METABOLITE PROFILING OF *Boesenbergia rotunda* TISSUE CULTURE CALLUS RELATED TO EMBRYOGENESIS AND PLANT REGENERATION

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METABOLITE PROFILING OF *Boesenbergia rotunda* TISSUE CULTURE CALLUS RELATED TO EMBRYOGENESIS AND PLANT REGENERATION

ABSTRACT

Boesenbergia rotunda or fingerroot ginger is commonly found in South East Asia and traditionally used to treat common illnesses. Interest in the medicinal properties of *B. rotunda* has led to the tissue culture studies of this plant. The exploitation of culture conditions can be expected to affect production of different calli types and cell metabolites. Hence, analysis of primary and secondary metabolites as well as hormones was performed by Ultra Performance Liquid Chromatography Mass Spectrometry to determine the biochemical changes related to embryogenesis and plant regeneration. This was complemented by histological characterization study by microscopy. Primary metabolite profiles showed higher levels of glutamine, arginine and lysine in *B. rotunda* embryogenic callus compared to non-embryogenic tissues (suspension cells, dry and watery calli). The metabolite markers for embryogenic competency were confirmed in sieved embryogenic cells. Rhizome had the highest flavonoid levels while shoot tips the lowest indicating that flavonoids in shoot tips may result from diffusion from the rhizome. The low endogenous auxin level in embryogenic callus suggests active auxin metabolism to stimulate cell division and elongation for embryogenesis. Histo-morphological study indicated that embryogenic callus can be characterized by the presence of starch granules, fibrils on cell surfaces and bright fluorescent spots after diphenylboric acid 2-aminoethylester staining. Cells in watery callus were non-proliferative, lacking fluorescent spots, nuclei and starch granules, however had apparently higher flavonoid levels, possibly due to higher stain specificity towards selected flavonoids in *B. rotunda*. Ultimately, the identification of primary metabolite and cell morphology markers in *B.*

rotunda cell cultures together with ongoing genomic studies can improve understanding of molecular processes related to embryogenesis and plant regeneration.

PROFIL METABOLIT *Boesenbergia rotunda* TISU KULTUR KALUS BERKAITAN DENGAN EMBRIOGENESIS DAN PERTUMBUHAN SEMULA

ABSTRAK

Boesenbergia rotunda atau tumbuhan temu kunci biasanya ditemui di Asia Tenggara dan digunakan secara tradisional untuk merawat penyakit - penyakit biasa. Ciri-ciri perubatan yang terdapat pada *B. rotunda* telah membawa kepada kajian kultur tisu tumbuhan ini. Namun begitu, eksploitasi keadaan kultur tisu boleh menyebabkan penghasilan pelbagai jenis kalus dan metabolit sel. Oleh itu, analisis metabolit primer, sekunder dan hormon telah dilakukan dengan menggunakan Spektrometri Jisim Kromatografi Cecair Berprestasi Tinggi untuk menentukan perubahan biokimia yang berkait dengan embriogenesis dan pertumbuhan semula. Ini disokong oleh kajian pencirian histologi menggunakan mikroskop. Profil metabolit primer menunjukkan tahap glutamin, arginin dan lisin yang lebih tinggi dalam *B. rotunda* kalus embriogenik berbanding tisu bukan embriogenik (sel ampaian, kalus kering dan kalus berair). Petanda kimia metabolit yang mengawal keupayaan embriogenik telah disahkan dalam sel-sel embriogenik melalui proses penyaringan. Rizom mempunyai tahap flavonoid tertinggi berbanding dengan pucuk tumbuhan yang paling rendah. Ini menunjukkan bahawa kemungkinan kandungan flavonoid dalam pucuk adalah disebabkan oleh resapan daripada rizom. Tahap auksin dalaman yang rendah pada kalus embriogenik mencadangkan bahawa metabolisme auksin sangat aktif dalam merangsang pembahagian dan pemanjangan sel untuk embriogenesis. Kajian histo-morfologi menunjukkan bahawa kalus embriogenik boleh dicirikan dengan kehadiran kanji, gentian halus pada permukaan sel dan tompok pendarfluor selepas pewarnaan dengan asid diphenylboric 2-aminoethylester. Sel-sel kalus berair merupakan sel-sel yang tidak berkembang dimana ia kekurangan tompok pendarfluor, nukleus dan granul kanji, sungguh pun ia mempunyai tahap flavonoid yang

lebih tinggi. Ini mungkin kerana pengkhususan pewarnaaan terhadap flavonoid yang tertentu pada *B. rotunda*. Akhirnya, pengenalan metabolit primer dan penanda morfologi sel dalam kultur sel *B. rotunda* bersama dengan kajian genomik yang berterusan boleh meningkatkan pemahaman dalam proses-proses molekul yang berkaitan embriogenesis dan pertumbuhan semula.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF SYMBOLS AND ABBREVIATIONS

Ca^{2+}	:	Calcium ion
cm	:	Centimetre
^{o}C	:	Degree Celsius
eV	:	Electron volt
g	:	Gram
$g.L^{-1}$	:	Gram per litre
h	:	hour
kHz	:	Kilohertz
kV	:	Kilovolt
uL	:	Microlitre
mL	:	mililitre
mm	:	milimetre
µm	:	Micrometre
$\mu g.mL^{-1}$	:	Microgram per mililitre
mg	:	miligram
$mg.L^{-1}$	:	miligram per litre
µM	:	Micromolar
min	:	Minutes
M	:	Molar
N_2	:	Nitrogen
ppb	:	Parts per billion
ppm	:	Parts per million
%	:	Percent
±	:	Plus minus
g	:	Relative centrifugal force
rpm	:	Rotation per minute
s	:	Second
V	:	Volt
v/v	:	Volume per volume
w/v	:	Weight per volume
2,4-D	:	2,4-dichlorophenoxy acetic acid
ABA	:	Abscisic acid
ACN	:	Acetonitrile
BAP	:	Benzylamino purine
BHT	:	Butylated hydroxyl toulene
CE-MS		Capillary Electrophoresis Mass Spectrometry
DC	:	Dry callus
DPBA	:	Diphenylboric acid 2-amino ethyl ester
DPPH	:	2,2-diphenyl-1-picrylhydrazyl
EC	:	Embryogenic callus
EC_S	:	Embryogenic cells sieved
ESI	:	Electron spray ionization
FA	:	Formic acid
GA	:	Gibberellins
GA_3	:	Gibberellic acid
GC-MS	:	Gas Chromatography Mass Spectrometry
H_2O	:	Water
IC	:	Inhibitory concentration

IAA	:	Indole-3-acetic acid
L	:	Conventionally propagated leaves
LC-TOFMS	:	Liquid Chromatography Time of Flight Mass Spectrometry
LLE	:	Liquid phase extraction
LC-MS	:	Liquid Chromatography Mass Spectrometry
MS	:	Mass Spectrometry
m/z	:	Mass to charge ratio
MRM	:	Multiple reaction monitoring
NAA	:	α-Napthaleneacetic acid
NMR	:	Nuclear Magnetic Resonance
NO	:	Nitric oxide
PAS	:	Periodic Acid-Schiff
PGE2	:	Prostaglandin E2
PGR	:	Plant growth regulators
QQQ	:	Triple Quadrupole
R	:	Regenerant (*in vitro* leaves)
Rh	:	Rhizome
SB	:	Shoot base
SC	:	Suspension cells
SEM	:	Scanning Electron Microscope
SPE	:	Solid phase extraction
TDZ	:	Thidiazuron
TNF-α	:	Tumor necrosis factor alpha
TOF	:	Time of Flight
TPA	:	Tetradecanoylphorbol-13-acetate
UPLC-MS	:	Ultra Performance Liquid Chromatography Mass Spectrometry
WC	:	Watery callus

LIST OF APPENDICES

CHAPTER 1

INTRODUCTION

The Zingiberaceae family, also known as the ginger family, consists of several genera including *Zingiber, Curcuma, Kaempferia* and *Boesenbergia*. Gingers are commonly grown in the tropical and subtropical climate of the South East Asia region such as Thailand, Indonesia, and Malaysia. These herbaceous plants are widely utilized in food and beverages, traditional medicines, and as decorative plants.

Boesenbergia rotunda (*B. rotunda*), a species of ginger found in Southeast Asia, India and China, is used in traditional medicines and as a flavouring in meals. A great deal of research on this plant had been carried out due to its positive medicinal properties, such as studies in isolation of novel compounds, investigation into its biological activities, and mass propagation through tissue culture techniques.

The research described in this thesis was conducted as part of a larger research programme (High Impact Research Programme UM.C/625/1/HIR/MOHE/SCI/19) that addresses the hypothesis that failure of *B. rotunda* suspension cells to regenerate into somatic embryos after long periods in *in vitro* culture is due to underlying molecular changes in the cells caused by deoxyribonucleic acid (DNA) methylation. To test the hypothesis, a multi-omics approach was undertaken to profile the genome, transcriptome, methylome and metabolome of *B. rotunda* samples. The metabolite profile data will be useful information as a basis for comparative profile study among various sample types and also a means to identify potential markers from the distinct metabolites or patterns within the profile.

Metabolites are small molecules ranging from 50 to 2000 Dalton (Da) and can be classified into primary metabolites and secondary metabolites. Primary metabolites include classes of sugars, lipids, amino acids, organic acids and phosphorylated organic

acids involved in fundamental functions such as growth, development, and reproduction. On the other hand, secondary metabolites consist of alkaloids, polyketides, terpenes and flavonoids involved mainly in plant defense mechanisms. Metabolite profiling can be performed using analytical platforms like Gas Chromatography Mass Spectrometry (GC-MS), Ultra Performance Liquid Chromatography Mass Spectrometry (UPLC-MS) and Capillary Electrophoresis Mass Spectrometry (CE-MS).

This thesis focused on metabolite profiling of seven *B. rotunda* sample types (conventionally propagated leaf, shoot base, embryogenic callus, dry callus, watery callus, suspension cells, and leaves from plants regenerated *in vitro*) using Ultra-Performance Liquid Chromatography Mass Spectrometry (UPLC-MS). The metabolite profiles of these tissues would allow the biochemical characterization of cell cultures that resisted growth under prolonged culture conditions, as well as identification of metabolite markers associated with embryogenesis. These data can be analyzed later together with gene expression and DNA methylation data for a more comprehensive study. The specific objectives of this thesis were:

1. To determine the metabolite profiles of various tissue types in *B. rotunda* by quantification of primary metabolites, secondary metabolites and hormones
2. To examine and document features of embryogenic and non-embryogenic calli of *B. rotunda* by scanning electron microscope (SEM), light and fluorescent microscopy using Periodic Acid-Schiff (PAS) reagent and diphenylboric acid-2-aminoethyl ester (DPBA), respectively

CHAPTER 2

LITERATURE REVIEW

2.1 *Boesenbergia rotunda* (*B. rotunda*)

The plant used in this study, *Boesenbergia rotunda,* is classified under the Zingiberaceae family and *Boesenbergia* genus. The common gingers, turmeric, melegueta pepper and galanga are examples of plant species classified under the same family. *B. rotunda* is commonly known as fingerroot ginger, Chinese keys or Chinese ginger (Figure 2.1). *B. rotunda* is an aromatic non-woody, monocotyledon plant with fleshy rhizomes. The pinkish-purple flowers are bisexual and have simple distichous leaves (Sirirugsa, 1999) with strong ascending veins. In addition, *B. rotunda* is consumed as a condiment in meals such as curries and soups (Tan *et al.*, 2012b). An example would be to cook the young leaves, shoots and rhizomes of *B. rotunda*, with coconut and spices (Facciola, 1990). It is also a traditional practice to blend the rhizomes of *B. rotunda* together and apply the paste to ease body pains and inflammation (Teron & Borthakur, 2013).

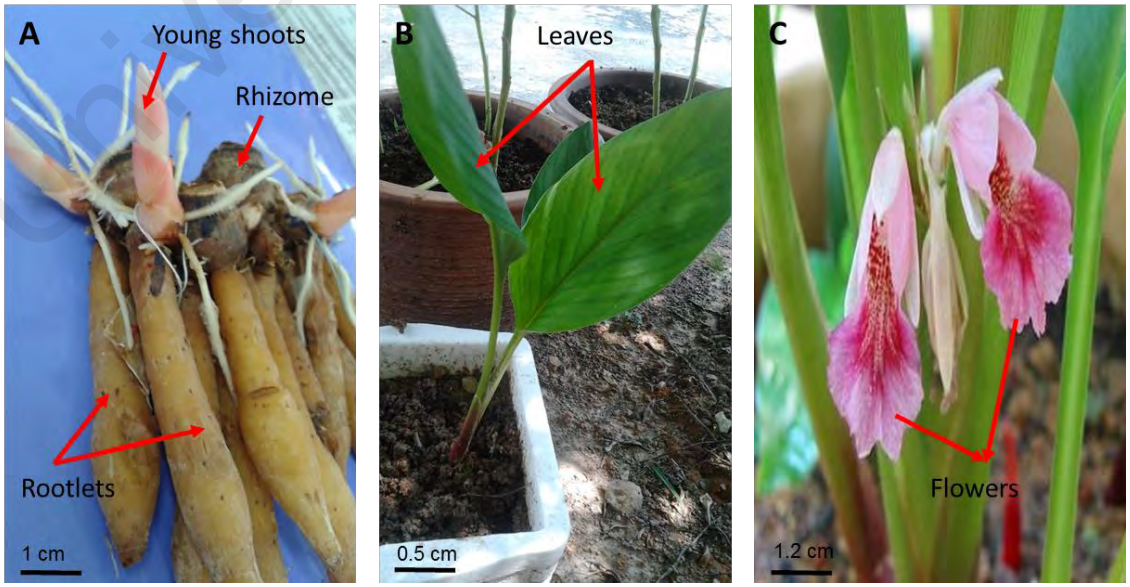

Figure 2.1: The plant organs of the *Boesenbergia rotunda* species; A: rhizome, young shoots and rootlets, B: leaves, C: flowers (author captured images)

2.2 Biological activities of *B. rotunda*

The importance of *B. rotunda's* various biological activities has been reported previously. *B. rotunda* is traditionally used as tonic for women after childbirth and to treat rheumatism, muscle aches, febrifuge, gout, stomach ulcers, and peptic ulcers (Tan *et al.*, 2012b). A recent study into the biological activities of essential oils showed that those from *B. rotunda* had better anti-bacterial activity against two Gram-positive (*S. aureus and B. cereus*) and two Gram negative bacteria species (*P. aeruginosa and E. coli*) compared to essential oils from *Curcuma mangga* (Baharudin *et al.*, 2015). Furthermore, a complete inhibition of larvicidal activity was observed with *B. rotunda* essential oils at an inhibitory concentration (IC_{50}) of 4.28% after 60 min of contact with the larvae of the mosquito vector for dengue and zika viruses, *Aedes aegypti* L. (Phukerd & Soonwera, 2013).

Apart from essential oils, flavonoid extracts from *B. rotunda* also exhibited promising anti-inflammatory, anti-microbial, and anti-viral effects (Tan *et al.*, 2012b). For example, panduratin A was identified to be the most potent cytotoxin against human pancreatic PANC-1 cancer cells compared to other six novel compounds isolated from *B. rotunda* (Win *et al.*, 2008). Furthermore, Kirana *et al.* (2007) showed that panduratin A at a concentration of 9 $\mu g.mL^{-1}$, completely inhibited growth of MCF-7 human breast cancer cells and HT-29 human colon adenocarcinoma cells. Another compound, boesenbergin A, was also found to exhibit anti-oxidative, cytotoxic and anti-inflammatory effects against three types of human adenocarcinoma cells (Isa *et al.*, 2012). In addition, pinostrobin extracted from rhizomes of *B. rotunda* exhibited anti-microbial (Bhamarapravati *et al.*, 2006), anti-ulcer (Abdelwahab *et al.*, 2011), anti-viral (Tewtrakul *et al.*, 2003) and anti-tumor (Morikawa *et al.*, 2008) properties. In the study conducted by Jing *et al.* (2010), extracts from rhizome of *B. rotunda* had the best cytotoxic activity

against the five cancer cell lines studied, compared to extracts from rhizome and leaves in other *Boesenbergia* species. Table 2.1 summarizes the five major flavanones and chalcones present in *B. rotunda,* and their reported biological activities.

Table 2.1: Biological activities reported for flavanones and chalcones from *B. rotunda*

Compound	Sources	Bioactivity	References
Panduratin A (Chalcones)	Rhizomes	Positive heptatoprotective activity in WRL-68 liver cell line, specifically in thioacetamide-induced cytotoxicty	(Salama *et al.*, 2013)
		Attenuated high fat diet-induced obesity in C57BL/6J mice by activating AMP-activated protein kinase as potent antiobesity agent	(Kim *et al.*, 2012)
		Antiangiogenic potential evidenced in two *in vivo* models, zebrafish (15μM) and murine Marine plug (5 μM) model induced with Panduratin A	(Lai *et al.*, 2012)
		Inhibited A549 human nonsmall cell lung cancer lines with IC_{50} of 4.4 $ug.mL^{-1}$	(Cheah *et al.*, 2011)
		Consumption of 50 $mg.kg^{-1}.day^{-1}$ stimulates AMP-activated protein kinase (AMPK) signaling that prevents lipid synthesis by increasing fatty acid oxidation activity	(Kim *et al.*, 2011)
		Patented the mouthwash formulation that is capable of reducing bad breath by 70-90%	(Hwang *et al.*, 2010)
		Capable of inhibiting biofilm formation by *Enterococci* bacteria that caused urinary and intestinal infection	(Rukayadi *et al.*, 2009a)
		Possess anti-microbial activities against clinical *Staphylococcus* strains	(Rukayadi *et al.*, 2009b)
		Inhibited nitric oxide (NO), prostaglandin E2 (PGE2) and tumor necrosis factor alpha (TNF-α) that causes inflammatory in human	(Tewtrakul *et al.*, 2009)
		High inhibition of *HIV-1 protease*, a promising drug target to combat HIV/AIDS compared to other bioactive compounds	(Cheenpracha *et al.*, 2006)

Table 2.1, continued

Compound	Sources	Bioactivity	References
Panduratin A (Chalcones)	Rhizomes	15 μM of panduratin A successfully inhibited lipid peroxidation in rat brain homogenate	(Shindo *et al.*, 2006)
		Treatment against prostate cancer cell lines in both parameters; time and dosage	(Yun *et al.*, 2006)
		Protective agent against *tert-butylhydroperoxide* (t-BHP) which is a precursor for lipid peroxidation activity	(Sohn *et al.*, 2005)
		Effective anticariogenic activity against oral bacteria (*Streptococcus mutans*) that caused tooth decay	(Hwang *et al.*, 2004)
		94% anti-inflammatory showed in 12-O-tetradecanoylphorbol-13-acetate (TPA) ear oedema in rats	(Tuchinda *et al.*, 2002)
	Roots and rhizomes	Potent antimutagenic effect against *Salmonella typhimurium* bacteria	(Trakoontivakorn *et al.*, 2001)
Cardamonin (Chalcones)	Rhizomes	Inhibited nitric oxide (NO), prostaglandin E2 (PGE2) and tumor necrosis factor alpha (TNF-α) that causes inflammatory in human	(Tewtrakul *et al.*, 2009)
		Cytotoxic activity against HL-60 cancer cell lines (human promyelocytic leukemia)	(Sukari *et al.*, 2007)
		High inhibition of *HIV-1 protease*, a promising drug target to combat HIV/AIDS compared to other bioactive compounds	(Cheenpracha *et al.*, 2006)
		Potent antimutagenic effect against *Salmonella typhimurium* bacteria	(Trakoontivakorn *et al.*, 2001)

Table 2.1, continued

Compound	Sources	Bioactivity	References
Pinostrobin (Flavanones)	Rhizomes	Reduction of ulcer area and mucosal content in rats	(Abdelwahab *et al.*, 2011)
		No toxicity observed in mice after consumption whereby similar haematological and histopathological parameters noted	(Charoensin *et al.*, 2010)
		Inhibition of Ca^{2+} signaling in yeast model that play roles cell regulation	(Wangkangwan *et al.*, 2009)
		Cytotoxic activity against HL-60 cancer cell lines (human promyelocytic leukemia)	(Sukari *et al.*, 2007)
		IC_{50} of 125 ug.mL^{-1} significantly reduced growth of *Helicobacter pylori* bacteria that caused gastritis	(Bhamarapravati *et al.*, 2006)
		94% anti-inflammatory showed in 12-O-tetradecanoylphorbol-13-acetate (TPA) ear oedema in rats	(Tuchinda *et al.*, 2002)
		Potent antimutagenic effect against *Salmonella typhimurium* bacteria	(Trakoontivakorn *et al.*, 2001)
Pinocembrin (Flavanones)	Rhizomes	No toxicity observed in mice after consumption whereby similar haematological and histopathological parameters noted	(Charoensin *et al.*, 2010)
		Cytotoxic activity against HL-60 cancer cell lines (human promyelocytic leukemia)	(Sukari *et al.*, 2007)
		94% anti-inflammatory showed in 12-O-tetradecanoylphorbol-13-acetate (TPA) ear oedema in rats	(Tuchinda *et al.*, 2002)
		Potent antimutagenic effect against *Salmonella typhimurium* bacteria	(Trakoontivakorn *et al.*, 2001)
Alpinetin (Flavanones)	Rhizomes	Cytotoxic activity against HL-60 cancer cell lines (human promyelocytic leukemia)	(Sukari *et al.*, 2007)

2.3 *In vitro* culture of *B. rotunda*

Tissue culture is a technique applied in agricultural biotechnology that allows a time-efficient means to mass-propagate desirable plant materials, as an alternative to conventional breeding and propagation programmes. Thorpe (2007) reported the application of tissue culture in five major areas: (1) the study of cell behavior (involved in cytology, primary metabolism, secondary metabolism and morphogenesis), (2) plant modification and improvement, (3) production of pathogen-free plants and for germplasm storage, (4) clonal propagation and (5) for the production of specific compounds of interest.

