HISTOLOGY TO PROVE THE OCCURRENCE SOMATIC EMBRYOGENESIS IN *Drosera burmannii*

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ABSTRACT

There are certain traits of carnivorous plants that have intrigued people for years. Carnivorous plants are marvellous in the way they portray natural selection. Carnivorous plants are admired for their ornamental value, however, their true value lies in their medicinal properties. Unfortunately, due to these traits, carnivorous plants have become endangered. Plant tissue culture techniques are being utilised to conserve these endangered species. *In vitro* propagation allows production of disease free clones of plants on a large scale. *In vitro* plant regeneration is carried out via organogenesis and somatic embryogenesis (SE). Direct somatic embryogenesis (DSE) is preferred as single cells are involved which reduces somaclonal variation and chimera formation. The aim of this research was to use histological study to observe and confirm pre- and post-globular developmental stages of SE in *D. burmannii*. Leaf explants of *D. burmannii* were previously induced on medium containing 0.01 mg/L thidiazuron (TDZ) to undergo SE. Leaf explants were fixed, embedded in paraffin wax, sectioned and stained to produce microscopic slides used in histological study. Stereo microscope analysis and histological study were carried out to observe morphological changes occurring in those leaf explants. The pre- and post-globular morphological structures such as globular, heart, torpedo and germinating were identified via stereo microscope and histology approach. Observation of outer and inner morphology of globular, heart, torpedo and germinating embryos confirmed that *D. burmannii* could be regenerated *in vitro* via SE. Even though it was found that *D. burmannii* does not have a cotyledon stage, from torpedo stage it goes directly into germinating stage. However, the presence of a germinating stage proved that regeneration via SE had occurred. It was concluded that *D. burmannii* regenerates via DSE and undergoes a unicellular pathway. Further research can be carried out to study nutritional factors to increase SE rate in *D. burmannii*.

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LIST OF ABBREVIATIONS

°C	degree Celsius
%	percentage
µm	micrometre
d	day
D. burmannii	*Drosera burmannii*
D. tokaiensis	*Drosera tokaiensis*
DSE	direct somatic embryogenesis
DWS	dish washing solution
EC	embryogenic cell
ECC	embryogenic competent cell
g	gram
H_2O_2	hydrogen peroxide
HCl	hydrochloric acid
hr	hour
ISE	indirect somatic embryogenesis
$KAl(SO_4)_2$	aluminium potassium sulphate
KI	potassium iodide
$MgSO_4$	magnesium sulphate
min	minute
MS medium	Murashige & Skoog (1962) medium
Na_2HPO_4	disodium phosphate
$Na_2S_2O_5$	sodium metabisulphite
$Na_2S_2O_3 \cdot 5H_2O$	sodium thiosulphate pentahydrate
$NaHCO_3$	sodium bicarbonate
NaH_2PO_4	monosodium phosphate
NBF	neutral buffered formalin
PAS	periodic-acid schiff

PGR	plant growth regulator
PP	polypropylene
s	second
SE	somatic embryogenesis
SERK	somatic embryogenesis receptor kinase
SO_2	sulphur dioxide
TBA	tert-butyl alcohol
TBO	toluidine blue O
TDZ	thidiazuron
TWS	Scott's tap water substitute
v/v	volume per volume concentration
w/v	weight per volume concentration

CHAPTER 1

INTRODUCTION

Carnivorous plants have intrigued people as far back as the nineteenth century. Charles Darwin had first described this phenomenon in his book titled "Insectivorous Plants" in 1875. The reason behind this was simply because the existence of carnivorous plants defied the common ideology that plants were harmless and immobile (Krol et al., 2011). To this day, approximately 600 species of carnivorous plants have been discovered around the world. Plants are classified as carnivorous based on several characteristics. Carnivorous plants have adapted over the years to trap and digest preys, and assimilate necessary metabolites for their growth (Adamec, 1997). Charles Darwin was fascinated by carnivorous plants because of how they portrayed natural selection (Albert, Williams, & Chase, 1992). Carnivorous plants evolved to survive in harsh environments such as soil lacking in nutrients (Banasiuk, Kawiak, & Krolicka, 2012).

