RESPONSE OF SELECTED CARNIVOROUS PLANTS TO THIDIAZURON AND RNA EXTRACTION OF *Drosera* × *tokaiensis*

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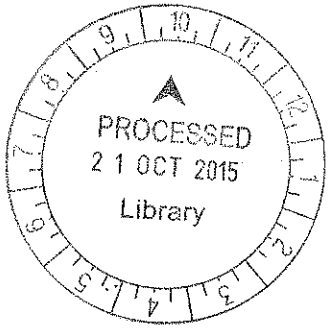

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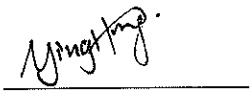
Lim Ying Houng	
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ABSTRACT

Carnivorous plants are undergoing extinction due to their wide exploitation for use in medical and horticultural industry, thus require *in vitro* culturing as an alternative method to preserve them and increase their market availability without endangering them. A range of concentrations of thidiazuron (TDZ) at 0.00, 0.01, 0.05, 0.20, 1.00 and 5.00 mg/L was tested on *Drosera* × *tokaiensis*, *Drosera burmanii* and *Dionea muscipula* to determine the optimum concentration for inducing somatic embryogenesis (SE) in each of the species. The cultures were treated for 30 days with one subculture at day 15. The response from TDZ treatment on the explants of the selected carnivorous plant species were determined by calculating the total number of somatic embryos formed. The results obtained were tested by using Analysis of Variance (ANOVA) and Dunnett's T3 test to detect the significant difference caused by the different concentration of TDZ. Different concentrations of TDZ resulted in a significant difference among explants of *D.* × *tokaiensi* and no significant difference among explants of *D. burmanii*. In particular, 0.01 mg/L of TDZ induced the highest number of somatic embryo in explants of *D.* × *tokaiensis* and 5 mg/L of TDZ induced highest number of somatic embryo in explants of *D. burmanii*. All explants of *D. muscipula* showed no induction of somatic embryo. To further confirm that somatic embryogenesis is taking place, detection of the expression of embryogenesis-related genes can be done. RNA extraction was performed for the assay in this study using the Analytik Jena RNA extraction Kit and cetyltrimethyl ammonium bromide (CTAB). Extracted total RNA from *D.* × *tokaiensis* were characterised by using agarose gel electrophoresis and UV spectrophotometry. RNA was successfully extracted by using the CTAB method. Two bands of 28S and 18S were visible in the intact total RNA samples. The total yield and quality of RNA extracted lower than expected probably due to pipetting error during 70% ethanol aspirating step.

TABLE OF CONTENT

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ANOVA	Analysis of Variance
cDNA	complementary deoxyribonucleic acid
CPs	Carnivorous Plants
CTAB	cetyltrimethyl ammonium bromide
°C	degree Celcius
Df	degree of freedom
D. burmanii	*Drosera burmanii*
D. muscipula	*Dionea muscipula*
DNA	Deoxyribonucleic acid
D. × *tokaiensis*	*Drosera* × *tokaiensis*
EDTA	ethylenediaminetetraacetic acid
EtBr	ethidium bromide
Fe	iron
g	gram
g/L	gram per litre
HCl	hydrochloric acid
kb	kilobase
kPa	kilopascal
L	Litre
LiCl	lithium chloride
µg	microgram
µg/µL	microgram per microlitre
µg/mL	microgram per millilitre
µL	microlitre
µM	micromolar
mg	milligram
mg/L	milligram per litre
mL	millilitre
mm	millimetre
mM	millimolar
M	Molar

mRNA	messenger ribonucleic acid
MS	Murashige and Skoog medium
NaCl	Sodium chloride
nm	nanometer
%	percentage
PCR	Polymerase Chain Reaction
pH	Hydrogen potential
pM	picomolar
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
rpm	revolutions per minute
SE	somatic embryogenesis
SS	sum of square
TAE	Tris-acetate-EDTA
TDZ	thidiazuron
UV	ultraviolet
v/v	volume per volume
w/v	weight per volume

1.0 CHAPTER 1:

INTRODUCTION

The order Caryophyllales contains the largest group of carnivorous plants (CP). They are flowering plants that are classified under one specialised form of diversity (Albert, Williams & Chase, 1992). Biologists are interested in CPs, as they have the potential to be used as medicinal herbs (Vasquez-Collantes, Rojas-Idrogo & Delgado-Paredes, 2014). For example, the largest family of CPs, Droseraceae, produces naphthoquinones that have a therapeutic effect on respiratory diseases such as tuberculosis, asthma and bronchial infections; studies had shown that naphthoquinones have antibacterial and antifungal properties (Vasquez-Collantes et al., 2014).