For *B. rotunda*, optimization of tissue culture conditions to promote callusing, embryogenesis and plant regeneration were carried out previously. Initial studies on *B. rotunda* tissue culture focused on increasing the biomass multiplication rate with use of growth regulators such as auxin: 2,4-dichlorophenoxyacetic acid (2,4-D), indole-3-acetic acid (IAA) and cytokinin: kinetin, benzylamino purine (BAP) supplemented together into the Murashige and Skoog media. A study by Tan *et al.* (2005) revealed that the utilization of a single medium, comprised of Murashige Skoog base media supplemented with 4.52 µM of 2,4-D, increased the embryogenic callus formation rate by 46% and this is essential for future research work on cell suspension cultures. Another study showed an increment of 50% in shoot regeneration with a Murashige Skoog media supplemented with 2.0 $mg.L^{-1}$ of BAP (Yusuf *et al.*, 2011). Yusuf *et al.* (2011) also reported that a mixture of auxin and cytokinin with concentrations of 3.0 $mg.L^{-1}$ 2,4-D and 2.0 $mg.L^{-1}$ BAP in the Murashige Skoog base media yielded the best embryogenic callus induction and plantlet regeneration from the meristem of *B. rotunda*. A more recent study by Wong *et al.* (2013) on *B. rotunda* cell suspension culture regeneration reported that the combination of 1.0 $mg.L^{-1}$ 2,4-D and 0.5 $mg.L^{-1}$ BAP supplemented in the Murashige Skoog base media yielded a 12-fold increase in cell volume during the culture period. Additionally in the

study, more than 50% of somatic embryos germinated successfully and were able to grow into plantlets on media supplemented with 3.0 $mg.L^{-1}$ BAP and 1 $mg.L^{-1}$ naphthalene acetic acid (NAA) (Wong *et al.*, 2013).

The tissue culture process also enables manipulation of clonal materials through various culture conditions or genetic engineering to produce desirable metabolites. For example, the production of anthocyanin increased in *Vitis vinifera* (wine grape) cell culture with the addition of jasmonic acid as an elicitor (Curtin *et al.*, 2003). Furthermore, the addition of precursor, phenylalanine in the cell culture system was shown to lead to accumulation of rosmarinic acid in *Coleus blumei* (painted nettle) plant culture (Petersen, 1997). In *B. rotunda*, Tan *et al.* (2012a) reported more than 100% increase in flavonoid yield upon addition of the precursor, phenylalanine and that the level of flavonoids increased dose-dependently with increased phenylalanine concentrations.

2.4 Plant Metabolites

Plant metabolites are small organic compounds that are the product of plant cell metabolism. There are two major classes of metabolites; primary and secondary. To date, no metabolite study has been reported for *B. rotunda* except for proteome and transcriptome studies which highlight the regulation of proteins and genes involved upon phenylalanine treatment, respectively (Md-Mustafa *et al.*, 2014; Tan *et al.*, 2012a). At present, only the bioactive compounds derived from secondary metabolites have been widely reported for *B. rotunda*.

2.4.1 Primary metabolites

Primary metabolites essentially regulate, or are the building blocks of overall plant growth and development while secondary metabolites have specific functions such as signaling or defense mechanisms against predators (Smetanska, 2008). Irchhaiya *et al.*

(2015) reported that the absence of primary metabolites can lead to death in plants but that this was not the case for secondary metabolites.

Primary metabolites are the major organic compounds synthesized by cells and are needed for physiological functions such as transpiration, photosynthesis and respiration (Roessner & Pettolino, 2007). Common primary metabolites are sugars, amino acids, organic acids, phosphorylated organic acids and lipids. Glucose is a source of energy for cellular respiration while amino acids are essential components of the peptides and proteins used in structural support, nutrient transport, enzymes and other cellular activities. Lipids function in secondary energy storage and as structural components of plants.

Primary metabolites are involved in important metabolic pathways that include glycolysis metabolism, amino acid metabolism, fatty acid metabolism and tricarboxylic acid (TCA) metabolism. In the TCA pathway, metabolites such as malic acid, succinic acid and citric acid are responsible for oxidation of respiratory substrates to drive adenosine triphosphate (ATP) synthesis (Sweetlove *et al.*, 2010). Additionally, in purine and pyrimidine metabolism, metabolites like adenine, guanine, uracil and thymine are involved as major energy carriers and precursors of nucleotide cofactor synthesis (Moffatt & Ashihara, 2002). Primary metabolites also function as precursors for secondary metabolite biosynthesis. Specifically for *B. rotunda*, the primary metabolite study in this thesis could enable the identification of essential metabolites needed for growth, development and as precursors of secondary metabolites of interest.

2.4.2 Secondary metabolites

Plant extracts contain secondary metabolites or compounds with potential biological activities. Secondary metabolites generally make up less than 1% of a plant's dry weight (Smetanska, 2008) and are not involved directly in plant growth and

development. Secondary metabolites can be categorized into groups of terpenes, alkaloids, phenolics, glycosides, polyketides and more. Alkaloids are well known for their toxicity to pests, microorganisms, herbivores, animals and human (Matsuura & Fett-Neto, 2015), and are part of the defensive mechanisms of plants (Kabera *et al.*, 2014; Lu *et al.*, 2012) while terpenes were reported as insecticides and defense responses against plant pathogens (Tholl, 2015). The plant based terpenoids have also been exploited by humans in the food industry (Caputi & Aprea, 2011) as flavours while in the cosmetic and perfumery industry as fragrances (Bauer *et al.*, 2008; Kumari *et al.*, 2014). In contrast, phenolic compounds have been reported to play central roles in plant defense against insects and pathogens (Barakat *et al.*, 2010; Daayf *et al.*, 2012; Lattanzio *et al.*, 2006; Sharma *et al.*, 2009). Phenolics cover a wide range of complex aromatic compounds of various classes with the most basic structure of a 6-carbon skeleton known as benzoquinones. Flavonoids are a group under the phenolic class with a basic 15-carbon skeleton structure that consists of two phenyl rings linked via one heterocyclic pyrane ring (Balasundram *et al.*, 2006; Baxter *et al.*, 1998; Harborne, 1989).

Flavonoids had been extensively studied since the early 20th century. Compounds isolated from this group have roles as pigments for flowers colouration and petals to attract pollinators. These compounds also function as a protective agent against UV radiation, possesses antimicrobial and antiherbivory effects (Dixon & Pasinetti, 2010). Flavonoids can be classified into various groups including anthocyanins, flavones, flavonols, chalcones, aurones, flavanones, dihydroflavonols, isoflavones, and biflavonoids (Iwashina, 2000) (Figure 2.2). Within each class, groups of flavonoids have different side chain substitutions that include hydroxyl, methoxy and glycosyl groups (Kumar & Pandey, 2013).

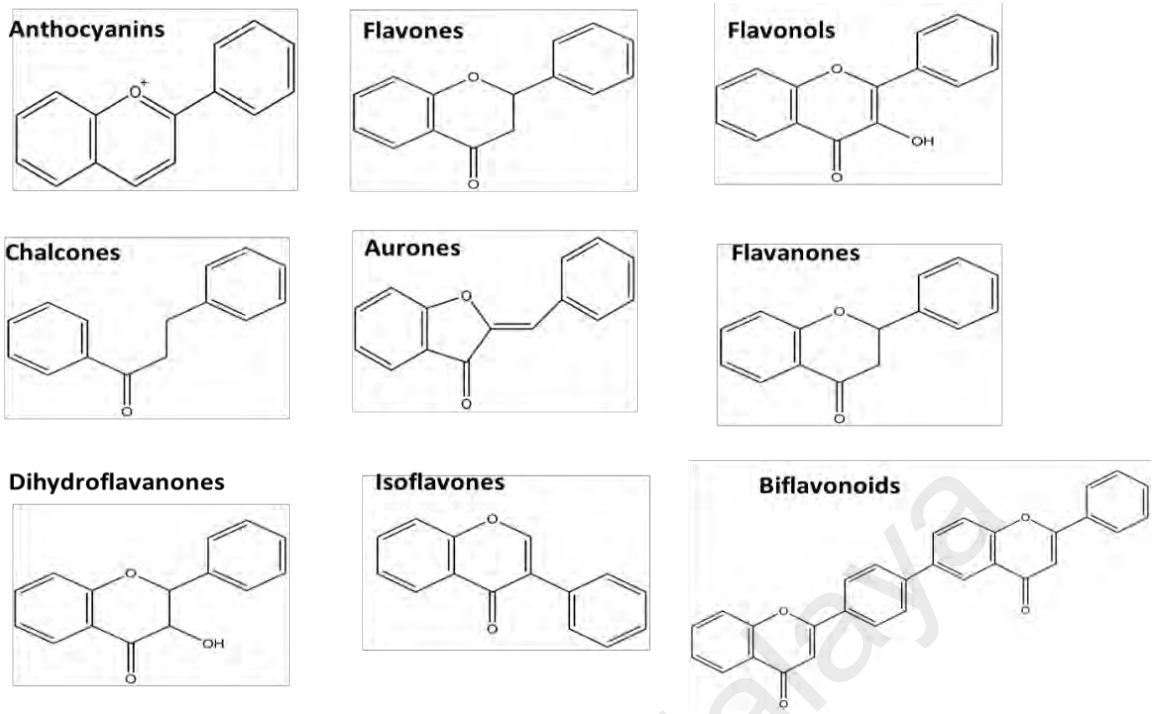

Figure 2.2: Various classification of flavonoids (Iwashina, 2000)

Flavonoid biosynthesis begins in the phenylpropanoid pathway with the amino acid phenylalanine as the precursor (Figure 2.3). Chalcone synthase (CHS) is the first specific enzyme in the flavonoid pathway and produces chalcone scaffolds from which all flavonoids are derived (Falcone Ferreyra *et al.*, 2012). Subsequently, a group of enzymes including isomerases, reductases and hydroxylases modify the basic flavonoid structure to form other flavonoid derivatives (Martens *et al.*, 2010). Finally, transferase enzymes alter the flavonoid backbone with sugars, methyl or acyl groups (Bowles *et al.*, 2005; Ferrer *et al.*, 2008).

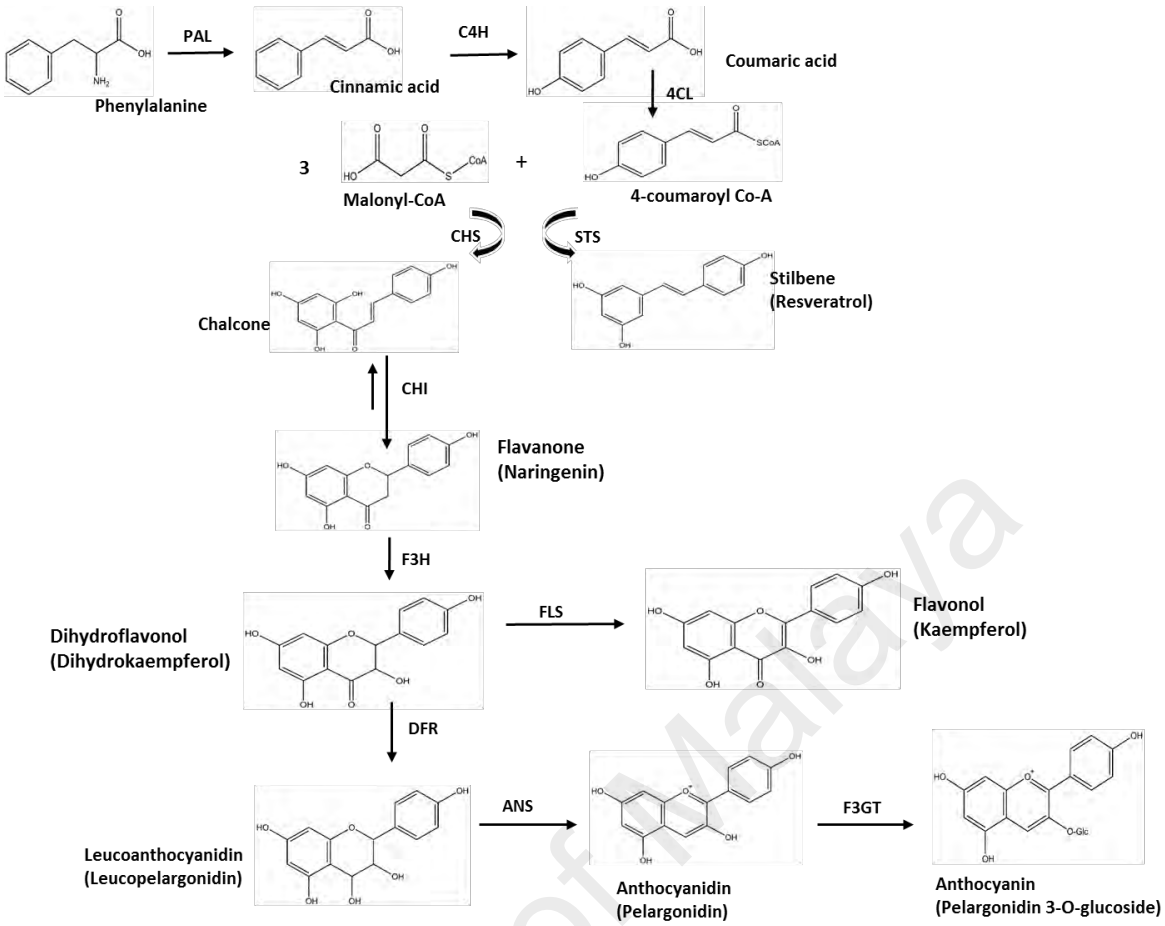

Figure 2.3: The pathway of flavonoid biosynthesis derived from phenylalanine as precursor. PAL: phenyl ammonium lyase, C4H: cinnamate 4-hydroxylate, 4CL: 4-coumaroyl-CoA ligase, CHS: chalcone synthase, STS: stilbene synthase, CHI: chalcone isomerase, F3H: flavanone 3-hydroxylase, FLS: flavonol synthase DFR: dihydroflavonol reductase, ANS: anthocyanidin synthase, F3GT: flavonoid 3-O-glucosyltransferase (Falcone Ferreyra *et al.*, 2012; Winkel-Shirley, 2001)

2.4.3 Plant hormones

Plant hormone profiles have been reported for several crops, including oranges (Almeida Trapp *et al.*, 2014), rosemary leaves (Müller & Munné-Bosch, 2011), *Arabidopsis* seeds (Kanno *et al.*, 2010), rice (Kojima *et al.*, 2009) and lettuce seeds (Chiwocha *et al.*, 2003) but not for *B. rotunda* to date. Plant hormones work together mutually in influencing and regulating overall plant growth and development (Gaspar *et al.*, 1996; Wang & Irving, 2011). Plant hormones are small diffusible molecules that easily penetrate cells (Wang & Irving, 2011) and are needed in small quantities. Plant hormones also act in mediating defense responses against pests and pathogens (Studham & MacIntosh, 2012). Typically plant hormones are classified into auxins, cytokinins, gibberellins, ethylene, abscisic acid, salicylic acid and jasmonic acid.

At the cellular level, auxins promote cell division, elongation, differentiation as well as the formation of organs from unorganized tissues. A recent study reported that auxin can also be used as a balanced cocktail with other growth regulators like cytokinins to enhance cell proliferation in *in vitro* propagation (Paque & Weijers, 2016). Naturally occurring auxins like indole-3-acetic acid (IAA), 4-chloroindole acetic acid (4-Cl-IAA) and phenylacetic acid (PAA) have been found in plants while the synthetic auxin like 2,4-D and NAA have IAA-like activity and are widely used in agriculture research. IAA is also reported to increase in Royal Gala apples throughout fruit development, with higher auxin content in seeds than in fruit cortex (Devoghalaere *et al.*, 2012). Additionally, exogenous application of IAA was found to enhance the expression of chalcone synthase (CHS) protein and increased total flavonoid content in grape berries (Luo *et al.*, 2016). IAA has also been reported to play a role in callus proliferation in white spruce (Kong *et al.*, 1997) and carrot calli (Michalczuk *et al.*, 1992b). Liu *et al.* (1998) reported the accumulation of endogenous IAA during adventitious root formation after exogenous treatment with naphthalene acetic acid (NAA) and IAA in soybean hypocotyl.

Furthermore, a four-fold increase in endogenous IAA content was observed in immature zygotic sunflower embryos under embryogenic conditions (Charrière *et al.*, 1999). Further evidence from the immuno-cytochemical localized IAA of sunflower somatic embryos at three phases of induction (before, during and after) supported that endogenous auxins could be one of the initial signals leading to somatic embryogenesis (Thomas *et al.*, 2002).

Cytokinins are responsible for chloroplast maturation, protein synthesis, leaf expansion (George *et al.*, 2008), lateral bud growth and delayed senescence (Gaspar *et al.*, 1996). Usually, auxins and cytokinins jointly regulate cell division with each hormone influencing different phases in the cell cycle (Gaspar *et al.*, 1996; Su *et al.*, 2011). A study reported that auxin and cytokinin control events of cell specifications like establishment of apical-basal axis in *Arabidopsis* (Möller & Weijers, 2009; Muller & Sheen, 2008). Veselý *et al.* (1994) also reported that auxins affect DNA replication while cytokinins control events leading to mitosis. A study on red globe grapes showed that endogenous cytokinin levels were reduced in rigid abnormal fruits after treatment with gibberellic acid (GA_3) and benzylamino purine (BAP) (He *et al.*, 2009). In addition, cytokinins also promoted cell division in the quiescent center of root apical meristem of *Arabidopsis* (Zhang *et al.*, 2013). Previous studies reported that cytokinins have an effect on callus growth rates but not on embryogenesis (Ernst & Oesterhelt, 1985; Jiménez & Bangerth, 2001). Cytokinins were also reported to affect fruit development of grape berries (Davies & Böttcher, 2009) and in oil palm mesocarp (Teh *et al.*, 2014). In the oil palm fruit development, high level of endogenous *trans*-zeatin was found at the early stage of fruit development but decreased rapidly at the fruit maturation stage (Teh *et al.*, 2014).

Other essential plant hormones are gibberellins, abscisic acid (ABA) and ethylene. Gibberellins (GA) stimulate developmental responses such as seed germination, fruit development, and sex determination, while ABA functions in the uptake of water and

ions, stomatal regulation, and leaf abscission (Moshkov *et al.*, 2008). In lettuce seeds, the endogenous level of GA increased with the increased in duration of light exposure (Toyomasu *et al.*, 1993). Gao *et al.* (2010) found an antagonistic interaction between GA and ABA in rapeseed. A similar interaction was observed in oil palm lipid biosynthesis in which high endogenous ABA with low GA was reported at the early maturation stage (Teh *et al.*, 2014). In *Arabidopsis*, the high ABA/GA ratio resulted in seed maturation, embryo growth and germination whereby high abundance of ABA was found when embryo enters the maturation stage (del Carmen Rodríguez-Gacio *et al.*, 2009; Finkelstein *et al.*, 2002). High endogenous levels of ABA were reported in embryogenic callus of carrots (Nishiwaki *et al.*, 2000) and white spruce (Kong *et al.*, 1997) but the opposite trend was shown in yellow alfafa (Ivanova *et al.*, 1994).

The essential role of ethylene is for fruit ripening, but also many aspects of complex plant growth development including seed germination, root development, shoot and root growth, flowering and formation of adventitious roots (Abeles *et al.*, 1992; Pech *et al.*, 2012). Usually, ethylene is biologically active at low concentrations from 0.01-1.00 ppm to trigger a response but the concentration varies in different plant species (Chang, 2016). For example, tomatoes and apples can generate tens of ppm ethylene (Chang, 2016).

2.4.4 Organic compounds from *B. rotunda*

Compounds reported to be present in *B. rotunda* are grouped into polyphenols, flavonoids and volatile organic compounds such as essential oils. These compounds are mainly extracted from the rhizomes of *B. rotunda*.

Baharudin *et al.* (2015) reported three major essential oils present in *B. rotunda* that constituted 0.24% fresh weight from the total of 0.27% fresh weight extracted from rhizome, and included nerol (0.11% fresh weight), *L*-camphor (0.10% fresh weight) and

cineole (0.03% fresh weight). However, Sukari *et al.* (2008) reported a higher percentage of camphor (58% of dry weight) from the total oxygenated monoterpene derivatives extracted in *B. rotunda* rhizome. Other essential oils that were successfully isolated from rhizomes of *B. rotunda* are geraniol, ocimene and camphene (Jantan *et al.*, 2001). Figure 2.4A shows the structure of major volatile organic compounds extracted from *B. rotunda*.

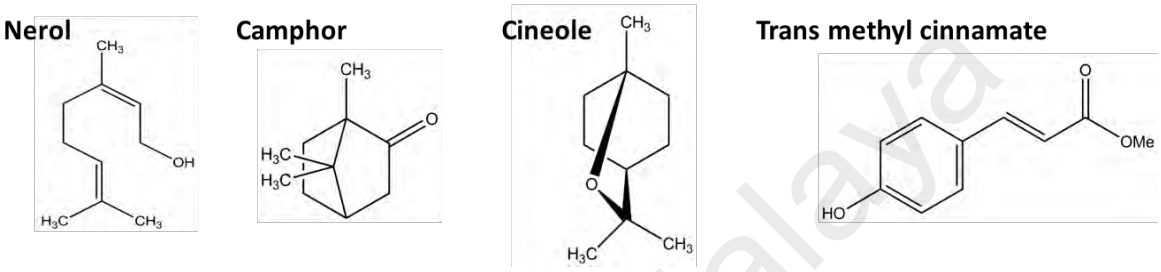

Figure 2.4A: Structures of major volatile organic compounds present in *B. rotunda* (Baharudin *et al.*, 2015)

Besides volatile organic compounds, various polyphenols were also extracted from *B. rotunda*. Jing *et al.* (2010) reported that hesperidin, naringin and kaempferol are the abundant polyphenols present in *B. rotunda*. Common polyphenols present in *B. rotunda* are as shown in Figure 2.4B.