Despite carnivorous plants being used for ornamental purposes, they not only have aesthetic value but also medicinal as well. In recent years, research has been conducted on carnivorous plants particularly on the *Drosera* genus commonly known as sundews with estimated 200 species. Conservation work is in progress for micropropagation of endangered species of *Drosera* such as *Drosera intermedia*. In Thai traditional medicine, *Drosera indica* is used to treat eczema and hepatitis; it contains plumbagin, a naphthoquinone which is a therapeutic agent (Thaweesak, Sakamoto, Tanaka, & Waraporn, 2011; Rejthar, Viehmannova, Cepkova, Fernandez, & Milella, 2014).

Unfortunately, due to human interference, carnivorous plants have become endangered. Because of their ornamental and medicinal value, carnivorous plants are in high demand. The medicinal properties of the *Drosera* genus come from the production of naphthoquinones and flavonoids, which are important raw materials for the pharmaceutical industry (Banasiuk et al., 2012). Human factors such as over-collection of plants, urbanisation, habitat destruction, and many more have put

valuable plant species at risk of extinction (Manole-Paunescu, 2014). Contrary to the conventional methods for growing plants, modern plant tissue culture techniques are preferred. *In vitro* propagation has become a key technique in ensuring survival of endangered plants and to fill the gap in supply and demand of commercially important plants (Irawati, 2013). *In vitro* propagation is utilised for mass production of disease free plant clones. This technique can be used to conserve the gene pool and gives superior varieties of plants (Rout, Mohapatra, & Jain, 2006). Environmental factors such as pests, climate, diseases, and geographical location are the problems which plague conventional methods for plant production (Banasiuk et al., 2012; Vinoth & Ravindhran, 2013). *In vitro* propagation is not constrained by these limiting factors. *In vitro* propagation can be carried out via organogenesis and somatic embryogenesis (SE) (Banasiuk et al., 2012; Vinoth & Ravindhran, 2013). Organogenesis exhibits chimera formation, and thus SE is the better alternative.

In SE, a haploid or diploid somatic cell differentiates into a whole new plant following the embryological stages without fusion of gametes (dos Santos, Mariath, Moco, & Bodanese-Zanettini, 2006). There are two ways in which SE can be initiated, either directly on explants known as direct somatic embryogenesis (DSE) or indirectly from unorganised tissues (callus) known as indirect somatic embryogenesis (ISE). However, the indirect route is not encouraged as the plants produced may not be genetically identical (Santacruz-Ruvalcaba, Gutierrez-Mora, & Rodriguez-Garay, 1998). Whereas, DSE is better as there is no callus formation leading to less somaclonal variation which also prevents chimera formation (Jimenez, 2001; Kamle, Bajpai, Chandra, Kalim, & Kumar, 2011). SE can be used for mass propagation of plants that are endangered or have medicinal and commercial value (Kamle et al., 2011).

Apart from the studies conducted at INTI International University (IIU), no studies have been conducted on the following research topic anywhere in the world from literature search. In the studies, histological approach was used to study the embryological development stages of SE in *Drosera tokaiensis* and *Drosera burmannii* (Sia, 2015; Kok 2015). For *D. burmannii* the early stages of embryological development were identified up to globular stage through DSE (Sia, 2015). However, the study regarding *D. burmannii* could not be completed due to time constraint even

though data was available on stages up to globular stage (Kok, 2016). Therefore, less data from *D. burmannii* was available as compared to *D. tokaiensis*. Studying *D. burmannii* could help to understand other routes of SE regeneration pathways in sundews in addition to those discovered in *D. tokaiensis*. Previous studies had identified globular stage in *D. burmannii*. Thus, gathering data on pre- and post-globular stages is important because only then, regeneration ability via SE can be proven in the species.

The aim of this research was to use histology to observe and confirm the different pre- and post-globular stages of SE in *D. burmannii*. *D. burmannii* leaf explants that were induced on medium containing 0.01 mg/L thidiazuron (TDZ) were fixed, embedded in paraffin wax, sectioned and stained. Embryological stages of development such as globular, heart, torpedo and germinating somatic embryos were identified from those stained sections.

