesis induction of *D.* × *tokaiensis* are not somatic embryos based on the absence of somatic embryogenesis morphological markers. Whereas the alternative hypothesis of this study is the nodular structures obtained from direct somatic embryogenesis are indeed somatic embryos based on the presence of morphological markers.

2.0 CHAPTER 2:

LITERATURE REVIEW

2.1 SUNDEW

The genus *Drosera*, commonly known as sundew, comprises one of the largest groups of carnivorous plants. There are at least 194 *Drosera* species discovered to date (McPherson, 2010). Sundew obtains their nutrients by using their stalked mucilaginous glands that cover their leaf surfaces to attract, trap and digest small creatures such as insects. The nectar gland within each 'tentacles' covering the leaf surfaces contains digestive enzymes which aim to digest the captured prey. Sundew is commonly found in wet habitats namely bogs, swamps, moist streambanks, marshes and so on where the acidic condition of soil reduces their nutrient absorption from the soil. Therefore, the nutrient uptake through their carnivorous behavior acts as an alternative way to obtain adequate amount of nutrients to sustain their lives.

2.1.1 Medicinal and Other Uses

As early as 12th century, sundew has been used as a medically important plant in phytotherapy. Matthaeus Platearius, an Italian doctor reported that sundew could be used as a remedy for coughs under the name *herba sole*. Some herbalists also recommend sundew tea for bronchitis, whooping coughs, dry coughs, asthma and ''bronchial cramps'' (Chakraborty & Bhattacharya, 2013). In addition, there were cases where patients who suffered from blood dysentery were administered with whole plant paste of certain sundew species such as *Drosera burmannii* Vahl (Mitra & Mukherjee, 2010) to cure the disease. Moreover, there was a study which indicated that extracts of the aerial parts of *Drosera peltata* Smith exhibited antimicrobial properties, making it to be an effective treatment against oral infectious diseases such as periodontitis and dental caries (Didry et al., 1998). Plumbagin isolated from the chloroformic extract was found to be the active ingredient responsible for antimicrobial properties of the sundew extracts. Some sundew extracts are also effective against antibiotic susceptible as well as the resistant strains of human