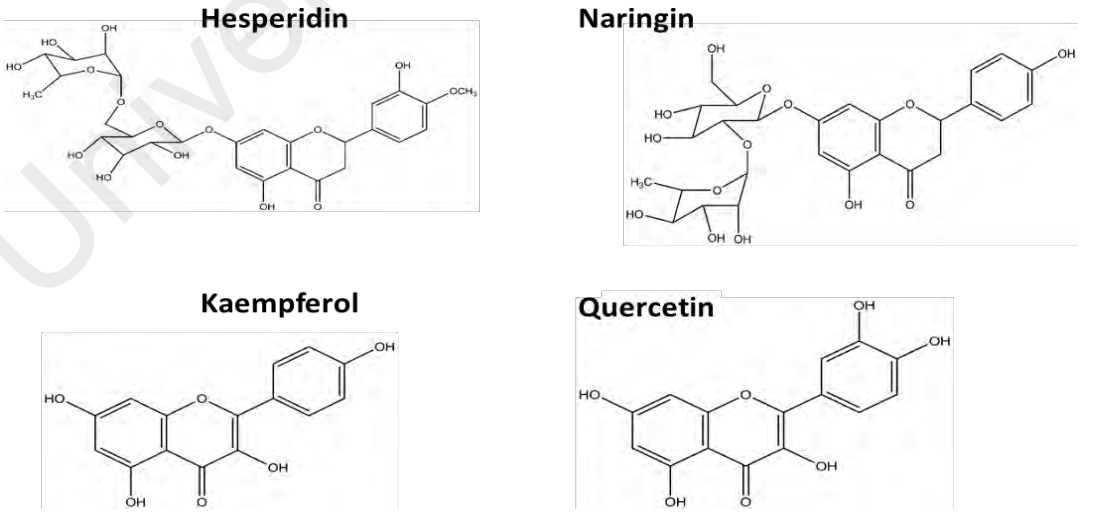

Figure 2.4B: Structures of major polyphenols present in *B. rotunda* (Jing *et al.*, 2010)

A wide range of flavonoids have been identified in *B. rotunda*, however only five flavonoid-related compounds (three flavanones and two chalcones) (Figure 2.4C) were selected as a focus in this study due to their previous reported biological activities (Tan *et al.*, 2012b). Panduratin and cardmonin are derivatives of chalcone, while pinostrobin, pinocembrin and alpinetin are derivatives of flavanones. Several researchers had successfully isolated pinostrobin and pinocembrin (Ching *et al.*, 2007; Sukari *et al.*, 2007; Tewtrakul *et al.*, 2003), as well as panduratin A (Cheenpracha *et al.*, 2006; Mahidol *et al.*, 1984; Shindo *et al.*, 2006; Trakoontivakorn *et al.*, 2001) from *B. rotunda*. Of the five compounds, only the biosynthesis pathway for pinostrobin and pinocembrin are known (Hwang *et al.*, 2003; Jiang *et al.*, 2005; Tan *et al.*, 2015).

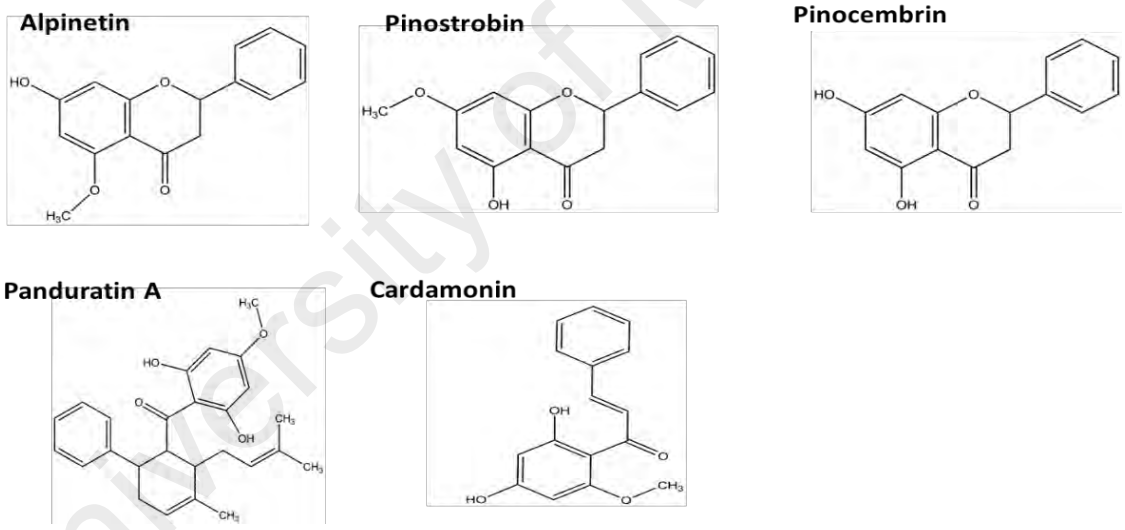

Figure 2.4C: Structures of flavanone (top) and chalcones (bottom) present in *B. rotunda* (Kiat *et al.*, 2006; Morikawa *et al.*, 2008)

2.5 Metabolite profiling

Metabolite profiling can now be performed with the increased sensitivity and selectivity of chromatographic methods and detectors available. Metabolite profiling can be classified into targeted and non-targeted approaches (Fiehn, 2006). In the targeted approach, specific metabolites are identified and quantified based on available standards, while in the non-targeted approach, all detectable metabolites are quantified relatively

using high resolution mass spectrometry. Further analysis to identify compounds of interest is then required, which may lead to the discovery of novel compounds.

Previous studies have reported the use of metabolomics platforms to investigate the biochemical changes in crops like sugarcane (Mahmud *et al.*, 2014), soybean (Aliferis *et al.*, 2014), tomato (Roessner-Tunali *et al.*, 2003), *Arabidopsis* (Fiehn, 2002) and oil palm (Neoh *et al.*, 2013; Teh *et al.*, 2013). Besides that, metabolomics platforms can be used to identify key biomarkers of embryogenesis from somatic embryos (Businge *et al.*, 2012; Dowlatabadi *et al.*, 2009), as well as to find metabolites associated with salt stress responses (Zhao *et al.*, 2014) and drought tolerance (Juhász *et al.*, 2014). Prior to this study, no findings were reported on the metabolite profile of various *B. rotunda* samples and the association with embryogenesis and plant regeneration.

Metabolite profiling can be performed using the following three steps; sample extraction, sample analysis (separation and detection) and data analysis. The two common analytical protocols for metabolite profiling are solid phase extraction and liquid-liquid extraction. Solid phase extraction utilizes a suitable adsorbent material such as cross linked-copolymers, graphitized carbons and specific n-alkylsilicas (Andrade-Eiroa *et al.*, 2016; Hennion, 1999) to separate the analyte into various fractions which helps to reduce the complexity of the sample matrix and increases the sensitivity of the analyte. A recent study showed that the usage of C18 adsorbent for solid phase extraction methods resulted in the best fractionation of polar and non-polar phases in urine samples compared to C8 adsorbent (Michopoulos *et al.*, 2015). In another study, the solid phase extraction method was coupled with a high resolution time-of-flight mass spectrometry (TOF-MS) to obtain greater insights of phenolic compounds that are present in black, green and white tea extracts (van der Hooft *et al.*, 2012). Theodoridis *et al.* (2011) also used a solid phase extraction method to facilitate separation of primary metabolites which had higher abundance compared to secondary metabolites present in grape extracts. For the liquid-

liquid extraction techniques, metabolites are extracted using various types of solvents. Common extraction solvents used are methanol and hexane for polar and non-polar metabolites, respectively (Hosp *et al.*, 2007; Kupchan *et al.*, 1973; Palama *et al.*, 2010; Strehmel *et al.*, 2014) or in mixtures with chloroform (Kim *et al.*, 2010; Teh *et al.*, 2013). Furthermore, an addition of butylated hydroxyl toluene (BHT) is also common during the extraction process to prevent oxidation of metabolites (Barnes *et al.*, 2016; Fan, 2012). Following from the extraction process, the dry extracts are then subjected for separation of individual components on the basis of affinity of analyte in the stationary and mobile phase. The stationary phase is either in solid or liquid form while the mobile phase is either in liquid or gas form. Common chromatographic instruments used for separation are gas chromatography (GC) and liquid chromatography (LC). The GC and LC utilize gases and liquids respectively, as mobile phase while both use columns as the stationary phase for compound separation. Several studies report separation strategies for polar and non-polar metabolites in plants and bacteria (Fiehn, 2006; Patti, 2011; Yanes *et al.*, 2011). Additionally, capillary electrophoresis (CE) is also used for chromatographic separation, based on electrophoretic mobility (charge of the molecule, viscosity and electron radius) and application of voltage. Subsequent to the instrumental analysis, the separated compounds are detected using various detectors available such as the mass spectrometry (MS), time of flight (TOF) and triple quadrupole (QQQ). Each of these detectors has unique features and functions that distinguish their application. Nowadays, the combination of various chromatographic instruments and detectors provide capabilities for separation and identification of complex sample matrices (Barding *et al.*, 2013; Kopka *et al.*, 2004; Metz *et al.*, 2007; Shulaev, 2006). Hence, careful consideration of these instruments prior to utilization is necessary to meet experimental objectives as each has its own advantages and disadvantages. Sample analyses can be carried out in quantitative or qualitative mode (Fiehn *et al.*, 2000; Sumner *et al.*, 2003; Verpoorte *et al.*, 2007). Full

quantification of samples can be performed with a set of prepared standards at various concentrations. Then, the samples are quantified using calibration curves developed from prepared standards. In contrast, the qualitative analyses utilize an internal standard to normalize errors that persist during extraction and instrumental analyses. Subsequent to the sample analyses, normalized data are reported in relative abundance. Tugizimana *et al.* (2013) reported that measurement of both qualitative and quantitative data in metabolomics study reflects the cellular state under defined conditions and provide understanding into cellular processes that control the biochemical phenotypes of the cells, tissues or whole organisms. Further, Dias *et al.* (2015) and Wiggins *et al.* (2016) both reported quantitative measurements in their study of chick pea and a small tropical shrub, respectively using metabolomics approach. Finally, the data obtained are interpreted and visualized using various statistical tools. One report reviewed several analytical methods in metabolomics approach from spectral processing to data analysis including univariate and multivariate analysis and finally biomarker discovery through performance assessment and model validation (Alonso *et al.*, 2015). Most instruments usually include data processing and alignment software to handle large mass spectrometry data such as Enhanced Chemstation Data Analysis software for Agilent (California, United States) GC-MS and Target Lynx software for Waters (Massachusetts, United States) UPLC-MS. The data interpretation can be performed using multivariate analysis like Umetrics SIMCA (Malmo, Sweden), ANOVA by IBM SPSS (New York, United States), R, MiniTab and others. These tools are useful and readily available. A recent study reported a developed R-Tool for metabolomics study to analyzed data from initial pre-processing to downstream association analysis (Liang *et al.*, 2016).

2.6 Histology of *B. rotunda*

Histology is an important study to discover, classify and describe morphological changes in the plant body and overall plant organization (Yeung, 1999). Histology is also a very useful tool for the study of plant morphogenesis (Wetmore & Wardlaw, 1951) in understanding the plant growth and differentiation mediated by cell division that resulted from a complex spatial and temporal hormonal control, which occurs through expression of multiple gene systems (de Almeida *et al.*, 2015). However in plant tissue culture, the plant morphogenesis link is disrupted (de Almeida *et al.*, 2015) where tissues are exposed to various culture conditions and this is so for *B. rotunda*. Therefore in this study, the metabolite data was complemented with histological study to provide a baseline knowledge towards understanding conditions and activities of cultured cells.

Histological studies provide microscopic details of cells while morphological studies determine gross structures, forms and features of multicellular organisms, including plants. Common microscopic instruments used in plant histology are light and fluorescent microscopy. Both types of study are performed through examination of cells under a microscope with the utilization of different stains to enhance features of cells, tissues and structures. The use of specific staining techniques can also highlight metabolic processes, localize compounds within cells and tissues as well as differentiate between living and dead cells (Table 2.2). A recent publication assessed past stains available and how staining techniques have been improved now (Alturkistani *et al.*, 2015). A recent study in sections of the hypocotyl of matured zygotic embryos of *Eucalyptus globulus* have revealed shoot formation via organogenesis instead of embryogenic with use of Periodic Acid-Schiff (PAS), toluidine blue and Sudan IV stain (Dobrowolska *et al.*, 2016). Further, Senthil Kumar and Nandi (2015) confirmed the formation of apical meristem and leaf primodium from the organogenic callus of *Asteracantha longifolia* Nees, a medicinal herb using crystal violet stain examined under a light microscope.

Hutzler *et al.* (1998) reported localization of phenolic compounds in cell walls, vacuoles and cell nuclei in Norway spruce trees using a confocal laser scanning microscope. Additionally, a histochemcial study in *Ocimum basilicum* (sweet basil) leaves reported presence of lipids which are concentrated in the glandular trichomes using Sudan IV stain (Amaral-Baroli *et al.*, 2016).

Table 2.2: Common stains and corresponding staining features and colour

Stain	Features	Colour	References
Eosin	Stain red blood cells, extracellular structures	Pink or red	(Bancroft JD 2013; Fischer *et al.*, 2008)
Haematoxylin	Stain nuclei	Blue	
PAS (Periodic Acid-Schiff)	Stain starch reserves	Redish purple	(Bancroft JD 2013; Baum, 2008)
Sudan red	Stain lipids	Redish orange	(Brundrett *et al.*, 1991)
Toluidine blue	Stain polysaccharides	Pinkish purple	(Retamales & Scharaschkin, 2014)
	Stain nuleic acid	Purplish	
	Stain phenolics (tannins, lignin)	Greenish blue	
Acetocarmine and Evan's Blue (double stain)	Stain embryogenic massses	Blue	(Gupta & Holmstrom, 2005)

In *B. rotunda*, three histological studies were previously reported using double stained red acetocarmine and Evan's blue (Gupta & Holmstrom, 2005). The embryogenic callus had absorbed the intense red of acetocarmine while the spongy callus were stained blue (Tan *et al.*, 2005). Yusuf *et al.* (2011) reported similar observations of embryogenic callus culture derived from shoot base tissue. In contrast, Wong *et al.* (2013) used the Periodic Acid-Schiff (PAS) reagent to view different stages of somatic embryo development from suspension culture of *B. rotunda.* Ultimately, the reported histological studies for *B. rotunda* provided a confirmation of the embryogenic competency in its callus culture.

Periodic Acid-Schiff reagent is used to view starch reserves. The starch in cells stains purplish pink while the nuclei stain blue. Usually, active and proliferative cells as found in embryogenic cells will contain rich starch granules. The embryogenic callus of coffee (Ribas *et al.*, 2011), olives (Mazri *et al.*, 2011), banana (Xu *et al.*, 2011) and date palm (SanÉ *et al.*, 2006; Zouine *et al.*, 2005) are examples of other crops studied with PAS staining. In an oil palm study with PAS stain, the shoot apical meristem of oil palm stained deep pink in the cell walls indicating that these cells are enriched with polysaccharides (Jouannic *et al.*, 2011).

Flavonoids (colour pigments) can also be stained using specific dyes to identify the location of these compounds in tissues. Buer *et al.* (2007) reported the use of diphenylboric acid 2-amino ethyl ester (DPBA) to stain mainly flavonols and flavonoids derivatives such as quercetin, kaempferol, naringenin and dihydroxy kaempferol, which can be differentiated by colour under fluorescent microscopy. Furthermore, the DPBA conjugates also fluoresce at various intensities; it was reported that quercetin-DPBA complex fluoresces eight times brighter than kaempferol-DPBA complexes (Peer *et al.*, 2001).

Plant histology studies can also be conducted using a scanning electron microscope (SEM). SEM provides information of cell surfaces to enable the determination of cell shapes, sizes and structures as an indicator of potential regeneration in tissue culture (Narciso & Hattori, 2010). SEM has also been used for plant pathology such as to characterize disease of the black resting sclerotia from the *Trichoderma asperellum*, fungus strain T34, a plant pathogen that infects lettuce (Cortadellas *et al.*, 2013). Roy *et al.* (1996) showed that a naturally occurring bacteria infecting spinach leaves is easily visible with SEM due to the absence of extracellular polysaccharides. Previous SEM studies of cell surface features which were documented with an association with embryogenesis, include extracellular matrix surface network and fibrils in rice

(Bevitori *et al.*, 2014), membranous, fibrillar structure and granules of mucilage-like material in oil palm (Palanyandy *et al.*, 2013) and regions covered with extracellular matrix which appeared as a thin membranous layer in kiwifruit (Popielarska *et al.*, 2006)

CHAPTER 3

METHODOLOGY

3.1 Plant source and plant materials

Boesenbergia rotunda (*B. rotunda*) samples were provided by other team members of the Centre for Research in Biotechnology for Agriculture (CEBAR) research group. Examples of the samples are shown in Figure 3.1. The tissue culture materials (i.e. SB, EC, DC and WC), the conventionally propagated leaf and the *in vitro* regenerated leaves were prepared as described in previous publications (Tan *et al.*, 2015; Yusuf *et al.*, 2011). The suspension cell samples were derived from embryogenic callus which fail to regenerate into somatic embryos after 12 months in culture (Wong *et al.*, 2013).

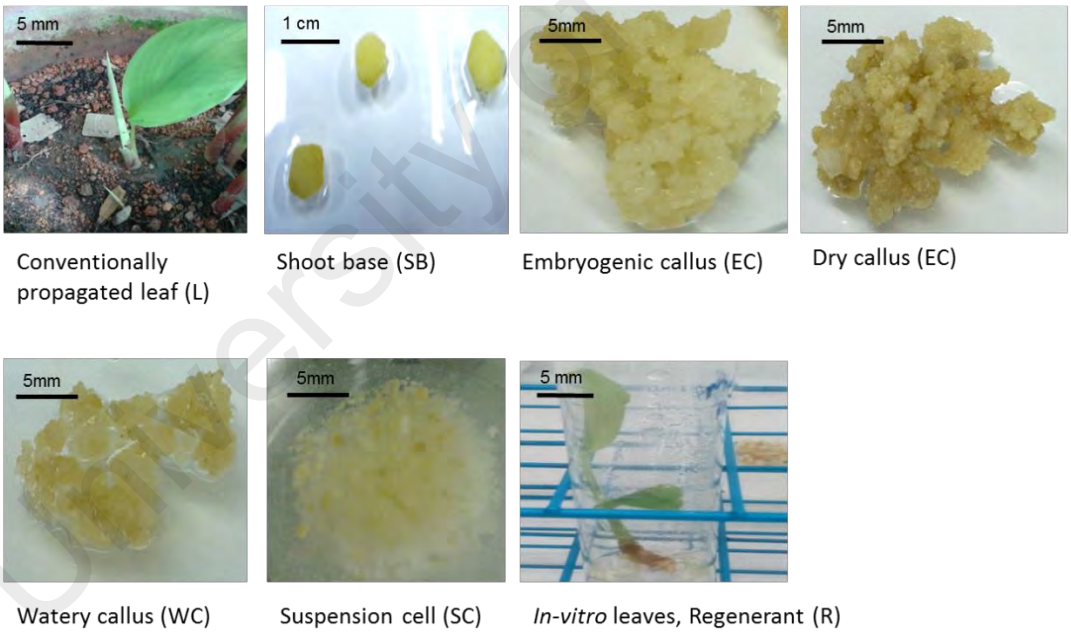

Figure 3.1: Samples collected for *B. rotunda* metabolite profiling analysis representing main development stages from source (leaf and shoot base), callus types and plant regeneration (author and Rezaul K. captured images).

The shoots of *B. rotunda* were allowed to grow in the dark to a specific length (Figure 3.2) for secondary metabolite analysis. The shoots were measured at three day intervals and harvested according to length category. Shoots were thoroughly washed and

cut into 5 cm lengths using a penknife, represented by T1, T2, T3, T4 and T5 as in Figure 3.2. The shoot sections were further sliced thinly and placed into tubes for further sample preparation.

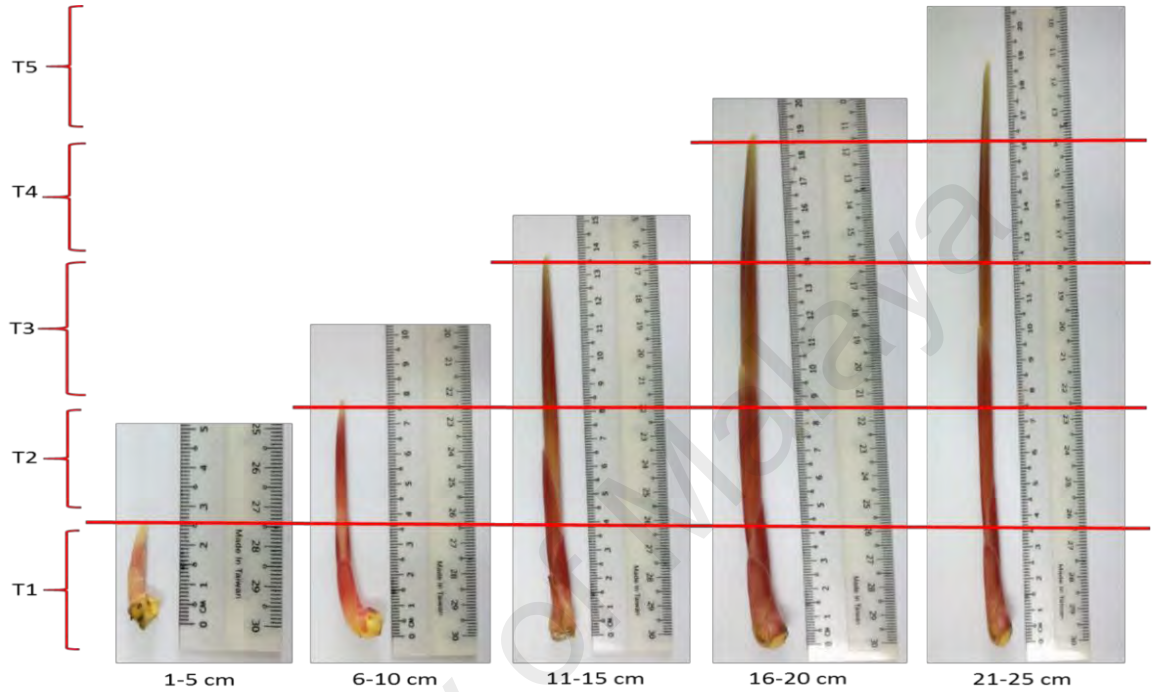

Figure 3.2: The five different shoot ages of *B. rotunda* based on length and divided into sections; T1-T5 (author and Y.S Tan own image).