CHAPTER 2

LITERATURE REVIEW

2.1 *IN VITRO* PROPOGATION OF SUNDEW

Darwin (1875), the scientist who discovered carnivorous plants stated that "I care more about *Drosera* than the origin of all the species in the world". To this day scientists are researching on *Drosera* genus the common sundew under Droseraceae family. *Drosera* evolved over the years to catch prey with the help of long tentacles on their leaves (Gibson & Waller, 2009). Despite being known for their ornamental beauty *Drosera* have medicinal value as well.

In Indian traditional medicine, leaves from *Drosera burmannii* mixed with salt are used for treatment of blisters (Yanthan, Kehie, Kumaria, & Tandon, 2017). Naphthoquinones and flavonoids are major compounds present in *D. burmannii* contributing to pharmaceutical value of the plant (Wang, Su, & Zeng, 1998). Dried *D. burmannii* plants called 'Herba Droserae' are being used in pharmaceutical industries in China and many European countries (Yanthan et al., 2017).

Yanthan et al. (2017), conducted a recent study in India for *in vitro* propagation of *D. burmannii*. Regeneration of *D. burmannii* via organogenesis was carried out to conserve the endangered species and to mass propagate for supplying raw materials to pharmaceutical industries.

2.2 SOMATIC EMBRYOGENESIS (SE)

Regeneration of plants can be carried out via SE another way of *in vitro* propagation. SE was first observed in *Daucus carota* (carrot) in 1958 and to this day, many plant species have been reported to display SE. Due to plants' attribute of cellular totipotency, SE is possible in many plant species (Ikeda-Iwai, Umehara, Satoh, & Kamada, 2003). In SE, *in vitro* regeneration of single somatic cells into whole plants occur where somatic cells undergo biochemical and morphological changes to form

somatic embryos (Sahijram & Bahadur, 2015). The morphological, biochemical, molecular and physiological changes in higher plants can be better understood by using SE as a model system (Karami, Aghavaisi, & Pour, 2009). SE occurs naturally in some plant species known as "apomixis" as seen in *Kalanchoe*, *Bryophyllum* and *Malaxis* species (Feher, 2005). SE can be induced *in vitro* in explants by using a plant growth regulator (PGR) or under stress condition (Feher, Pasternak, & Dudits, 2003). SE can also be induced by using TDZ.

2.3 THIDIAZURON (TDZ)

In 1976 in Germany, TDZ or N-phenyl-N-(1,2,3-thiadiazol-5-ylurea) was developed and used as a cotton defoliant (Lu, 1993). In 1982, it was reported that TDZ had cytokinin-like activity in *Phaseolus lunatus* callus cultures (Mok et al., 1982). According to Mok et al. (1982), among several thiadiazolylurea derivatives, TDZ demonstrated very high cytokinin-like activity which exceeds even that of zeatin. TDZ is a substituted phenylurea compound (Murthy, Murch, & Saxena, 1998) and does not have the purine ring found in zeatin (Lu, 1993). Thomas and Katterman (1986), discovered that in soybean, TDZ stimulated cell division. TDZ also stimulated radish cotyledon expansion and in tobacco leaf discs TDZ induced adventitious shoot formation (Thomas & Katterman, 1986).

In micropropagation and plant tissue culture techniques TDZ has been successfully used as a PGR (Thomas & Katterman, 1986). TDZ exhibits cytokinin and auxin-like effects on differentiation and growth of explants (Guo, Abbasi, Zeb, Xu, & Wei, 2011; Murthy et al., 1998). Low concentration of TDZ promotes axillary proliferation whereas high concentration of TDZ stimulates somatic embryo, adventitious shoot and callus formation (Visser, Qureshi, Gill, & Saxena, 1992). Medium containing TDZ produces shoot numbers equivalent to or greater than medium with purine-type cytokinins (Lu, 1993).

Perfect shoot formation was seen in *Artemisia annua* L. leaf and stem explants grown on Murashige & Skoog (MS; 1962) medium containing TDZ (Lualon, De-Eknamkul, Tanaka, Shoyama, & Putalun, 2008). 70% successful cases of SE were observed when MS medium was used as compared to other basal media (Bhojwani &