However, CPs are undergoing extinction due to large-scale collection from the wild and destruction of their natural habitat for development (Anthony, 1992). Thus, in this research, *in vitro* propagation method was used as an alternative method to conserve and regenerate them. CPs selected in this research were the sundews, *Drosera* × *tokaiensis* and *Drosera burmanii, and* the Venus fly trap, *Dionea muscipula. D. muscipula* is the only species of Venus fly trap and it is a highly endangered species listed in the Appendix II of the Convention on International Trade in Endangered Species (Luken, 2005).

In vitro propagation method involves aseptic culture of an explant in a sterile tube or flask that provides a controlled condition for the growth of the explant. Plant tissue culture is more preferred compared to other conventional methods such as cutting and planting, as it involves aseptic techniques that prevent the invasion of microorganism (Mineo, 1990), producing a large number of disease-free identical clones. Conventional propagation methods of plant regeneration are prone to pest and disease because the growth condition is not in a complete control. In addition, the amount of healthy plants produced will be much lesser than *in vitro* propagation method.

In vitro plant regeneration includes organogenesis, a process of initiating the development of plant organs, and somatic embryogenesis, a method of obtaining embryogenic tissues from somatic cell in explants for regeneration. In order to carry out *in vitro* propagation and study the morphology responses of these CPs, a plant growth regulator is essentially required.

Thidiazuron (TDZ) is a multi-dimensional plant growth regulator (Guo, Abbasi, Zeb, Xu & Wei, 2011), made up of substituted phenylurea compound, was widely used in plant tissue culture for micropropagation (Murthy, Murch & Saxena, 1998). TDZ was used to stimulate the growth of explants of the selected carnivorous plant species, and their morphological responses towards TDZ were observed. Hopefully the results from the *in vitro* propagation of CPs would aid the conservation of these endangered CP species.

To further confirm somatic embryogenesis was taking place, detection of the gene expression involved in early stage of embryogenesis could be envisioned. In order to do this, RNA extraction method from a selected plant species, *D.* × *tokaiensis* was established. RNA is an important biological material in molecular studies which indicate the expression of a set of genes including the genes predominant or specific to somatic embryogenesis.

The aim of this experiment was to investigate the morphological responses, both organogenesis and embryogenesis, of different Caryophyllales members towards different concentrations of TDZ. Besides, experiment was also carried out to establish an RNA extraction method on a selected plant species, *D.* × *tokaiensis*.

2.0 CHAPTER 2:

LITERATURE REVIEW

2.1 CARNIVOROUS PLANTS (CPs)

Carnivorous plants are defined as plants that attract, capture, digest and absorb nutrients from their preys, mostly invertebrates. CPs acquired unique ability such as attracting, trapping, retention, digesting and absorption of the prey as nutrient for their growth (Albert et al., 1992). Hence, they are able to live in a wet and nutrient-poor ecosystem with low nitrogen, sulphur and phosphorus content (Bekesiova, Nap & Mlynarova, 1999). Researchers had proven that CPs evolved from the mechanism of infolding of leaves with the upper surface becoming the inner part of pitcher traps, to the derivation of trigger hairs of *Dionaea* species and shoot-leaf indistinctness in *Utricularia* species (Albert et al., 1992). The specialised morphology and trapping mechanism of CPs led them to become one of the plants that botanist and horticulturist are interested in. Also, they are being chosen as a model system for evolutionary studies.

Besides exhibiting a special nutritional requirement, they also have the potential to be used as medicinal herbs (Vasquez-Collantes et al., 2014). However, CPs are undergoing extinction due to large-scale collection from the wild and their natural habitat are being exploited for development (Anthony, 1992).

2.1.1 Selected Species from Droseraceae

The plant order Caryophyllales consists of members of flowering plants with a highly integrated interaction between form and function (Albert et al., 1992). Certain Caryophyllales members at family level are also called CPs or insectivorous plants (Vasquez-Collantes et al., 2014). Many CPs are classified under the order Caryophyllales, and then sub-classified into different plant families such as Nepenthaceae, Drosophyllaceae, Dioncophyllaceae and Droseraceae. Darwin had provided the detailed research evidence for carnivory in several genera. After that,

around 600 species of CPs had been identified and they are classified into 6 different angiosperm subclasses.