3.2 Chemicals and Reagents

Primary and secondary metabolite standards were purchased from Sigma-Aldrich (Missouri, United States) and ALB Technology Limited (Kowloon, Hong Kong) except for panduratin. The panduratin standard was obtained from Prof Rais Mustafa, Faculty of Medicine and Dr. Lee Y.K, Faculty of Science, University of Malaya. The hormone standards were purchased from OlChemIm Ltd (Olomouc, Czech Republic). The standards were dissolved to known concentrations prior to analysis and used as is.

3.3 Metabolite extraction protocols

3.3.1 Primary and Secondary Metabolites

A single extraction protocol was used to extract both primary and secondary metabolites. Rhizome, shoot base, shoot section (T1 to T5), conventionally propagated leaf, regenerated *in vitro* leaves, callus and suspension cell samples each had three biological replicates. The samples were ground to a fine powder under a stream of liquid nitrogen. Finely powdered samples weighing 200 mg each were used for the extraction process. Samples were extracted using 2 mL of 80% (v/v) methanol (MeOH) with 0.1% (w/v) butylated hydroxy toluene (BHT). Ribitol (200 ppm) was added to the solvent mixture as an internal standard. Next, the mixture was placed in a vortex (Biosan V-32 model, Riga, Latvia) for 30 s followed by an incubator shaker (MaxQ 4000 SHKE4000-8CE model, Massachusetts, United States) for 5 min at 500 rpm. Soon after, the mixture was sonicated (Elmasonic S120H model, Singen, Germany) at 37 kHz for 5 min at 10ºC. Subsequently, the mixture was placed into a centrifuge Eppendorf 5810R model (Hamburg, Germany) for 10 min at 4ºC, 3100 x *g*. The supernatant was pipetted into a new clean tube. The process of extraction was repeated twice, each with fresh 80% (v/v) MeOH only (Neoh *et al.*, 2013). All the dry extracts were combined and dried using an evaporator Genevac EZ-2 model (Ipswich, United Kingdom).

3.3.2 Hormones

Hormone classes analyzed included cytokinins, gibberellins, auxins, salicylates, jasmonates and abscisic acid (ABA). Shoot base, leaves, callus and suspension cell samples each had three biological replicates. Fine powdered samples weighing 100 mg were extracted using 1 mL of MeOH:isopropanol (20:80, v/v) mixture with 1% (v/v) glacial acetic acid. Next, the mixture was sonicated (Elmasonic S120H model, Singen, Germany) at 37 kHz for 20 min at 4ºC to 7ºC. Then, the mixture was placed into a

centrifuge Eppendorf 5810R model (Hamburg, Germany) for 5 min at 4ºC, 3100 *g*. The supernatant was transferred into a new clean tube. The process of extraction was repeated twice, each with fresh solvent mixture added (Muller & Munne-Bosch, 2011). All the dry extracts were combined and dried using an evaporator Genevac EZ-2 model (Ipswich, Germany).

3.4 Analysis using Ultra Performance Liquid Chromatography-Mass Spectrometry (UPLC-MS)

3.4.1 Primary Metabolites

Dry extracts were first dissolved in 100 µL 50% acetonitrile (ACN). Dry extracts were analyzed in triplicate for each biological replicate using a Waters Acquity (Massachusetts, United States) UPLC system coupled with a Xevo Triple Quadrupole Mass Spectra (Massachusetts, United States) detector. The separation was performed using an Acquity UPLC® HSS T3 column (1.8 µm, 2.1 mm x 100 mm) with solvent A [0.1% formic acid (FA) in water (H_2O)] and solvent B (0.1% FA in ACN), according to the protocol. The elution gradient was as follows: initial at 95% solvent A; 0-3 min linear gradient to 60% solvent A; 3-5 min linear gradient to 5% solvent A; 5.0-5.1 min linear gradient to 95% solvent A and hold to 7 min. The flow rate was set to 0.3 $mL.min^{-1}$ with an injection volume of 3 µL. Both positive and negative electron spray ionization (ESI) mode were used in the mass detector with desolvation temperature of 350ºC while the capillary voltage was set to 2.9 kV. The total acquisition time was 15 min. The mass spectrometry parameters were optimized for detection of each metabolite using multiple reaction monitoring (MRM) (Appendix A). Data were normalized using an internal standard and dry extract weight. The data are reported in relative abundance.

3.4.2 Secondary Metabolites

Five secondary metabolites of interest, namely panduratin, pinocembrin, pinostrobin, alpinetin, and cardamonin were selected based on reported biological activities as well as readily available standards. Dry extracts were first dissolved in 100 µL 50% ACN. Dry extracts were analyzed in triplicate using a Waters Acquity (Massachusetts, United States) UPLC system coupled with a Xevo Triple Quadrupole Mass Spectra (Massachusetts, United States) detector. The separation was performed using an Acquity UPLC® BEH C18 column (1.7 µm, 2.1 mm x 100 mm) with solvent A (0.1% FA in H_2O) and solvent B (0.1% FA in ACN). The elution gradient was as follows: initial at 60% solvent A; at 0-10 min linear gradient to 10% solvent A and hold to 2 min; 12.0-12.5 min linear gradient to 60% solvent A and hold to 2.5 min. The flow rate was set to 0.3 $mL.min^{-1}$ with an injection volume of 3 µL. Positive ESI mode was used in the mass detector with a desolvation temperature of 350°C while the capillary voltage was set to 3.5 kV. The total acquisition time was 15 min. The mass spectrometry parameters were optimized for detection of each metabolite using MRM (Appendix B). Calibration curves for each standard were prepared and data were quantified in percent dry extracts and percent wet weight.

3.4.3 Hormones

Dry extracts were first dissolved in 100 µL of 50% MeOH. Dry extracts were analyzed in triplicate using a Waters Acquity (Massachusetts, United States) UPLC system coupled with a Xevo Triple Quadrupole Mass Spectra (Massachusetts, United States) detector. The separation was done using a UPLC® HSS T3 column (1.8µm, 2.1 mm x 100 mm) with solvent A (0.1% FA in H_2O) and solvent B (0.1% FA in MeOH). The elution gradient was as follows: initial at 99.9% solvent A; at 0-3 min linear gradient to 70% solvent A; 3-8 min linear gradient to 100% solvent B and hold to 2 min; 10-13

linear gradient to 70% solvent A; 13-14 min linear gradient to 99.9% solvent A and hold to 1 min. The flow rate was set to 0.25 $mL.min^{-1}$ with an injection volume of 3 µL. Both positive and negative ESI mode were used in the mass detector with desolvation temperature of 330°C while the capillary voltage was set to 4.5 kV. The total acquisition time was 10 min. The mass spectrometry parameters were optimized for detection of each metabolite using MRM (Appendix C). Calibration curve for each standard was prepared, and data were quantified in parts per billion (ppb).

3.5 Statistical Analysis

Data from UPLC-MS were processed using Target Lynx™ software (Waters, Massachusetts, United States). Also, clustering analysis was performed using Principal Component Analysis (PCA) and Orthogonal Partial Least Square Analysis (OLPS-DA) by Umetrics (Malmo, Sweden). ANOVA by IBM SPSS Statistics (Version 20) and t-test algorithm of Excel 2000 by Microsoft analysis were carried out to identify significant differences with a 95% confidence level.

3.6 Scanning electron microscopy

Samples were fixed using 4% glutaraldehyde for 2 days at 4°C followed by washing with 0.1 M sodium cacodylate buffer at intervals of 30 min (repeated thrice). Next, the samples were post-fixated with 1% osmium tetroxide for 2 h at 4°C. Subsequently, samples were washed thrice again with 0.1 M sodium cacodylate buffer for 30 min each before the dehydration process using a series of acetone water mixtures (35% acetone, 50%, 75% and 95% acetone) for 45 min each. After that, the samples were incubated in 100% water for 1 h (repeated thrice). Samples were then dried in a Bal-Tec CPD 030 (Schalksmuhle, Germany) critical point dryer at 40°C for 90 min, mounted on stubs and gold coated before viewing. Finally, samples were examined under a Jeol JSM-6400 (Tokyo, Japan) scanning electron microscope, with X-ray analyzer.

3.7 Light microscopy

Semi-thin sections were prepared by a service provided at the Advanced Biotechnology and Breeding Centre (ABBC), Malaysian Palm Oil Board (MPOB) as described in recent publication (Wong *et al.*, 2013). Sections were stained with Periodic Acid-Schiff (PAS) reagent before examination under light microscopy using an Olympus BX51 model (Tokyo, Japan). Estimation of the number cells per unit area in each sample was performed using the analySIS FIVE LS Research (Version 5) by Olympus Soft Imaging Solutions (Munster, Germany).

3.8 Fluorescence microscopy

Semi-thin sections (prepared as in 3.7) were stained with a mixture of 0.25% (w/v) diphenylboric acid 2-aminoethylester (DPBA) and 0.1% (w/v) Triton X-100 for 15 min before viewing under an Olympus BX51 model (Tokyo, Japan) fluorescent microscope (Sheahan & Rechnitz, 1992). The excitation and emission wavelengths are 400-410 nm and 455 nm, respectively (U-MNV2 mirror unit).

CHAPTER 4

RESULTS

4.1 Primary metabolite profiles of *Boesenbergia rotunda* (*B. rotunda*) samples

A total of 51 targeted primary metabolites were analyzed in seven samples; conventionally propagated leaf (L), shoot base (SB), embryogenic callus (EC), dry callus (DC), watery callus (WC), suspension cells (SC) and regenerated *in vitro* leaves (R) of *B. rotunda* (Figure 4.1).

The primary metabolite analysis was performed using Ultra Performance Liquid Chromatography-Mass Spectrometry (UPLC-MS) and the data were normalized based on dry extract weight (Table 4.1), and reported as relative abundance. Each of the primary metabolites were grouped into respective metabolic pathways including amino acids, glycolysis, pentose phosphate, polyamines, purine and pyrimidine, tricarboxylic acid cycle (TCA), purine and pyrimidine and shikimate. Generally, the relative abundance of the primary metabolites varied significantly between the different *B. rotunda* samples. The dry and watery callus had relatively the lowest abundance of primary metabolites profiled compared to the other samples (Table 4.1). Embryogenic callus had the highest abundance of most primary metabolites, followed by shoot base. In particular, EC was observed to have comparatively higher levels of amino acids than shoot base with abundance ratio of 0.1 – 61 times. Phenylalanine and tryptophan are precursors for secondary metabolite and hormone metabolism, and were observed to be at higher levels in embryogenic callus than in shoot base, with abundance ratio of 13:1 and 16:1 respectively (Table 4.1).

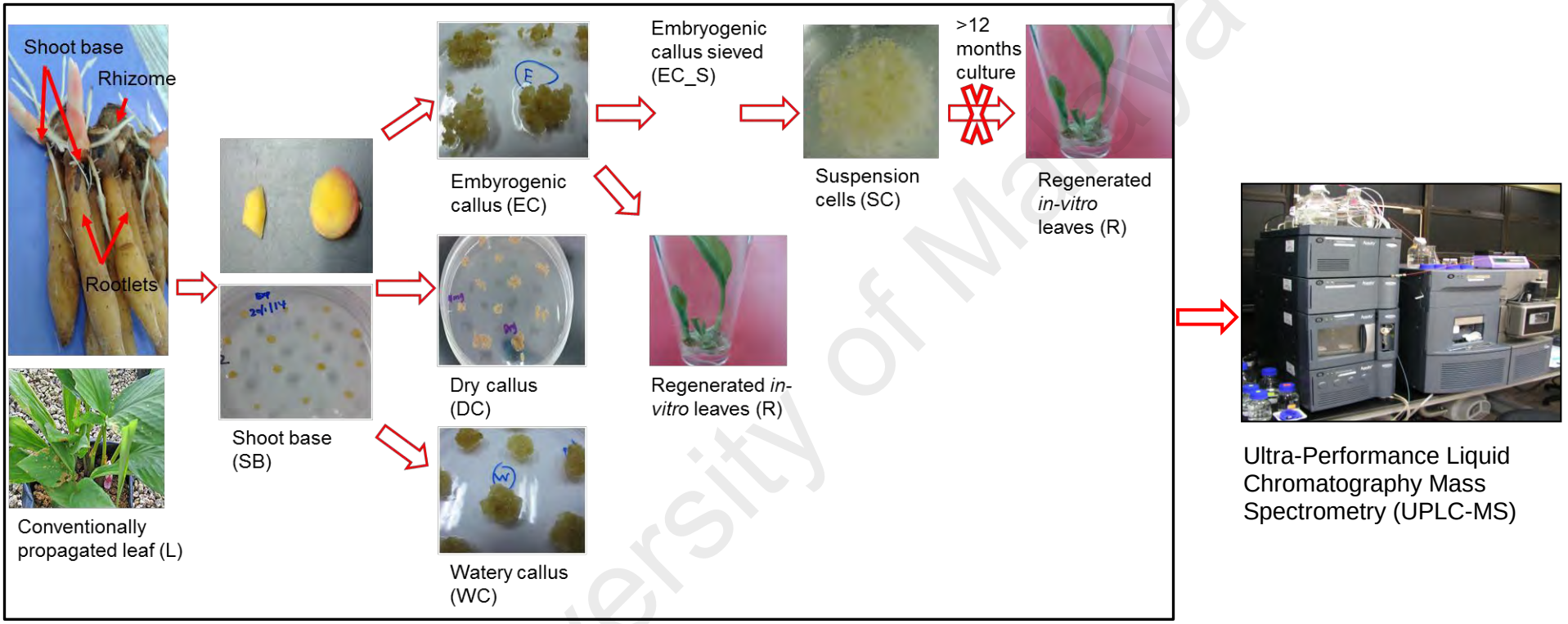

Figure 4.1: Workflow of various *B. rotunda* samples (author and Rezaul K. own images).

Table 4.1: Relative abundance of primary metabolites and their associated pathways in seven samples types of *B. rotunda* (n=3 biological replicates); L: conventionally propagated leaf; EC: embryogenic callus; DC: dry callus; WC: watery callus; SC: suspension cells, R: regenerated *in vitro* leaves; ND: not detected; ± indicates the standard deviation.

Metabolites	Pathways	Leaf (L)	Shoot base (SB)	Embryogenic callus (EC)	Dry callus (DC)	Watery callus (WC)	Suspension cells (SC)	Regenerant (R)
Glycine (Gly)	**Amino acid**	ND	ND	0.0037 ± 0.0017	ND	0.00073 ± 0.00018	0.0100 ± 0.0027	ND
Homoserine	**Amino acid**	1.06 ± 0.14	0.80 ± 0.27	1.62 ± 0.29	0.00274 ± 0.00079	0.026 ± 0.020	1.255 ± 0.073	4.11 ± 0.21
Glutamine (Gln)	**Amino acid**	31.2 ± 3.2	27.7 ± 3.9	130 ± 26	0.0551 ± 0.0036	0.161 ± 0.065	19.3 ± 1.4	360 ± 22
Histidine (His)	**Amino acid**	4.07 ± 0.87	5.2 ± 1.5	94 ± 19	0.0238 ± 0.0094	0.139 ± 0.065	30.2 ± 2.1	25.7 ± 1.2
S-adenosyl methionine	**Amino acid**	0.91 ± 0.32	0.29 ± 0.13	2.84 ± 0.37	ND	ND	0.298 ± 0.034	0.87 ± 0.12
Spermine	**Amino acid**	ND	ND	0.42 ± 0.20	0.0070 ± 0.0018	0.0138 ± 0.0024	0.067 ± 0.019	0.439 ± 0.087
Arginine (Arg)	**Amino acid**	14.6 ± 3.6	10.0 ± 3.3	370 ± 69	0.070 ± 0.017	0.55 ± 0.31	85.0 ± 5.8	113.0 ± 6.6
Alanine (Ala)	**Amino acid**	ND	ND	0.467 ± 0.088	ND	0.0020 ± 0.0003	0.12 ± 0.01	ND
Asparagine (Asn)	**Amino acid**	1.15 ± 0.17	0.81 ± 0.38	0.43 ± 0.15	ND	0.0184 ± 0.0016	0.231 ± 0.019	1.99 ± 0.15
Aspartic acid (Asp)	**Amino acid**	8.12 ± 0.57	5.9 ± 2.7	3.75 ± 0.93	0.00513 ± 0.00087	0.065 ± 0.047	2.18 ± 0.11	29.1 ± 1.5
Glutamic acid (Glu)	**Amino acid**	38.3 ± 5.5	23.4 ± 6.7	2.44 ± 0.27	0.028 ± 0.077	0.25 ± 0.14	1.43 ± 0.15	32.6 ± 4.8
Serine	**Amino acid**	ND	ND	0.0169 ± 0.0051	ND	ND	0.0087 ± 0.0017	ND
Proline (Pro)	**Amino acid**	0.82 ± 0.21	0.28 ± 0.14	3.06 ± 0.66	0.0013 ± 0.0002	0.015 ± 0.012	0.644 ± 0.071	1.61 ± 0.16
Phenylalanine (Phe)	**Amino acid**	0.138 ± 0.085	0.116 ± 0.055	1.48 ± 0.35	ND	0.01544 ± 0.00065	2.48 ± 0.31	0.355 ± 0.063
Valine (Val)	**Amino acid**	1.39 ± 0.26	0.96 ± 0.21	7.6 ± 1.6	0.0187 ± 0.0029	0.049 ± 0.045	7.67 ± 0.66	6.86 ± 0.64
Tyrosine (Tyr)	**Amino acid**	2.15 ± 0.46	0.7 ± 0.29	4.22 ± 0.82	0.0091 ± 0.0024	0.0075 ± 0.0010	2.01 ± 0.21	2.32 ± 0.35
Trptophan (Trp)	**Amino acid**	4.7 ± 1.4	1.7 ± 0.4	26.7 ± 7.0	ND	ND	21.1 ± 2.7	5.90 ± 0.84
Hydroxyproline	**Amino acid**	ND	ND	0.170 ± 0.036	ND	0.002601 ± 0.000083	0.23 ± 0.03	0.074 ± 0.016
Lysine (Lys)	**Amino acid**	53.3 ± 5.0	47.5 ± 7.1	190 ± 33	0.0676 ± 0.0068	0.24 ± 0.12	30.1 ± 1.7	590 ± 29
Methionine (Met)	**Amino acid**	ND	ND	0.036 ± 0.013	ND	ND	0.0150 ± 0.0027	ND
Antranilate	**Amino acid**	27.1 ± 4.6	0.083 ± 0.026	5.08 ± 0.83	ND	0.0116 ± 0.0023	1.500 ± 0.084	16.0 ± 1.0
Adenine	**Amino acid**	0.61 ± 0.17	0.24 ± 0.13	2.06 ± 0.47	0.00461 ± 0.00081	0.0066 ± 0.0027	0.90 ± 0.10	0.673 ± 0.070

Table 4.1, continued

Metabolites	Pathways	Leaf (L)	Shoot base (SB)	Embryogenic callus (EC)	Dry callus (DC)	Watery callus (WC)	Suspension cells (SC)	Regenerant (R)
Creatine	**Amino acid**	ND	ND	0.0086 ± 0.0028	ND	0.0050 ± 0.0027	0.0051 ± 0.0015	ND
Glycerol-3-phosphate	**Glycolysis**	2.1 ± 1.2	1.5 ± 1.4	3.2 ± 1.1	0.048 ± 0.035	0.118 ± 0.045	1.06 ± 0.39	0.59 ± 0.25
Fructose-6-phosphate	**Glycolysis**	49 ± 25	12.1 ± 4.3	22 ± 14	0.40 ± 0.19	0.70 ± 0.34	0.18 ± 0.11	48 ± 15
Fructose-1,6-phosphate	**Glycolysis**	2.2 ± 1.4	0.180 ± 0.037	0.46 ± 0.15	0.073 ± 0.071	0.059 ± 0.037	0.151 ± 0.061	3.5 ± 1.6
Gluconic acid	**Pentose Phosphate**	1.58 ± 0.74	0.53 ± 0.32	0.79 ± 0.36	0.19 ± 0.11	0.27 ± 0.17	0.59 ± 0.25	0.73 ± 0.35
Erythrose-4-phosphate	**Pentose Phosphate**	0.97 ± 0.47	0.21 ± 0.09	0.38 ± 0.25	0.0196 ± 0.0054	0.0383 ± 0.020	0.046 ± 0.024	0.95 ± 0.33
Xylulose-5-phosphate	**Pentose Phosphate**	0.37 ± 0.21	0.205 ± 0.089	0.32 ± 0.17	ND	0.0096 ± 0.0074	0.136 ± 0.037	0.37 ± 0.16
Ribulose-5-phosphate	**Pentose Phosphate**	1.04 ± 0.64	0.73 ± 0.41	1.20 ± 0.46	ND	0.0208 ± 0.0095	0.84 ± 0.26	1.64 ± 0.56
6-phosphogluconic acid	**Pentose Phosphate**	4.6 ± 2.8	0.500 ± 0.068	ND	0.46 ± 0.31	0.97 ± 0.70	ND	2.33 ± 0.98
Putrescine	**Polyamines**	ND	ND	0.043 ± 0.011	ND	ND	ND	0.179 ± 0.026
GABA	**Polyamines**	0.452 ± 0.072	0.46 ± 0.30	7.5 ± 2.5	0.00191 ± 0.00061	0.01321 ± 0.00086	6.16 ± 0.44	0.590 ± 0.053
Citrulline	**Polyamines**	1.50 ± 0.32	0.88 ± 0.30	43.0 ± 7.7	0.0083 ± 0.0013	0.076 ± 0.052	6.91 ± 0.66	9.62 ± 0.83
Ornithine (Orn)	**Polyamines**	ND	ND	0.66 ± 0.10	ND	0.0256 ± 0.0024	0.330 ± 0.022	11.85 ± 0.73
Guanine	**Purine and pyrimidine**	250 ± 50	52 ± 20	7.04 ± 0.64	0.158 ± 0.044	0.197 ± 0.098	8.17 ± 0.95	16.1 ± 1.5
Uracil	**Purine and pyrimidine**	0.16 ± 0.05	0.121 ± 0.028	0.089 ± 0.057	ND	0.00313 ± 0.00059	0.094 ± 0.017	ND
Thymine	**Purine and pyrimidine**	13.6 ± 3.7	2.44 ± 0.93	0.38 ± 0.06	0.0062 ± 0.0021	0.0077 ± 0.0046	0.477 ± 0.068	0.79 ± 0.11
Hypoxanthine	**Purine and pyrimidine**	0.178 ± 0.055	0.152 ± 0.069	0.121 ± 0.025	ND	ND	0.034 ± 0.006	0.094 ± 0.011
Ribose-5-phosphate	**Purine and Pyrimidine**	0.80 ± 0.39	0.73 ± 0.33	1.1 ± 0.4	ND	0.028 ± 0.016	0.79 ± 0.23	1.48 ± 0.51
Shikimic acid	**Shikimate**	0.011 ± 0.005	0.0071 ± 0.0034	ND	ND	ND	ND	ND
Shikimate-3-phosphate	**Shikimate**	0.22 ± 0.09	1.24 ± 0.77	0.18 ± 0.12	ND	ND	0.52 ± 0.23	0.45 ± 0.18

Table 4.1, continued

Metabolites	Pathways	Leaf (L)	Shoot base (SB)	Embryogenic callus (EC)	Dry callus (DC)	Watery callus (WC)	Suspension cells (SC)	Regenerant (R)
Malic acid	**TCA cycle**	240 ± 86	140 ± 45	130 ± 61	0.133 ± 0.078	0.56 ± 0.21	160 ± 60	190 ± 60
2-Oxoisovaleric acid	**TCA cycle**	26 ± 16	14.3 ± 7.9	16 ± 12	ND	0.11 ± 0.06	18 ± 11	20 ± 10
cis-Aconitic acid	**TCA cycle**	3.3 ± 1.5	2.6 ± 1.3	0.99 ± 0.64	ND	ND	5.7 ± 2.5	0.78 ± 0.32
Citric acid	**TCA cycle**	19.7 ± 9.2	16.6 ± 8.5	11.0 ± 6.5	ND	ND	24 ± 10	5.3 ± 2.7
Oxaloacetic acid	**TCA cycle**	0.46 ± 0.34	0.035 ± 0.028	ND	ND	ND	ND	0.12 ± 0.10
α-ketoglutaric acid	**TCA cycle**	1.9 ± 1.1	0.37 ± 0.27	0.066 ± 0.052	ND	0.0136 ± 0.0086	0.62 ± 0.52	0.55 ± 0.34
Isocitric acid	**TCA cycle**	10.6 ± 6.3	7.6 ± 4.2	4.7 ± 3.2	ND	ND	15.2 ± 9.5	2.8 ± 1.5
3-Phosphoglyceric acid	**TCA cycle**	33 ± 18	10.4 ± 3.6	2.26 ± 0.78	4.6 ± 2.5	6.5 ± 3.4	3.6 ± 1.2	18.5 ± 5.3
Lactic acid	**Others**	0.33 ± 0.18	0.22 ± 0.11	0.21 ± 0.11	0.206 ± 0.081	0.18 ± 0.10	0.22 ± 0.10	0.29 ± 0.17

The Partial Least Square Discriminant Analysis (PLS-DA) score plot (Figure 4.2) showed three clusters with shoot base and regenerated *in vitro* leaves grouped individually, while the other five samples appeared clustered together (Figure 4.2). Subsequent sections of this chapter describe the results of the specific comparative study among the *B. rotunda* samples based on primary metabolites profiled.

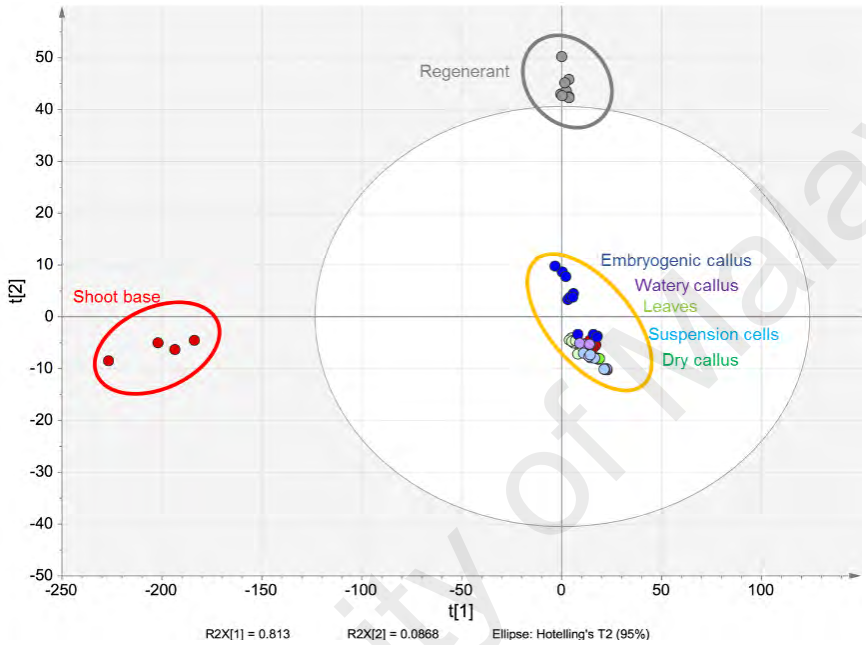

Figure 4.2: Three clusters observed from Partial Least Square Discriminant Analysis (PLS-DA) in seven different samples of *B. rotunda* (n=3 biological replicates); Red ellipse: shoot base (SB), grey ellipse: regenerated *in vitro* leaves (R), yellow ellipse with green: dry callus (DC), purple: watery callus (WC), blue: embryogenic callus (EC), light green: conventionally propagated leaf (L) and light blue: suspension cells (SC).

4.1.1 Comparison of primary metabolite profiles in conventionally propagated leaf and regenerated *in vitro* leaves of *B. rotunda*

Conventionally propagated leaf samples (L) were compared with regenerated *in vitro* leaves (R) based on primary metabolites profiled. Orthogonal Partial Least Square Discriminant Analysis (OPLS-DA) was carried out to enable clustering analysis between both L and R samples. The conventionally propagated leaf and regenerated *in vitro* leaf samples had different metabolite profiles, as expected since despite being similar leaf tissues, they were grown under different environmental conditions. This is evident from

the separated cluster observed in Figure 4.3A. Concentrations of glutamine and lysine were 10 times more abundant in R sample than in L as shown in the S-plot in Figure 4.3B. Additionally, L and R samples exhibited significantly different (p-value<0.05) concentrations of metabolites such as cis-aconitic acid, citric acid and oxaloacetic acid that involved in tricarboxylic acid (TCA) cycle (Table 4.1).

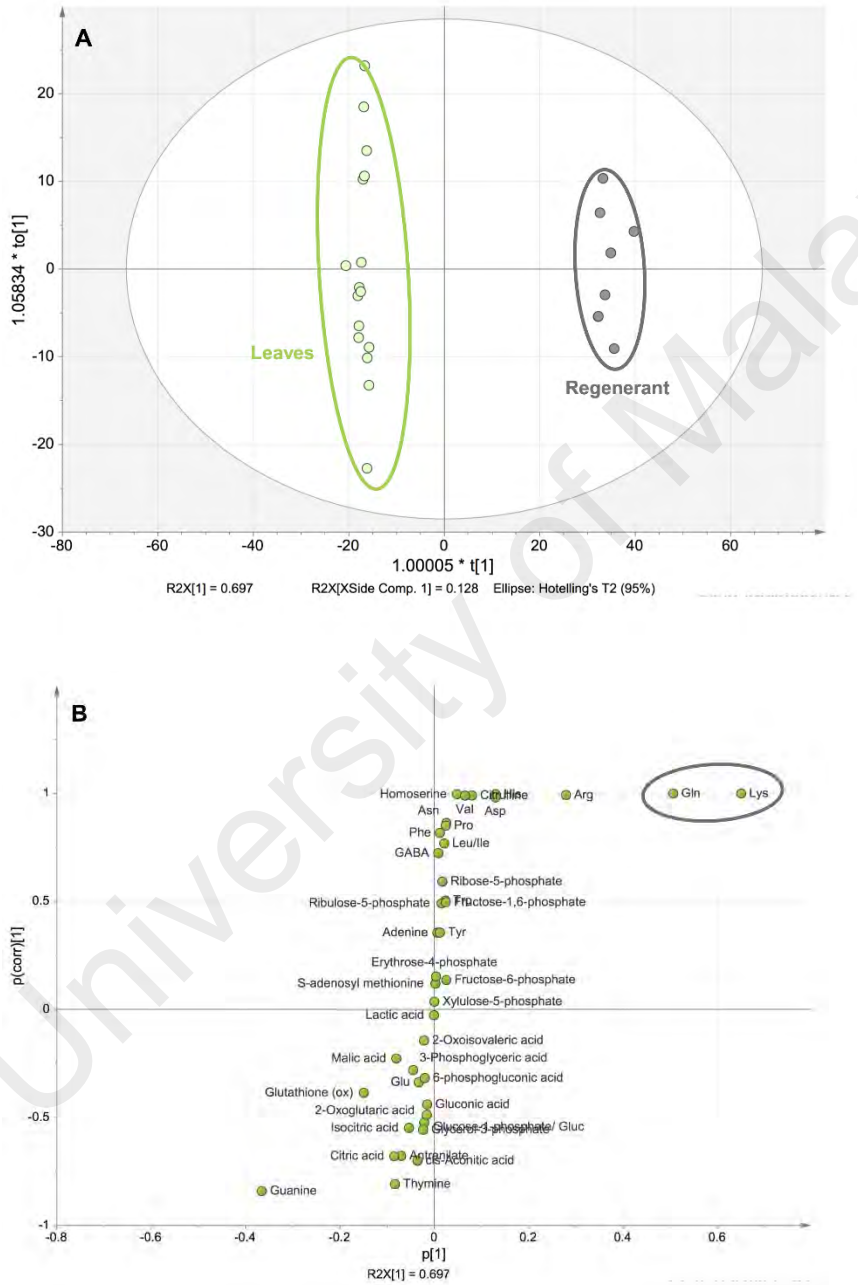

Figure 4.3: Primary metabolite variables associated to two different leaves samples of *B. rotunda* (n=3 biological replicates). A: Orthogonal Partial Least Square Discriminant Analysis (OPLS-DA) in two different samples of *B. rotunda*. B: Grey ellipse in the S plot highlights metabolites associated with *in vitro* regenerated leaves (R) versus conventionally propagated leaf (L) with p-value<0.05. L: light green, R: grey

4.1.2 Comparison of primary metabolite profiles in the shoot base and the three calli types of *B. rotunda*

The three calli types; EC, DC and WC were formed from the shoot base (SB) of *B. rotunda* using different concentrations of 2,4-D as reported (Tan *et al.*, 2005; Yusuf *et al.*, 2011). The primary metabolite profile of embryogenic callus was compared with two other non-embryogenic calli, dry and watery callus as a reported study showed the competence of EC to proliferate and grow (Tan *et al.*, 2005). Data were normalized based on dry extract weight (Table 4.1), although a similar trend could be observed when normalized with the estimated cell density (cells.mm^{-2}) for each sample (SB, EC, DC and WC) (Table 4.2). Among the three calli types, WC had the lowest estimated cell density than in EC and DC. Unsupervised principal component analysis (PCA) revealed three major clusters (Figure 4.4). DC and WC were grouped together, while SB and EC were clustered individually.

Table 4.2: Estimation of cell density (cell.mm^{-2}) in shoot base, embryogenic and non-embryogenic calli in *B. rotunda* (n=3 biological replicates). SB: shoot base; EC: embryogenic callus; DC: dry callus; WC: watery callus; ± indicates the standard deviation.

Tissues	Estimate cell number	Width of observation (µm)	Length of observation (µm)	Area (mm^2)	Cell density (cell/mm^2)
Shoot base (SB)	87 ± 7	104.8 ± 3.8	86 ± 3	0.00905 ± 0.00063	9680 ± 1454
Embryogenic callus (EC)	152.7 ± 9.5	107.4 ± 1.9	83.0 ± 1.5	0.00891 ± 0.00012	17126 ± 909
Dry callus (DC)	170 ± 7	107.4 ± 2.2	85.8 ± 3.9	0.00921 ± 0.00027	18312 ± 695
Watery callus (WC)	44.7 ± 4.9	106.5 ± 2.9	85.91 ± 0.86	0.00915 ± 0.00032	4896 ± 696

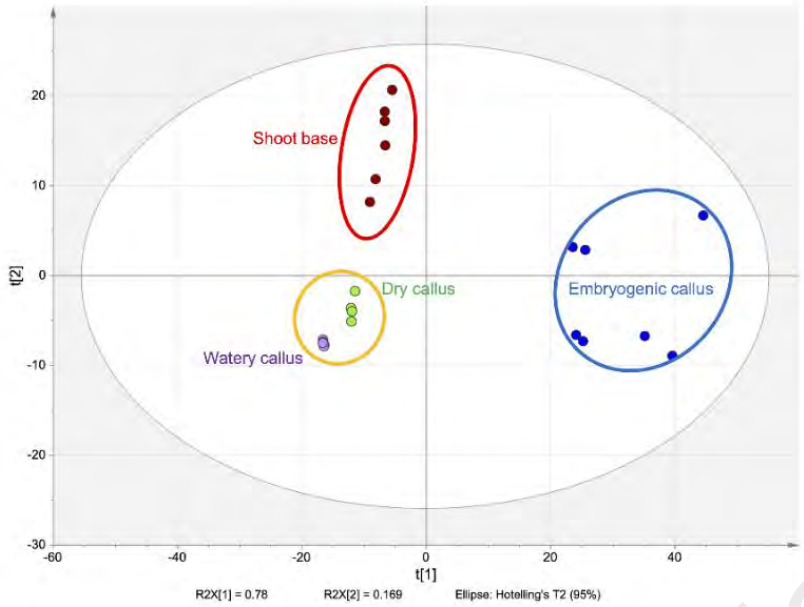

Figure 4.4: Principal Component Analysis (PCA) plot showing three clusters in callus and shoot base samples from *B. rotunda* (n=3 biological replicates). Blue ellipse: embryogenic callus (EC); yellow ellipse with green; dry callus (DC) and with purple: watery callus (WC); and red ellipse: shoot base (SB).

From the PCA plot, an OPLS-DA score plot was performed to study the correlation between the three different callus types (EC, DC and WC) based on primary metabolite profiled as shown in Figure 4.5. Evidently, Figure 4.5A and B both show a clear distinction between EC and the two non-embryogenic calli, DC and WC. Amino acids arginine, glutamine and lysine were significantly higher in EC (Figure 4.5C and D) than in the non-embryogenic calli. Specifically comparing DC and WC (Figure 4.5E and F), revealed two separated clusters with arginine to be an outlier in the S plot associated with WC.

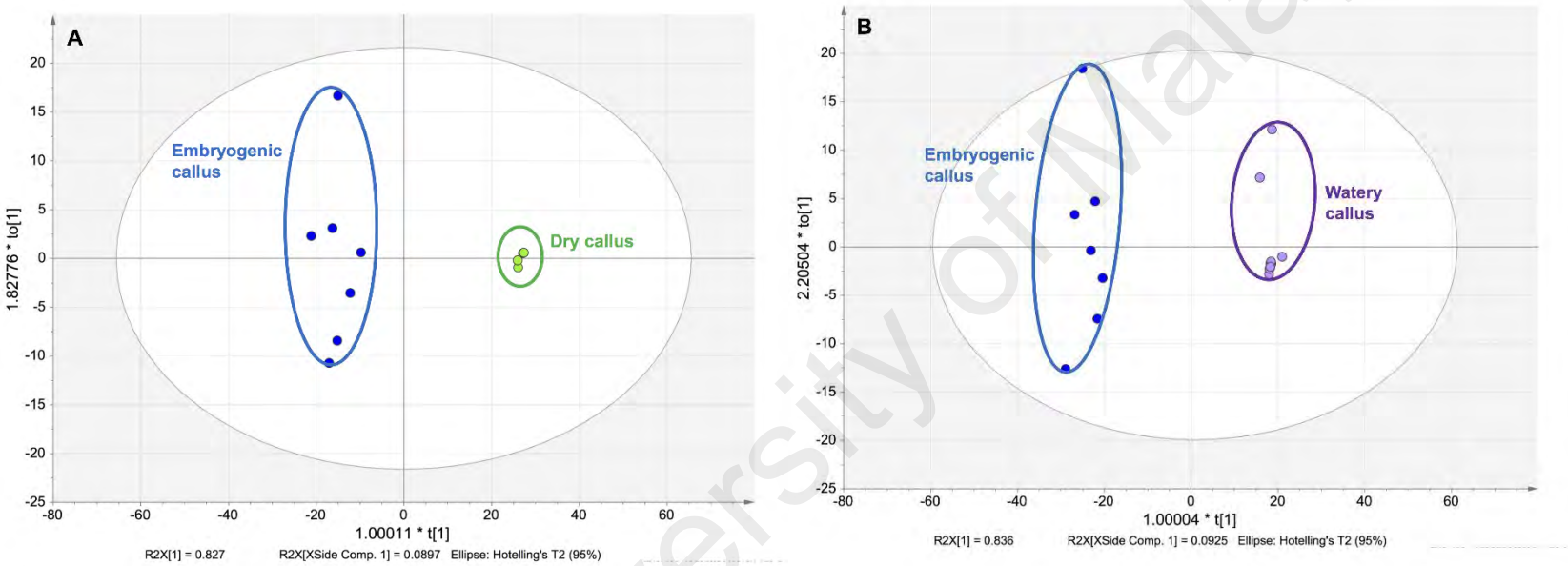

Figure 4.5: Primary metabolite variables associated with different callus types from *B. rotunda* (n=3 biological replicates). A: Orthogonal Partial Least Square Discriminant Analysis (OPLS-DA) plot for embryogenic callus (EC) and dry callus (DC); B: OPLS-DA plot for EC and watery callus (WC); C: Blue ellipse in the S plot highlights metabolites associated with EC versus DC with p-value<0.05, D: Blue ellipse in the S plot highlights metabolites associated with EC versus WC with p-value<0.05, E: OPLS-DA plot for DC and WC; F: S plot showing metabolite comparison between DC and WC. DC: green, WC: purple, EC: blue

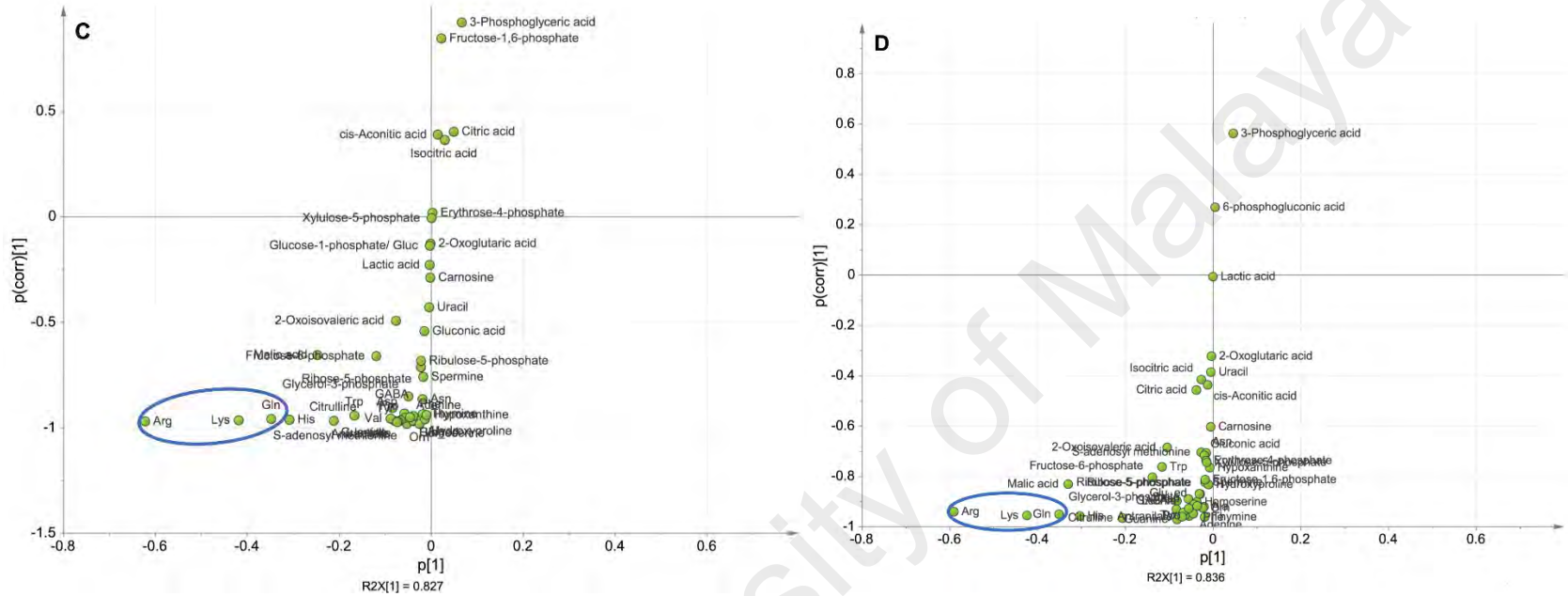

Figure 4.5, continued

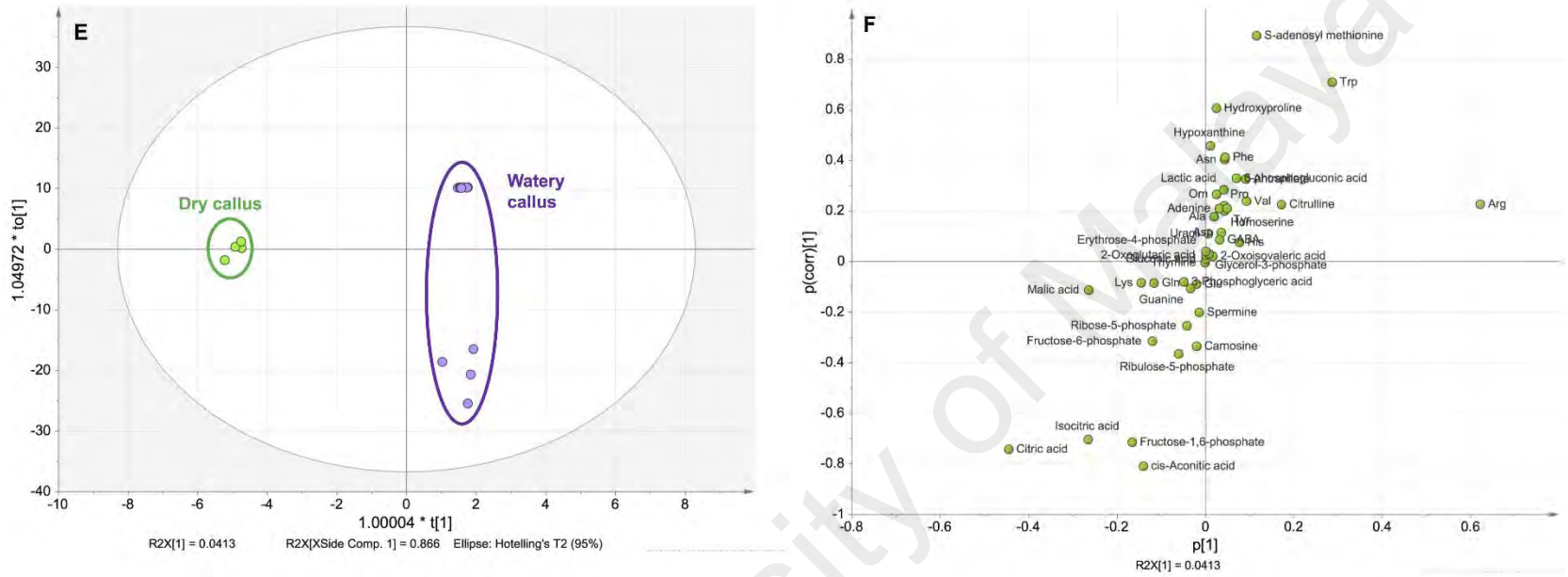

Figure 4.5, continued

4.1.3 Validation of primary metabolite markers in association with embryogenesis of *B. rotunda*

The primary metabolite markers, especially amino acids glutamine, arginine and lysine that were found to correlate well with embryogenesis in *B. rotunda* from the earlier OLPS-DA plots (Figure 4.5) were validated in sieved embryogenic cells. As embryogenic callus comprised a mixture of a high proportion of embryogenic cells and some non-embryogenic cells, embryogenic callus was sieved using a 425 μm stainless steel sieve to enrich embryogenic cells farther and to confirm the primary metabolite concentration observed in callus tissue based on morphology. The sieved embryogenic cells (EC_S) had more than 2 times the concentration of metabolite markers, glutamine and lysine compared to the embryogenic callus, with the exception of arginine (Figure 4.6) while the dry and watery calli had the lowest abundance of the primary metabolite markers.