CPs selected in this research were the sundews, *D.* × *tokaiensis* and *D. burmanii* and the Venus fly trap, *Dionea muscipula.* Historically, there were four genera classified under the Droseraceae and they are the *Drosera, Drosophyllum, Aldrovanda* and the Venus fly trap, *Dionea* (Rivadavia, Kondo, Kato & Hasebe, 2003). *Drosera,* the sundew, is one of the most widespread genera of CPs with around 150 species distributed in Africa, Australia and South America. Including *D. burmanii,* there were only three *Drosera* species found in Peninsular Malaysia (Hoshi, Shirakawa, Takeo & Nagano, 2010). *Drosera* has an active flypaper trap with mobile glandular hairs that is used to capture their prey (Hoshi et al., 2010). *Drosera* species produce secondary metabolites which were proven to have antifungal, antimicrobial and various medicinal effects (Bekesiova et al., 1999). Naphthoquinones are important bioactive compounds produced by leaves and roots of the *Drosera* species (Rejthar, Vlehmannova, Cepkova, Fernandez & Milella, 2014). These compounds include a flavonoid that was active against cancer, plumbagin that could be used to treat bronchial infection, microbial infection, tuberculosis, malaria and asthma, and 7-methyljuglone that was highly toxic to fungal pathogen (Jayaram & Prasad, 2006). For this reason, the gene pool of Droseraceae has become interest in research.

D. × *tokaiensis* is a natural hybrid between *Drosera spatulata* and *Drosera rotundifolia* as *in situ* hybridisation analysis showed that *D.* × *tokaiensis* consisted of chromosomes from both *D. spatulata* and *D. rotundifolia* (Hayakawa, Hamachi, Ogawa, Minaniya, ... & Fukuda, 2012). The population of *D. burmanii* has diminished gradually in size due to environmental factors and the public demand due to their medicinal value (Jayaram & Prasad, 2006). In Asia, exporters collect *D. burmanii* from the wild without taking appropriate measures to conserve and propagate them. According to the Conservation of Nature and Natural Resources (IUCN), *D. burmanii* is listed under the vulnerable category (Jayaram & Prasad, 2006). However, they are considered as one of the endangered species as no stringent conservation measures have been taken by the government and environmental regulatory agencies (Jayaram & Prasad, 2006).

The only species of Venus fly trap, *D. muscipula* displayed a trapping mechanism called the snap trap (Rivadavia et al., 2003). Recent research stated that this adaptation of *D. muscipula* provided more than 75% of its nitrogen requirement (Luken, 2005). *D. muscipula* was listed as a Species of Concern between South Carolina and North Carolina where there is a long-term decline in population. (Luken, 2005). In addition, it is also listed as endangered in Appendix II of the Convention on International Trade in Endangered Species (Luken, 2005). Therefore, it is essential to conserve and regenerate these carnivorous plants via *in vitro* propagation method.

2.2 INTRODUCTION TO PLANT TISSUE CULTURE

Plant tissue culture refers to the culture of explants aseptically under a defined and controlled environment and nutritional conditions (Hussain, Qarshi, Nazir & Ullah, 2012). Factors such as temperature, pH of medium, supplement of nutrients and the light intensity will be controlled during culturing of explants. Plant tissue culture technique is also known as micropropagation technique. It has the ability to produce identical plants with superior quality such as better disease resistance and stronger stress tolerance capacities. It produces disease-free clones of the desired plants in large numbers within a short period of time. The genotypes of the resultant clones are completely identical to the parental plant. And in recent years, plant tissue culture technology had been widely used not just for propagation of plant, but also to use these cloned plants in the production of secondary metabolites, elimination of disease and improvement of plants (Hussain et al., 2012).

2.2.1 Benefits of Plant Tissue Culture

Other than micropropagation, there are other conventional methods of propagation such as grafting, cutting, seedling, air-layering and so on. However, plant tissue culture is the preferred regeneration method compared to other conventional methods. This is due to the rapid propagation process of plant tissue culture that is able to produce a large number of virus-free clones. Research had shown that culturing under controlled conditions led to an increase in total yield up to 150% of virus-free potatoes (Hussain et al., 2012). The growth conditions of conventional propagation methods are not in a complete control and also prone to pest and disease. In addition, the