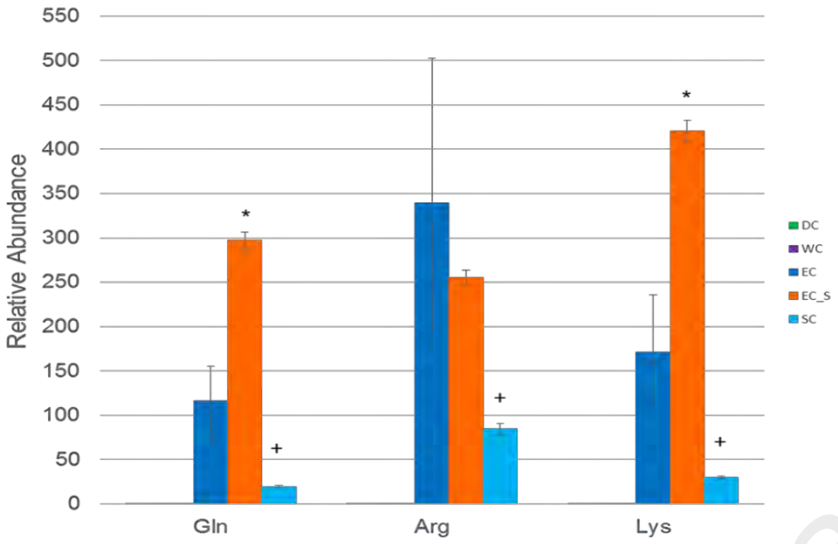

Figure 4.6: Relative abundance of metabolite markers in *B. rotunda* samples (n=3 biological replicates); Green: dry callus (DC); purple: watery callus (WC); blue: embryogenic callus (EC); orange: sieved embryogenic cells (EC_S); light blue: suspension cells (SC). Error bars indicate standard deviation, asterisks represent p-value<0.05 by student T-test between EC and EC_S, pluses represent p-value<0.05 by student T-test between EC and SC

4.1.4 Comparison of primary metabolite profiles in the embryogenic callus and suspension cells of *B. rotunda*

Subsequent to the above comparative study, another OPLS-DA scores plot was performed to enable classification of regenerative sample (ie. embryogenic callus) versus the non-regenerative sample (ie. suspension cells) from the primary metabolites profiled as a previous study reported that suspension cells of *B. rotunda* failed to regenerate into new plants after prolonged culture conditions (Wong *et al.*, 2013). Results showed that SC clustered away from EC, which is regenerative and capable of embryogenesis (Figure 4.7A). The amino acids glutamine, arginine and lysine were observed to be higher in EC while no primary metabolite markers were found to be associated with SC (Figure 4.7B). The amino acid concentrations in EC were more than 5-fold higher in glutamine and lysine, while 3-fold higher in arginine than in SC (Figure 4.6).

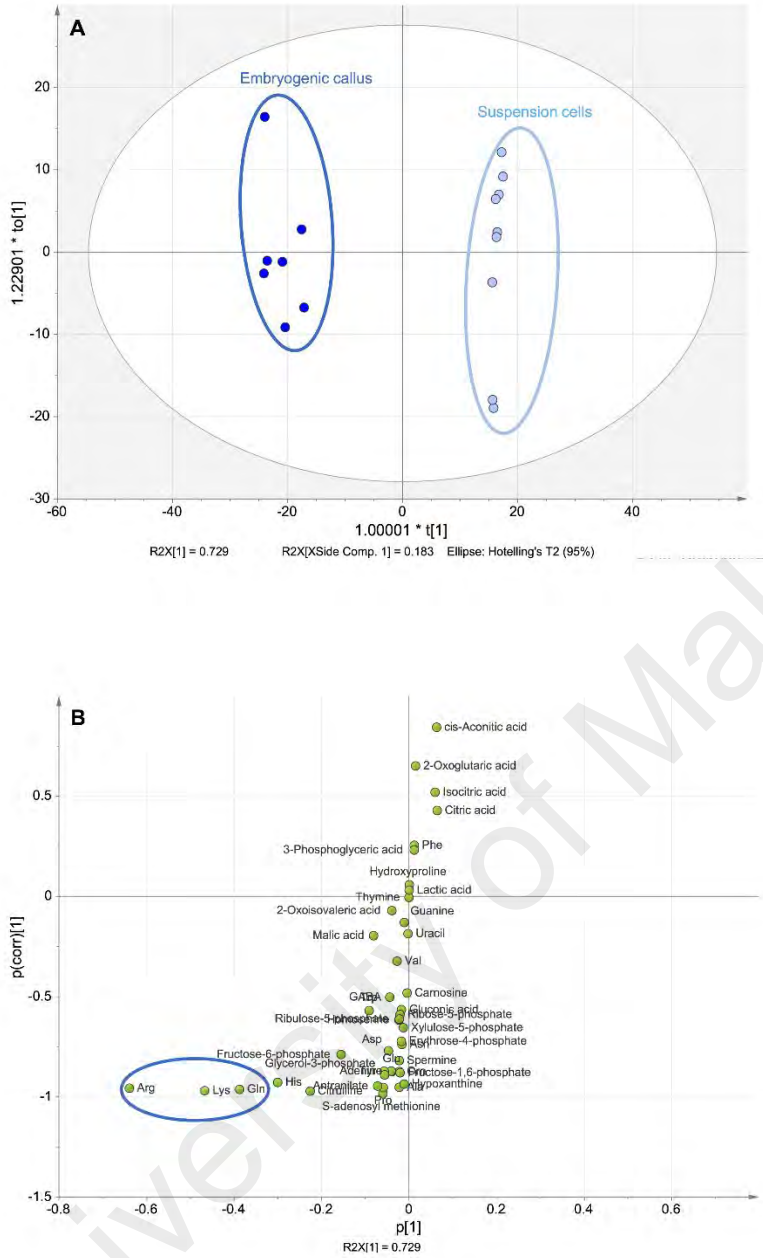

Figure 4.7: Primary metabolite variables associated with plant regeneration in EC and SC of *B. rotunda* (n=3 biological replicates). A: OPLS-DA plot for embryogenic callus (EC) and SC; B: Blue ellipse in the S plot highlights metabolites associated with EC versus SC with p-value<0.05. SC: light blue, EC: blue.

4.2. Secondary metabolite analysis in *B. rotunda* samples

The secondary metabolite analysis in seven sample types of *B. rotunda* were focused on flavonoid-related compounds (three flavanones and two chalcones) using UPLC-MS (Table 4.3). The secondary metabolite concentrations were normalized based on percent dry extract and percent wet weight. Generally, shoot base (SB) and regenerated

in vitro leaves (R) samples had the highest concentration of secondary metabolites as reported among the seven samples (Table 4.3). Shoot base sample had the highest panduratin concentration while the regenerated *in vitro* leaves sample had the highest cardamonin concentration regardless in percent dry extract or in wet weight (Table 4.3). The percent dry extract concentration in both L and R samples were not statistically different for four metabolites; panduratin, pinocembrin, alpinetin and cardamonin except pinostrobin. Among the three callus types (EC, DC and WC), EC had significantly the lowest abundance of panduratin in percent dry extract than in both DC and WC. In contrast, insignificant dry extract concentration of secondary metabolites were reported in suspension cells and the two non-embryogenic callus (dry and watery callus) (Table 4.3). The concentration of five secondary metabolites were not significantly different between the four sample types; EC, DC, WC and SC in percent wet weight (Table 4.3 and Figure 4.8B).

Table 4.3: The distribution of the five secondary metabolites in various samples in *B. rotunda* (n=3 biological replicates); ± indicates the standard deviation and different letters represent significant differences for each metabolite at 95% confidence level by Tukey's test.

Normalization	Tissue types	Panduratin	Pinocembrin	Alpinetin	Pinostrobin	Cardamonin
% Dry extract	Leaves (L)	9.46E-04 ± 2.96E-05^{a}	8.51E-04 ± 1.51E-04^{a}	8.85E-04 ± 4.97E-05^{a}	8.12E-04 ± 1.29E-05^{a}	2.88E-03 ± 1.02E-03^{a}
	Shoot base (SB)	2.71E-03 ± 4.20E-05^{b}	6.80E-04 ± 3.38E-04^{a}	8.30E-04 ± 4.12E-04^{a}	1.51E-03 ± 5.95E-04^{b}	1.45E-03 ± 3.76E-04^{b}
	Embryogenic callus (EC)	6.69E-05 ± 1.32E-05^{c}	3.40E-05 ± 6.39E-06^{b}	5.22E-05 ± 1.07E-05^{b}	6.01E-05 ± 1.10E-05^{c}	1.99E-05 ± 3.60E-06^{c}
	Dry callus (DC)	1.49E-04 ± 6.89E-05^{d}	7.48E-05 ± 3.37E-05^{b}	1.17E-04 ± 5.27E-05^{b}	1.35E-04 ± 6.37E-05^{c}	4.38E-05 ± 2.09E-05^{c}
	Watery callus (WC)	1.77E-04 ± 5.52E-05^{d}	9.24E-05 ± 2.87E-05^{b}	1.47E-04 ± 4.53E-05^{b}	2.09E-04 ± 9.19E-05^{c}	6.25E-05 ± 2.30E-05^{c}
	Suspension culture (SC)	1.51E-04 ± 3.75E-05^{d}	7.73E-05 ± 1.76E-05^{b}	1.17E-04 ± 2.98E-05^{b}	1.48E-04 ± 2.51E-05^{c}	4.88E-05 ± 8.46E-06^{c}
	Regenerant (R)	9.76E-04 ± 9.77E-05^{a}	8.25E-04 ± 2.29E-04^{a}	1.02E-03 ± 1.95E-04^{a}	1.67E-03 ± 5.04E-04^{b}	4.01E-03 ± 2.12E-03^{a}
% Wet weight	Leaves (L)	4.37E-06 ± 1.98E-07^{a}	3.94E-06 ± 7.59E-07^{a}	4.09E-06 ± 3.04E-07^{a}	3.75E-06 ± 9.72E-08^{ab}	1.34E-05 ± 4.94E-06^{a}
	Shoot base (L)	3.56E-05 ± 3.04E-05^{b}	5.75E-06 ± 3.16E-06^{a}	7.03E-06 ± 3.85E-06^{b}	1.42E-05 ± 9.08E-06^{c}	1.55E-05 ± 1.13E-05^{a}
	Embryogenic callus (EC)	3.33E-07 ± 4.54E-09^{a}	1.70E-07 ± 6.38E-09^{b}	2.60E-07 ± 1.55E-09^{c}	3.00E-07 ± 1.30E-08^{a}	9.96E-08 ± 6.97E-09^{b}
	Dry callus (DC)	3.33E-07 ± 5.77E-09^{a}	1.67E-07 ± 1.14E-09^{b}	2.61E-07 ± 1.75E-09^{c}	3.00E-07 ± 7.71E-09^{a}	9.70E-08 ± 3.20E-09^{b}
	Watery callus (WC)	3.51E-07 ± 2.58E-08^{a}	1.83E-07 ± 1.82E-08^{b}	2.92E-07 ± 4.29E-08^{c}	4.23E-07 ± 1.90E-07^{a}	1.26E-07 ± 4.31E-08^{b}
	Suspension culture (SC)	3.58E-07 ± 1.74E-08^{a}	1.85E-07 ± 1.35E-08^{b}	2.78E-07 ± 1.13E-08^{c}	3.61E-07 ± 5.19E-08^{a}	1.18E-07 ± 1.69E-08^{b}
	Regenerant (R)	4.50E-06 ± 3.91E-06^{a}	3.98E-06 ± 3.66E-06^{a}	4.80E-06 ± 4.27E-06^{ab}	8.04E-06 ± 7.44E-06^{b}	2.01E-05 ± 2.05E-05^{a}

All the secondary metabolites tested were present at a significant level (p-value <0.05) with more than 10 times greater abundance in shoot base (SB) than in the three callus type; EC, DC and WC (Figure 4.8A). Watery callus (WC) had the highest total secondary metabolite concentration of 0.00069% dry extract while embryogenic callus (EC) had the lowest, 0.00023% dry extract (Figure 4.8A). Dry callus (DC) had an intermediate level at 0.00052% of the total dry extract. Comparatively, SB still had the highest concentration of secondary metabolites while the three callus types had relatively similar abundances of all five secondary metabolites studied.

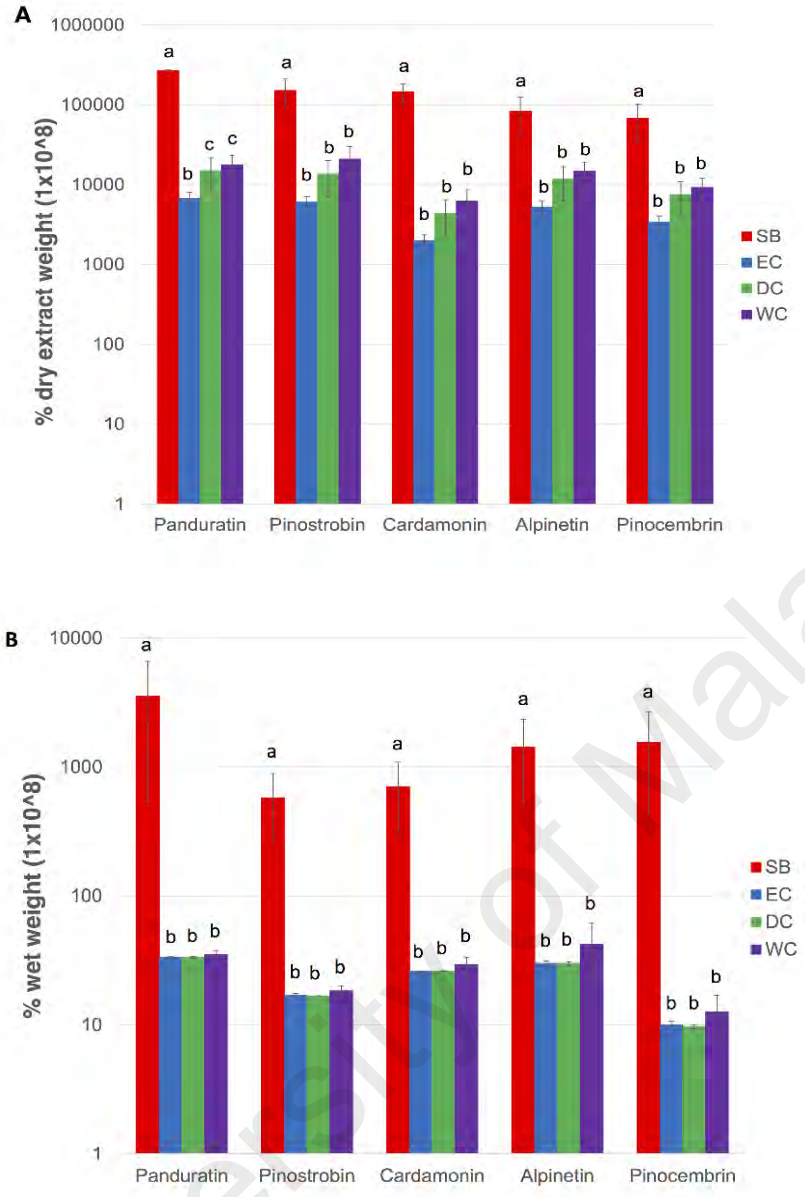

Figure 4.8: Quantitative analysis of the five secondary metabolites in *B. rotunda* callus and shoot base tissues (n=3 biological replicates); A: values expressed in percent dry weight. B: values expressed in percent wet weight. Red: shoot base (SB); blue: embryogenic callus (EC); green: dry callus (DC); purple: watery callus (WC). Error bars indicate standard deviation and different letters represent significant differences for each metabolite at 95% confidence level by Tukey's test.

4.2.1 Distribution of five flavonoids in various sections and ages of *B. rotunda*

The experiment was designed to investigate the relative concentrations of five secondary metabolites (panduratin, pinostrobin, pinocembrin, cardamonin, and alpinetin) at different ages and sections of shoots; rhizome (Rh), SB and shoot sections (T1 to T5) (Figure 3.2). Results showed a steady increase in growth of the shoot sections of *B.*

rotunda (Figure 4.9) as the shoots mature. The young shoots of *B. rotunda* developed an average shoot growth of 0.2 cm per day and increased to 0.3 cm and finally to 0.5 cm daily at matured stage.

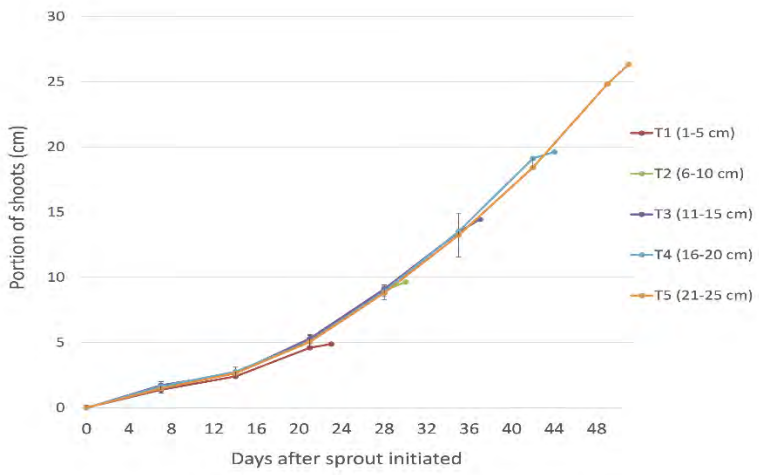

Figure 4.9: Five separate growth curve of shoots in *B. rotunda* (n=5 biological replicates); Blue: T1, red: T2, green: T3, purple: T4, light blue: T5. Error bars indicate standard deviation.

A heatmap was generated to view the trend of secondary metabolite concentrations in the different shoot sections (vertical; Rh to T5) and the different ages of tissues (horizontal; 1-5 cm to 21-25 cm). For the different shoot sections of *B. rotunda*, a general trend of decreasing secondary metabolite concentrations were observed with rhizome (Rh) having the highest concentration followed by shoot base (SB) and finally T1 to T5 (Figure 4.10). The further away the shoot section from Rh, the lower the concentration of secondary metabolites, except for alpinetin concentration which had higher concentration in T2 (0.58% dry extract) than in T1 (0.37% dry extract) at shoot length; 6-10 cm (Figure 4.10). Another exception was observed at shoot length; 21-25 cm whereby the abundances of panduratin and alpinetin in shoot base (SB) were more than one fold higher than in rhizome (Rh).

The concentration of secondary metabolites varies with age of *B. rotunda* shoots. The secondary metabolites in young shoot base (SB) showed insignificant concentration but increased significantly except cardamonin as the shoot base matured with at least 10 times higher concentration from 0.09% dry extract (at 1-5 cm shoot length) to 1.01% dry extract (at 21-25cm shoot length) (Figure 4.10). On the other hand, the relatively young

rhizome (Rh) (shoot length <10 cm) had significant panduratin concentration but the levels became insignificant as rhizome tissue matured (shoot length >16cm). Specifically for pinocembrin and cardamonin metabolites, the concentration trend decreased from young to mature T3 tissues with significant abundance observed at matured T3 tissue (shoot length >16cm). Additionally, the matured T4 tissue had insignificant concentrations for all five secondary metabolites.

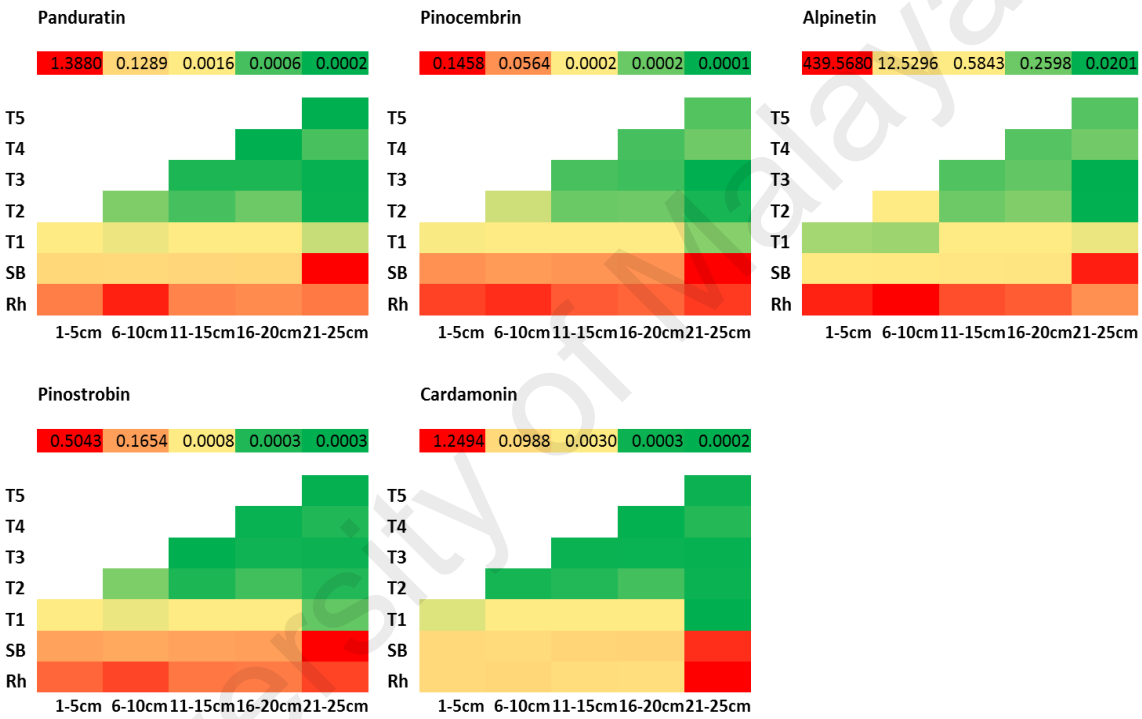

Figure 4.10: Heatmap of the five flavonoids concentration (% dry extract) in various shoot sections and ages of *B. rotunda* (n=3 biological replicates); Red: highest concentration, green: lowest concentration. Scale bar numbers denotes concentrations (% dry extract) for each metabolites.

The trend of five secondary metabolite concentrations was visualized using a line graph of a young shoot length; 6-10 cm shown in Figure 4.11. Significantly, higher concentrations of all five secondary metabolites was observed in the rhizome (Rh) tissue, with the concentrations for each metabolite decreasing from the shoot base and along the more distal root samples. Rhizome was observed to have 60 times more alpinetin than in

shoot base. Other metabolites such panduratin, pinostrobin and pinocembrin had at least one fold higher values in rhizome than in shoot base.

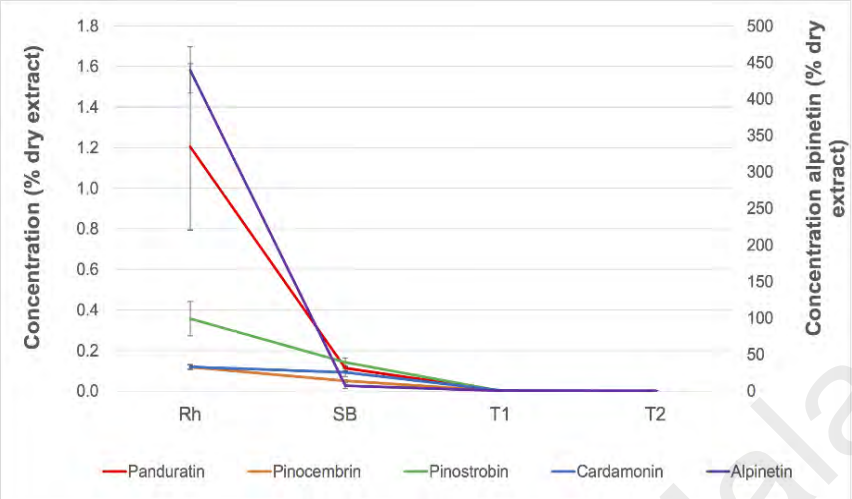

Figure 4.11: Concentration (% dry extract) of secondary metabolites at 6-10 cm shoot section of *B. rotunda* (n=3 biological replicates); Rh: rhizome, SB: shoot base, T1: region of shoot 1-5 cm distal from the shoot base, T2: region of shoot 6-10 cm distal from the shoot base. Error bars indicate standard deviation.

4.3 Hormone analysis in *B. rotunda* samples

Plant hormone analysis was performed for all seven samples of *B. rotunda*. As most of the classes of hormones (e.g. gibberellins, ABA, cytokinins) were not detected, only auxins, jasmonates and salicylates data are shown. 2,4-dichlorophenoxyacetic acid (2,4-D), an auxin was applied exogenously to the media to form different calli types (Tan *et al.*, 2005; Yusuf *et al.*, 2011) using the following concentrations; dry callus (DC) (4 $mg.L^{-1}$), embryogenic callus (EC) (3 $mg.L^{-1}$) and watery callus (WC) (1 $mg.L^{-1}$). Results showed that the intracellular concentration of 2,4-D was statistically similar in DC (1444 ppb ± 495.3) and WC (1126 ppb ± 136.8) (Figure 4.12). In contrast, 2,4-D was not detected in EC and SC (detection limit of 1 ppb), despite there being an intermediate concentration of 2,4-D in the EC and SC medium. Another common type of auxin used as plant growth regulator is indole-3-acetic acid (IAA). A range of IAA concentrations from 20-240 ppb was shown in the six samples, except for the conventionally propagated

leaf (L). The endogenous hormone indole-3-acetic acid (IAA) had a decreasing concentration trend in *B. rotunda* as follows: DC (565 ± 261 ppb) > WC (420 ± 232 ppb) > SB (379 ± 148 ppb) > SC (372 ± 185 ppb) > EC (160 ± 20 ppb) > R (157 ± 29 ppb) (Figure 4.12). Concentration of IAA was three times more in DC than in EC.

Endogenous jasmonic acid and methyl jasmonate showed similar trends in which they were only detected in conventionally propagated leaf (L) and in regenerated *in vitro* leaf (R) samples. The concentration in R samples was significantly higher (p-value < 0.05) than in L for jasmonic acid and methyl jasmonate. For jasmonic acid, L and R samples had concentrations of 234 ± 33 ppb and 389 ± 55 ppb, respectively (Figure 4.12). On the other hand, R sample had more than 2-fold higher methyl jasmonate than in L sample. As for the salicylate (benzoic acid) concentration, the six samples types were statistically similar except for SB. SB had the highest benzoic acid concentration of 2547 ± 476 ppb while WC had the lowest concentration of 712 ± 284 ppb.

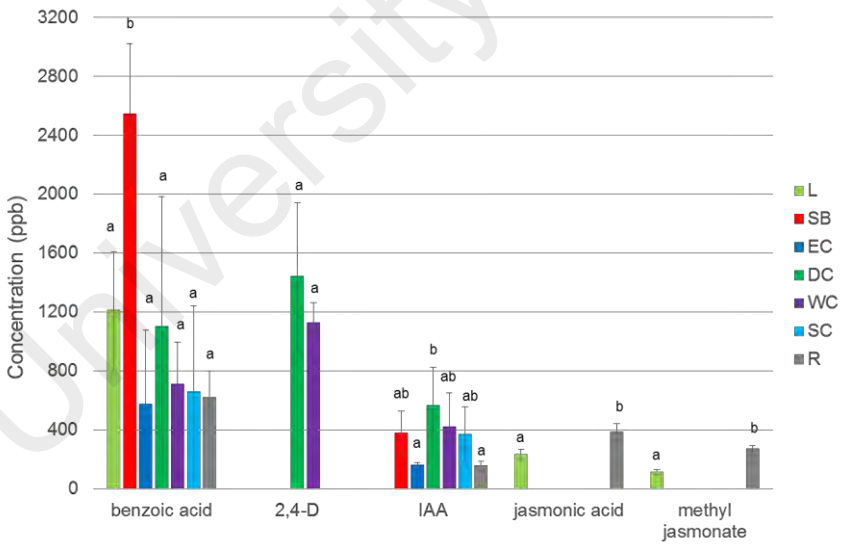

Figure 4.12: Intracellular hormones concentrations (parts per billion) in dry extracts of *B. rotunda* (n=3 biological replicates); Light green: conventionally propagated leaf (L), red: shoot base (SB), blue: embryogenic callus (EC), green: dry callus (DC), purple: watery callus (WC), light blue: suspension cells, grey: regenerant (R). Error bars indicate standard deviation and different letters represent significant differences at 95% confidence level by Tukey's test

4.4 Histo-morphological analysis of shoot base and three callus types of *B. rotunda*

The histo-morphological study was carried out for shoot base (SB) and the three callus types. Morphologically, EC were pale-yellowish, globular and friable callus while DC were yellowish, friable, nodular and dry (Figure 4.13B and C). Although WC had a yellowish colour similar to that of EC and DC, its morphology was very different from the other callus types. WC were spongier than either DC or WC and wet in appearance (Figure 4.13D).

In addition to being able to differentiate the physical morphologies of each callus type through naked eye, utilization of scanning electron microscope (SEM) provided more detailed information about the cell surface. Both EC and DC had more regular shapes and sizes than did WC, which had irregular shapes and sizes (Figure 4.13F to H). Moreover, EC had more fibrils on cell surface than did DC cells, which were more rounded and compact. Figure 4.15E showed that the cells of the SB were organized and, regularly-shaped and -sized and that some cells were starting to accumulate starch granules.

The fluorescence study was conducted using diphenylboric acid 2-aminoethylester (DPBA) stain to visibly locate flavonol abundance, especially quercetin, kaempferol and related flavonoid derivatives. Results showed the presence of thin yellowish-green lining of cell membranes across all four sample types (Figure 4.13I to L). However, only EC and DC showed the presence of fluorescent greenish-blue spots (Figure 4.13J and K) indicating the presence of localized flavonoids while no fluorescence was observed in WC (Figure 4.13L), which had higher flavonoid concentrations (Figure 4.13I and L) than EC and DC samples.

Apart from the fluorescence study, histological examination using light microscopy was also performed on the shoot base (explant) and the three types of callus

of *B. rotunda* stained with the Periodic Acid-Schiff (PAS) reagent to view starch reserves. EC and DC had dense cells with prominent dark blue clusters (Figure 4.13N and O) while WC had cells with irregular shapes and sizes and lacked blue clusters. Moreover, the presence of purplish-red spots in SB, EC, and DC (Figure 4.13M to O) indicated the presence of starch grains. The SB sample of *B. rotunda* also showed the presence of vascular bundles surrounded by parenchyma cells (Figure 4.13M).

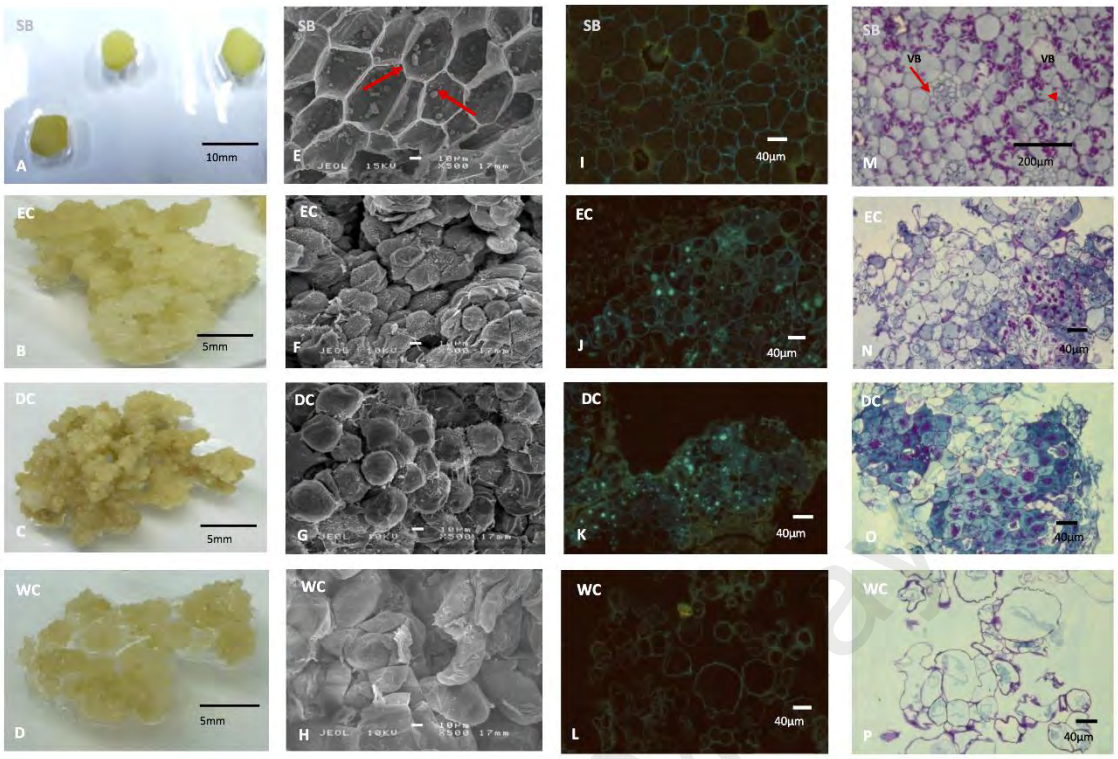

Figure 4.13: Morphology and histology of *B. rotunda* shoot base and callus. **A-D**: morphology of samples; A: cross section of 1 cm x 1 cm shoot base tissue; B: friable pale yellowish callus; C: compact, dense and dry callus, D: spongy and wet callus; **E-H**: SEM images (100x magnification); E: regular-shaped and -sized cells with arrows showing the presence of starch; F: regular-shaped cells with fibrils; G: rounded, compact cells; H: elongated and irregular-shaped cells; **I-L**: morphology of each sample viewed under fluorescent microscopy with diphenylboric acid 2-aminoethylester (DPBA) stain (100x magnification); I: fluorescent yellowish-green lining of cell membrane, J: fluorescent greenish blue spots observed with yellow lining of cell membrane; K: fluorescent greenish-blue spots observed with yellow lining of cell membrane; L: yellowish lining of cell membrane; **M-P**: morphology of each sample viewed under light microscopy with Periodic Acid-Schiff (PAS) stain (100x magnification); E: organized and compact cells with presence of vascular bundles (VB) and purplish-red starch granules; F: presence of dark blue clusters indicates active cell division and red-purplish starch granules; G: presence of dark blue clusters indicates active cell division and purplish-red starch granules; H: irregular-shaped and -sized cells without starch granules. SB: shoot base; EC: embryogenic callus; DC: dry callus; WC: watery callus

CHAPTER 5

DISCUSSION

Plant tissue culture is mainly utilized in agriculture biotechnology to increase yield, to improve quality of plants, to produce uniform planting materials and important bioactive secondary compounds. However, application of plant tissue culture can pose several issues including abnormalities and somaclonal variation which may affect plant yield, embryogenesis and regeneration.

In *Boesenbergia rotunda* (*B. rotunda*), plant tissue culture study was performed primarily due to the increased demand for plant material as a result of positive medicinal properties reported from the derived secondary metabolite extracts. However, a recent study by Wong *et al.* (2013) reported that homogenous suspension cultures of *B. rotunda* maintained for periods of several months fail to regenerate into somatic embryos and hence new plants. The failure of suspension culture to regenerate into plantlets through somatic embryos is possibly due to underlying molecular changes in the cells. It could be hypothesized that these molecular changes would be reflected by cellular metabolite levels during tissue culture, which may relate to embryogenesis. To test the hypothesis, a metabolmics approach was undertaken. Accordingly, this thesis specifically investigated the biochemical profiles of various *B. rotunda* samples representing different stages of culture, embryogenesis and regeneration (Figure 4.1). Additionally, manipulation of plant cell culture conditions in *B. rotunda* can be expected to affect cell metabolism and thus might have an impact on the production of specific secondary metabolites of interest.

In the present study, the metabolite profile of suspension cells under prolonged culture conditions (more than 12 months) was compared with that of embryogenic callus to understand recalcitrance in plant regeneration since the suspension cells were initiated from embryogenic callus. Additionally, the metabolite profiles of three callus types

(embryogenic callus which is the embryogenic type, dry callus and watery callus which are non-embryogenic types) that were derived from the shoot base of *B. rotunda* were compared to identify metabolite markers associated with embryogenesis. Furthermore, the histo-morphological study supported the characterization of different *B. rotunda* samples using microscopy techniques.

5.1 Distinct metabolite profiles were observed for *B. rotunda* samples

The abundance of most of the primary metabolites in various *B. rotunda* samples studied were very different from one another (Table 4.1 and Figure 4.2), with embryogenic callus having the most abundant primary metabolites. Watery callus of *B. rotunda* did not exhibit embryogenic competency, as no calli developed into embryos, as reported by Tan *et al.* (2015). The primary metabolite profile for suspension cells showed a cluster that was separated from that for embryogenic callus of *B. rotunda* (Figure 4.6). Failure in regeneration of *B. rotunda* suspension cells is likely to be because the cells are stressed under prolonged culture conditions. As a result of stress more secondary metabolites were produced in suspension cells, resulting in higher concentrations of more than 2-fold of these compounds than in embryogenic callus (Table 4.3). Previous studies reported that the presence of stresses including temperature, humidity, light intensity, water supply and plant growth regulators can impact qualitative and quantitative production of secondary metabolites (Akula & Ravishankar, 2011; Gaspar *et al.*, 2002). A study in two wheat leaves cultivars, Aikang 58 (AK) and Chinese Spring (CS) reported an increase in total flavonoids content in CS cultivar and higher expression level of genes involved in flavonoids biosynthesis under water deficiency stress (Ma *et al.*, 2014). An increased in flavonoid accumulation was also reported in *Hydrocotyle bonariensis* (pennywort) leaves under exposure of auxin and cytokinin conditions (Masoumian *et al.*, 2011). A genetic study is currently underway to determine changes in gene expression

that can complement the metabolomics data towards understanding the roles of various metabolic activities in recalcitrance to regeneration of *B. rotunda* suspension cells. A previous study in *Arabidopsis* reported that suspension cells were mixoploid and the amount of DNA in cells varied under prolonged culture conditions (Sedov *et al.*, 2014).

The conventionally propagated leaf (L) samples were grown in an open condition with direct exposure to the environment (12 h daylight with a temperature range of 25-30^{o}C) while the regenerated *in vitro* leaf samples (R) were propagated in a closed, controlled environment (16 h day/ 8h dark with temperature at 25^{o}C). Therefore it is likely that the different environmental conditions experienced by these samples may result in the segregated clusters and different metabolic profiles (Figure 4.3) although both were derived from the same physiological structure (i.e. leaves) of *B. rotunda*. Additionally, the conventionally propagated leaf samples were harvested at a matured age as compared to the young regenerated *in vitro* leaves and this may further add to the different metabolic profiles observed. Robinson *et al.* (2007) reported that environment effects on metabolome were greater than genetic variation effects in Douglas fir trees. Similarly, studies on the two important crops, maize (Frank *et al.*, 2012) and rice (Matsuda *et al.*, 2012) indicated that metabolite variations were greater between samples grown under different environmental conditions, such as location and seasons, compared to differences between samples with genetic variations between strains and between wild type and genetically modified varieties.

5.1.1 Amino acid requirement in plant regeneration and embryogenesis of *B. rotunda*

Amino acids are essential components for plant growth and development. For *B. rotunda*, high abundance of certain amino acids possibly encouraged cell differentiation

and division leading to embryogenesis and plant regeneration. Specifically, the abundance of glutamine and lysine was more than five-fold higher in embryogenic callus than in suspension cells (which were non-regenerative following prolonged culture) while arginine was more than 600-fold higher in the embryogenic callus than in the dry and watery calli (the non-embryogenic) samples (Table 4.1 and Figure 4.5). The very low abundance of arginine and glutamine in the non-embryogenic samples of *B. rotunda* could be a result of cells not metabolizing well, thus showing low overall callus growth. This finding is complemented together with cell features observed from the histo-morphological study (Figure 4.13) which will be discussed later in section 5.4. Interestingly, the highly significant level of glutamine and lysine (Figure 4.7) in the sample of sieved embryogenic cells of *B. rotunda*, which had a higher proportion of embryogenic cells than embryogenic callus samples further confirmed the importance of these amino acids for callus growth and proliferation. Additionally, the low concentrations of the amino acid markers especially glutamine, arginine and lysine found in suspension cells (Figure 4.7) could be indicative of failure in plant regeneration. The amino acid, lysine has been reported to promote rice plantlet regeneration through exogenous application (Pongtongkam *et al.*, 2004) while glutamine, together with arginine, has been reported to play major roles in tissue culture proliferation and growth: A study on white pines (*Pinus strobes*) revealed that endogenous levels of glutamine and arginine were associated with early development of zygotic embryos (Feirer, 1995). Another study in Japanese conifer (*Cryptomeria Japonica*) reported high accumulation of glutamine in embryogenic callus (Ogita *et al.*, 2001). Khan *et al.* (2014) have reported a 3-fold increase of endogenous glutamine in somatic embryos of milk thistle. Glutamine was reported to be a nitrogen source in calli of carrots (Kamada & Harada, 1984) and heart vine (Jeyaseelan & Rao, 2005) as well as a precursor of other amino acids

(Newsholme *et al.*, 2003). In addition, a higher glutamine content was reported in callus than in regenerated shoots of bamboo (Ogita, 2005).

Similar to the results for *B. rotunda* (Table 4.1 and Figure 4.5), higher levels of arginine were reported in somatic embryos than in non-embryogenic callus of milk thistle (Khan *et al.*, 2014). Arginine is an important precursor for polyamine biosynthesis, via the arginine decarboxylase pathway (Minocha *et al.*, 2004). The association of polyamine with embryogenesis were reported in Norway spruce (Malá *et al.*, 2009; Minocha *et al.*, 1993) and eggplant (Singh Y. & Manchikatla, 1998). The major products of polyamine biosynthesis are putrescine, spermidine and spermine. In *B. rotunda*, putrescine was found only in embryogenic callus which are embryogenic competent but was absent in the other non-embryogenic samples (Table 4.1). Previous studies have reported putrescine in promoting plant regeneration through somatic embryogenesis in cotton (Sakhanokho *et al.*, 2005) and sugarcane (Reis *et al.*, 2016). Additionally, up-regulation of two arabinogalactan proteins (AGPs) due to putresine treatment was reported as a possible action related to somatic embryogenesis in sugarcane (Reis *et al.*, 2016). Further studies in cotton (Poon *et al.*, 2012) and white oak (Mallón *et al.*, 2013) have confirmed that exogenous addition of AGPs in tissue culture medium of embryogenic callus stimulates somatic embryogenesis.

The three important metabolite markers found (glutamine, arginine and lysine) were validated (Figure 4.7) and well correlated with embryogenic competency in *B. rotunda* samples. Therefore, it is possible that these compounds could be exogenously applied in culture media to observe if this results in an increase of embryogenesis rate compared to the existing media formulation for *B. rotunda* since no studies were reported previously. Nonetheless, careful optimization in the cultured media is necessary to ensure high success rate. At the least, these amino acids could be used as good indicators for cell embryogenic competency.

5.2 Are secondary metabolites produced in cultured callus cells of *B. rotunda*?

The secondary metabolites, specifically flavonoids were most concentrated in the shoot base and rhizome of *B. rotunda* (Table 4.3, Figure 4.8 and 4.10), in agreement with a previous comparative study by Tan *et al.* (2015) and Yusuf *et al.* (2013). The trend of decreasing secondary metabolite concentrations in rhizome to shoot base and finally to T5 (Figure 4.10 and 4.11) of *B. rotunda* suggests that flavonoids had diffused away from the rhizomes, along the shoots towards the developing tips. Harborne (2013) reported that most flavonoid investigations were concentrated in rhizome because this organ had most flavonoids identified. Therefore, rhizome is likely to be the major source of secondary metabolite production as this organ has differentiated tissue, than in undifferentiated tissue such as calli (embryogenic and non-embryogenic) and suspension cells of *B. rotunda*. Previous reports reviewed that high levels of secondary metabolites were produced in organs of differentiated tissue especially in shoot and root culture (Jedinák *et al.*, 2004; Rao & Ravishankar, 2002).

The very low abundance of flavonoids quantified in cultured callus cells regardless of dry and wet weight (Figure 4.8) raises the question of whether the flavonoids were biosynthesized at relatively low levels in calli cells or if the flavonoids were residual from the shoot base explant. It is important to note that if flavonoid biosynthesis is not active in the cultured callus cells, multiplying the cells may not produce the secondary metabolites as desired even through the use of the fastest growing callus type, which was embryogenic callus in this case. Additionally, if the metabolites are residual from the original explant, the more cycles of cell division (longer time in propagation), the lower the secondary metabolite concentration in the callus cells. Therefore, future experiments to demonstrate if the flavonoid biosynthesis occurs in the cultured callus cells or explant, as this knowledge could benefit the large scale production of secondary metabolites of

interest. The ongoing transcriptome study of *B. rotunda* may provide data on the expression of genes in the related metabolic pathways to support the above hypothesis. Thus far from the secondary metabolite data for *B. rotunda*, it is reported that low level of flavonoid content was associated with healthy, active and proliferative cells like the embryogenic callus in contrast to the recalcitrant cells which had higher flavonoids content in the non-embryogenic calli (the dry and watery calli) (Figure 4.8). Additionally, the level of secondary metabolites in suspension cells resembled the levels observed in the non-embryogenic callus in *B. rotunda* (Table 4.3).

5.2.1 Biosynthesis of secondary metabolites in *B. rotunda* samples

Secondary metabolites are produced as part of a plant's defense mechanism (Kabera *et al.*, 2014). The biosynthesis of flavonoids occurs via the phenylpropanoid pathway with phenylalanine as the precursor. Previous studies in *B. rotunda* reported on activation of certain genes and proteins involved in the biosynthesis of phenylpropanoid pathway upon utilization of phenylalanine as precursor through transcriptome (Md-Mustafa *et al.*, 2014) and proteome (Tan *et al.*, 2012a) analysis. From the metabolite profiling data, along with low secondary metabolite levels, the level of phenylalanine in embryogenic callus was 95 times higher than in the non-embryogenic calli (the dry and watery calli) (Table 4.1), possibly indicating lower conversion of phenylalanine to flavonoids in embryogenic calli despite activated primary metabolism noted earlier. Similarly in the non-embryogenic calli of *B. rotunda*, low phenylalanine concentration was noted with highest flavonoid content. The highest accumulation of flavonoids was observed in the watery callus (Table 4.3) compared to the other two callus samples; embroyogenic and dry calli. However, future investigation is warranted to understand the underlying metabolism of phenylalanine in the phenylproanoid pathway in *B. rotunda* cultured samples. Previous studies in conifers (Dubravina *et al.*, 2005) and red spiderling

herb (Chaudhary & Dantu, 2015) reported the effects of browning and subsequently poor growth in calli due to high phenolic content. Similar results of higher phenolic content were observed in non-embryogenic callus of chick pea (*Cicer arietinum*) (Naz *et al.*, 2008), walnut (*Juglans regia*) (Rodriguez, 1982) and alfalfa (*Medicago sativa*) (Dubravina *et al.*, 2005) compared to levels in embryogenic calli, in agreement with the data for *B. rotunda* in this study.

5.3 The importance of auxin in somatic embryogenesis of *B. rotunda*

The synthetic auxin 2,4-dichlorophenoxyacetic acid (2,4-D) is widely used as a plant growth regulator in plant tissue culture. Like other auxins, 2,4-D initiates growth of calli by promoting cell differentiation to form overall calli morphologies. When induced with 2,4-D, calli of *B. rotunda* showed totipotency (Tan *et al.*, 2005; Wong *et al.*, 2013; Yusuf *et al.*, 2011), as reported for *Arabidopsis* (Raghavan, 2004) and carrots (Komamine et al., 1992). The low concentration of 2,4-D (detection limit of 1 ppb) observed in embryogenic callus of *B. rotunda*, was possibly due to the active metabolism and utilization of this plant growth regulator to promote the formation of embryos (Figure 4.14). Intracellular auxin was not detected in conventionally propagated leaf (L) and regenerated *in vitro* leaves (R), since 2,4-D is not a naturally occurring auxin and was not exogenously applied to the culture media. The regenerated *in vitro* leaves were grown under growth regulator-free media as reported by Wong *et al.* (2013). Commonly, the concentration of 2,4-D in plant culture media is optimized for different plant species and explants on callus formation A study in carrots by Zimmerman (1993) showed inhibited growth of calli after the globular stage of callusing in the presence of 2,4-D in some carrot cell lines. For *B. rotunda*, the concentration of 2,4-D had been optimized to induce selected callus types mainly to increase the abundance of selected bioactive secondary metabolites (Tan *et al.*, 2005; Yusuf *et al.*, 2011).

The naturally occurring auxin, indole-3-acetic acid (IAA), may encourage embryogenesis in *B. rotunda* samples. The low level of intracellular IAA observed in embryogenic callus suggests that IAA may be actively metabolized to stimulate embryogenesis. Several studies found contradictory data on levels of IAA needed to encourage somatic embryogenesis; high levels of IAA were needed to encourage somatic embryogenesis in sugarcane (Guiderdoni *et al.*, 1995), wheat (Jiménez & Bangerth, 2001), and maize (Jiménez & Bangerth, 2001), while insignificant difference in IAA levels in the embryogenic and non-embryogenic callus were reported in carrot (Michalczuk *et al.*, 1992a) and oil palm (Besse *et al.*, 1992), which suggest that the intracellular IAA level may not be the only factor for embryogenic events. It was reported that the presence of exogenous 2,4-D may influence IAA metabolism in carrot cell lines (Michalczuk *et al.*, 1992a; Michalczuk *et al.*, 1992b). In the case of *B. rotunda*, a moderate level of exogenous 2,4-D leads to a formation of embryogenic callus with low intracellular IAA levels (Figure 4.12). Additionally, the low endogenous level of IAA in regenerated *in vitro* leaves (R) of *B. rotunda* could be due to the active involvement in vegetative growth (Figure 4.14). Previous studies in orchid cultivars reported that action of IAA alone or in conjunction with other phytohormones in the culture media promotes callusing, shooting and rooting, while inhibiting germination and flowering (Faria *et al.*, 2013; Hossain & Dey, 2013; Novak *et al.*, 2014).

5.3.1 IAA concentrations vary for different *B. rotunda* samples

The study of IAA concentration in *B. rotunda* samples is essential to provide baseline knowledge on the embryogenic competency from the endogenous concentration of IAA. The naturally occurring auxin, IAA is derived from the precursor tryptophan in the tryptophan-dependent IAA biosynthesis pathway (Ljung, 2013). Several tryptophan dependent pathways are postulated including the indole-3-acetamine (IAM) pathway, the

indole-3-pyruvic acid (IPA) pathway, the tryptamine (TA) pathway and the indole-3-acetyldoxime (IAOX) pathway (Ljung, 2013; Mano & Nemoto, 2012; Pollmann *et al.*, 2006; Woodward & Bartel, 2005). In *B. rotunda*, the embryogenic callus and suspension cells samples had concurrent results in which a high concentration of the precursor tryptophan were noted, with low concentration of the product, IAA (Table 4.1 and Figure 4.12). Despite this, the embryogenic callus and suspension cells had different sample characteristics; embryogenic callus had the potential to undergo embryogenesis and later plantlet regeneration unlike the suspension cells that had no competency to regenerate into new plant under prolonged culture condition. In contrast, the non-embryogenic calli (dry and watery callus) had relatively low concentration of tryptophan with high level of IAA. This is in good agreement with the report on the tissue culture of milk thistle explants, which had a higher level of tryptophan in somatic embryos, possibly due to establishment of an auxin gradient required for embryo differentiation (Khan *et al.*, 2014). In rice (*Oryza sativa*) culture, large quantities of embryogenic calli were obtained in culture media containing tryptophan (Chowdhry *et al.*, 1993). Similar studies with various rice cultivars reported that the presence of tryptophan stimulated plant regeneration (Shahsavari, 2011; Wijesekera *et al.*, 2007). Additionally, a report on the culture of wild cherry by Sung (1979) indicated that high abundance of tryptophan decreases the endogenous level of IAA in the presence of 2,4-D due to auxin self-regulation or interference of IAA synthesis with 2,4-D, which could corroborate the results obtained in embryogenic callus of *B. rotunda*.

5.3.2 Presence of other hormones in *B. rotunda* samples

Wounding that occurred during *B. rotunda* leaf sampling may be the cause of accumulation of jasmonic acid (JA) and methyl jasmonate (MeJA) in conventionally propagated leaf and regenerated *in vitro* leaves samples (Figure 4.12). Ryan (2000)

reported the increase of jasmonates due to wounding of tomato leaves from the linolenic acid biosynthesis pathway via the action of systemin signaling. In plant tissue culture of potatoes and tomatoes, the presence of JA retarded callus formation (Ravnikar & Gogala, 1990), and promoted dormancy and senescence (Tung *et al.*, 1996), respectively. The concentration differences of benzoic acid was insignificant across all four *in vitro* cultured samples of *B. rotunda* with embryogenic callus having the lowest concentration of 575 ± 501 ppb. Eventually, the study of benzoic acid pathways could potentially shed light to the role played in *B. rotunda* cell culture from the high concentration reported in shoot base sample.

5.4 Presence of fibrils and starch reserves indicated embryogenic competency in *B. rotunda* cell culture

Microscopy was carried out to characterize the detailed morphology of cells in the shoot base and the three callus types of *B. rotunda* sampled in this study. At an early stage of callusing, cells from embryogenic and dry calli were hard to distinguish. Prominent differences were only observed at a later stage of callusing, at about the 3rd -4th week of culture when dry callus became hard callus clumps that resisted growth upon sub-culture, while structures in embryogenic callus were observed as globular, translucent spheres which differentiated and developed into somatic embryos. Similar morphologies of *B. rotunda* embryogenic callus had been reported elsewhere (Tan *et al.*, 2005; Yusuf *et al.*, 2011; Yusuf *et al.*, 2013). Moreover, embryogenic cell cultures from other plants such as banana (Jalil *et al.*, 2008), coffee (Quiroz-Figueroa *et al.*, 2002) and potatoes (Sharma & Millam, 2004) also exhibited similar morphology as *B. rotunda* embryogenic callus. In contrast, the unique morphology of watery callus was easily identified at the early stage of callus initiation (Figure 4.13D).

A scanning electron microscope (SEM) can be used as tool to validate the characteristics of callus types, a complementary study to the light microscopy technique. Through SEM analysis, the observed presence of a membranous layer and fibrils on the cell surface of embryogenic callus suggests that the *B. rotunda's* embryogenic callus had potential morphogenic capacity besides the validated amino acid metabolite markers; glutamine, arginine and lysine as reported. Similar morphology had been reported in embryogenic callus of kiwifruit (Popielarska *et al.*, 2006). The absence of fibrils is indicative of non-embryogenic calli, as reported in studies of sugarcane (Rodríguez *et al.*, 1996) and *Citrus* hybrid callus (Chapman *et al.*, 2000), concurring with the observation for watery callus sample of *B. rotunda* (Figure 4.13H). Popielarska *et al.* (2006) reported that presence of fibrils possibly derived from pectins, had been reported to play a role in cell-to-cell adhesion and the control of cell wall ionic status and porosity. Baluška *et al.* (2003) further reports that pectin oligosaccharide fragments released from cell walls function as signaling molecules in the regulation of overall developmental processes. The presence of starch granules was observed only in shoot base sample which derived from rhizome of *B. rotunda* under examination of scanning electron microscopy. Similar findings of starch granules were reported in the rhizomes of mango ginger (Policegoudra & Aradhya, 2008) and the rhizomes of switchgrass (Sarath *et al.*, 2014).

5.4.1 Fluorescence study in the three callus types of *B. rotunda*

To date, there are no reports on the usage of diphenylboric acid-2-aminoethyl ester (DPBA) stain to study the correlation of fluorescence intensity observed through DPBA staining with flavonols and flavonoids derivatives in cells of *B. rotunda* but previous studies have been reported in the model plant *Arabidopsis* (Buer *et al.*, 2007; Peer *et al.*, 2001; Saslowsky *et al.*, 2005). Observation under fluorescent microscopy in the three calli types (embryogenic, dry and watery calli) suggests that the DPBA dye could have

different specificity for particular flavonoids in *B. rotunda* (Figure 4.13J to L). The five secondary metabolites studied were not categorized under flavonols but classified into groups of chalcones (panduratin and cardamonin) and flavonones (pinostrobin, pinocembrin and alpinetin). A previous study reported that DPBA stained accumulated flavonols in the endosperm of *Arabidopsis* seeds (Endo *et al.*, 2012). Another study also reported that DPBA emission intensity is greater for flavonols, dihydroflavonols and the non-glycosylated flavonoids than for glycosylated flavonoids (Murphy *et al.*, 2000; Ogo *et al.*, 2016; Sheahan & Rechnitz, 1992). In *Arabidopsis*, Peer *et al.* (2001) reported that DPBA-stained flavonoid complexes had unique fluorescing intensities using fluorescein isothiocyanate (FTIC) filters measured at different wavelengths. Peer *et al.* (2001) also found that kaempferol, quercetin and naringenin chalcone were stained in yellow-green (520 nm), gold (543 nm), and yellow (527 nm), respectively. *B. rotunda* watery callus on the other hand had no detectable fluorescence (Figure 4.13L) but had the highest total 5 secondary metabolite concentrations as measured by Ultra Performance Liquid Chromatography Mass Spectrometry (UPLC-MS); the metabolites studied are the flavonoid-related compounds with concentration of 6.9×10^{-4} % dry extract for watery callus (Table 4.3), a contradictory result to that for embryogenic callus of 2.3×10^{-4} % dry extract and 5.2×10^{-4} % dry extract in dry callus. This could be due to different affinity of various flavonoids for the DPBA stain. For *B. rotunda*, part of the flavonoid biosynthesis pathway in the embryogenic callus could still be active from the observed fluorescence of stained sample despite the low concentration of the five secondary metabolites studied (Table 4.3). Poustka *et al.* (2007) reported that DPBA does not fluoresce with anthocyanin, a glycosylated flavonoid derivative. Further detailed investigation would require the use of flavonoid-specific stains or laser induced ionization mass spectrometry techniques.

5.4.2 Presence of starch in shoot base, embryogenic and dry callus of *B. rotunda* from Periodic Acid-Schiff (PAS) reagent

For examination under Periodic Acid-Schiff reagent (PAS), presence of dark blue clusters were observed in both embryogenic and dry calli (Figure 4.13N and O) despite the fact that only embryogenic callus leads to further plant development. This finding is similar to that reported for embryogenic callus of oil palm (Sarpan *et al.*, 2011) and date palm (Zouine *et al.*, 2005), where dark blue stains were observed at meristematic regions. *B. rotunda* watery callus on the other hand, did not have dark blue stains, which indicated that watery callus cells were not actively dividing and absent of primary metabolite indicators; amino acids glutamine, arginine and lysine.

The presence of purplish-red spots in shoot base, embryogenic and dry callus (Figure 4.13M to O) indicated the presence of starch grains which conserve energy for overall culture growth and development. Previous studies on somatic embryos of date palm (Zouine *et al.*, 2005) and embryogenic callus of banana (Xu *et al.*, 2011) also reported similar coloured structures of starch reserves using PAS. Nonetheless, utilization of PAS stain is useful as a complementary study in determination of embryogenic competency in *B. rotunda* if used together with primary metabolite markers (i.e. glutamine, arginine and lysine).

CHAPTER 6
CONCLUSION AND RECOMMENDATIONS

Primary metabolites, secondary metabolites, and hormones were profiled using an UPLC-MS for seven *B. rotunda* samples; namely conventionally propagated leaf (L), shoot base (SB), embryogenic callus (EC), dry callus (DC), watery callus (WC), suspension cells (SC) and regenerated *in vitro* leaves (R). Each sample was found to have a distinct primary metabolite profile but watery and dry calli had lowest abundance among all the samples studied.

The embryogenic and non-embryogenic callus of *B. rotunda* were distinguished using 51 targeted primary metabolites. Embryogenic callus in *B. rotunda* had higher levels of primary metabolites in general, especially the amino acids glutamine, arginine, and lysine, compared to suspension cells, dry callus and watery callus. The elevated level of metabolite markers for embryogenesis such as glutamine and lysine in *B. rotunda* culture was confirmed in sieved (enriched) embryogenic cells samples. The concentration of specifically the amino acids marker provides a very clear prediction of embryogenic competency of cells and will be a useful tool in future studies to accurately categorize cell types. Future practical assays to identify specific metabolite markers can be performed using high throughput liquid chromatography mass spectrometry and optical spectroscopy using Raman.

Embryogenic callus, dry callus, watery callus and suspension cells had significantly lower concentrations of secondary metabolites, specifically flavonoids, compared to shoot base samples. The highest abundance of flavonoids was observed in the rhizome sample of *B. rotunda* suggesting that the rhizome could be the main production site for flavonoid compounds. An ongoing transcriptome study of equivalent samples could help to explore this further. If flavonoid biosynthesis is activated in

cultured cells, then mass propagation of cultured cells especially the fastest growing embryogenic callus would be useful in increasing the production of desirable secondary metabolites. Additionally, the active and proliferative cells like the embryogenic callus had low concentration of the five secondary metabolites studied (flavonoid related compounds) as opposed to the high abundance of flavonoid related compounds found in watery callus but having unhealthy and dead cells in *B. rotunda*.

The importance of the hormone, auxin, in embryogenesis was affirmed from the apparent lack of 2,4-dichlorophenoxy acetic acid and indole-3-acetic acid levels in embryogenic callus which suggests an active metabolism to promote cell division and elongation. In contrast, the non-embryogenic competent tissues such as dry callus, watery callus and suspension cells were likely less efficient in auxin metabolism. For *B. rotunda*, it was observed from the embryogenic callus sample that an intermediate level of 2,4-D (3 $mg.L^{-1}$) with low IAA level (160 ± 20 ppb) encouraged embryogenesis. The accumulation of jasmonates and methyl jasmonates in conventionally propagated leaf and regenerated *in vitro* leaves samples were possibly due to wounding that occurred during sampling. Future work on the biosynthesis of salicylates; specifically benzoic acid in *B. rotunda* culture is necessary to determine possible mechanisms for the relatively high concentration reported in shoot base compared to the other samples.

Histo-morphological characterization differentiated the shoot base and three callus samples, embryogenic, dry and watery calli of *B. rotunda*: scanning electron microscopy showed that embryogenic callus had more fibrils on the cell surface, while bright fluorescent spots were observed after diphenylboric acid 2-aminoethylester staining compared to watery callus. Using the Periodic-acid Schiff stain, shoot base, embryogenic and dry calli were observed to have starch reserves stained in purplish red spots, in contrast to a lack of starch in watery callus. Although watery callus had the highest concentration of secondary metabolites among the *in vitro* cultured callus

quantified by UPLC-MS, histological profiling indicated that these cells were non-proliferative, lacking in nuclei and localized starch.

The data from this study will be integrated together with gene expression and methylome profiles by other researchers in the group, to enable the identification of genetic mechanisms that affect recalcitrance and regeneration of suspension cells in *B. rotunda*. Future work should investigate the exogenous application of the embryogenic metabolite markers into culture media to increase embryogenesis rate. These markers can also be used as an indicator of embryogenic competency in tissues. Transcriptome studies of the flavonoid biosynthetic pathway may provide insight into the relatively low levels of secondary metabolites observed in calli, and its implications for possible culture production of selected flavonoid compounds.

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LIST OF PUBLICATION AND PAPER PRESENTED

Publication

1. Ng, T. L. M., Karim, R., Tan, Y. S., Teh, H. F., Danial, A. D., Ho, L. S., … Harikrishna, J. A. (2016). Amino acid and secondary metabolite production in embryogenic and non-embryogenic callus of fingerroot ginger (*Boesenbergia rotunda*). PLOS ONE, *11*(6).

Oral presentation

1. 2nd International Conference on Life Science and Sustainability (2016), Penang Malaysia
   Title: Biochemical characterization of embryogenic and non-embryogenic calli in *Boesenbergia rotunda*