THE EFFECTS OF ELICITORS AND PRECURSOR ON *IN VITRO* CULTURES OF *SAUROPUS ANDROGYNUS* FOR SUSTAINABLE METABOLITE PRODUCTION AND ANTIOXIDANT CAPACITY IMPROVEMENT

WEE SIN LING, PhD

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ABSTRACT

Sauropus androgynus, commonly known as 'sweet shoot' is an underutilized crop known for its high nutritive values and medicinal properties. To date, scientific studies assessing the potentially important benefits of sweet shoot for use as medicinal plants are still limited, with only six studies reporting on *in vitro* propagation and nine articles describing the production of secondary metabolites. The present study revealed that shadehouse-grown plants contained a low yield of bioactive phytochemicals, such as phenolic (61.20 µg/10g FW), flavonoid (193.62 µg/10g FW), naringenin (128.01 µg/10g FW), quercetin (1.56 µg/10g FW) and kaempferol (274.85 µg/10g FW). Moreover, it also had very low antioxidant activity in DPPH (54.03%) and FRAP (397.56 µg/10g FW) assay. The elicitation of cultured tissues is therefore necessary to improve the production of phytochemical compounds and to increase the antioxidant capacity in sweet shoot. In this study, four different types of cultured tissues (*in vitro* shoot cultures, light-induced callus, dark-induced callus and somatic embryos) were selected to achieve this goal, followed by the extraction of phytochemicals from these cultured tissues treated with elicitors and precursor for better production of phytochemicals.

For *in vitro* shoot induction, nodal explants cultured on semi-solid MS medium supplemented with 2.0 mg/l 6-benzylaminopurine (BAP) and 0.5 mg/l indole-3-acetic acid (IAA) produced the highest number of shoots (7 shoots per explant) with longer shoot length (5.74 cm). For light-induced callus induction, leaf explants grown in illuminated conditions with semi-solid MS medium enriched with 2.0 mg/l α-naphthalene acetic acid (NAA) and 1.0 mg/l kinetin showed good proliferation from the leaf explants (71.67%) with the highest callus fresh weight (4.53 g) and highest callus expansion rate (18.50 cm^2). Likewise, leaf explants induced in dark condition on semi-solid MS medium containing similar hormone composition, also displayed the highest callus fresh weight of 4.54 g and callus expansion rate of 13.85 cm^2.

Three-month-old dark-induced callus were transferred onto liquid MS medium fortified with a different concentration of NAA and kinetin to further

induce somatic embryos. After three weeks of callus inoculation, as high as 83.33% of embryogenic cell cultures achieved its maximum density of 5.2 ml in liquid MS medium supplemented with 2.0 mg/l NAA and 1.0 mg/l kinetin. In histodifferentiation medium (liquid MS medium containing 1.0 mg/l NAA and 0.5 mg/l kinetin), a mean number of 15.60, 14.80 and 13.20 embryos per g callus of globular, heart-shaped and torpedo-shaped embryos developed respectively after 9 weeks of embryo induction. The torpedo-shaped embryos were then inoculated into hormone-free MS medium and 90% of these embryos successfully differentiated into cotyledonary embryos after 3 weeks of maturation. These results showed a complete ontogeny of sweet shoot somatic embryo from the globular stage to heart-shaped, torpedo-shaped and cotyledonary stage. After two months of shoot initiation on MS medium supplemented with 2.0 mg/l BAP and 0.5 mg/l IAA, a relatively high percentage (>75%) of shoot proliferation occurred from nodal derived shoot (6.74 shoots per explant), light-induced callus (6.23 shoots per callus) and somatic embryos (6.45 shoots per embryo) of sweet shoot. These well proliferated shoots were then subjected to root initiation in half strength MS medium containing 1.0 mg/l IAA and high percentage of root formation was successfully achieved in 90% of the plantlets after 10 days of culture. For hardening off, the rooted plantlets were transferred to culture jars containing purified water and maintained at ambient conditions for one month. High survival rate (>76.67%) was achieved in perlite:compost mixture (1:1) after one month of acclimatization in shadehouse.

To enhance the production of phytochemicals and antioxidant capacity in tissue cultures of sweet shoot, elicitor and precursor treatments served as alternative methods in influencing the biosynthetic pathway for the accumulation of phytochemicals. In this study, shadehouse-grown plants and cultured tissues of sweet shoot were treated individually with different concentrations of methyl jasmonate (MJ), salicylic acid (SA) and phenylalanine (Phe) for a treatment period of 3 weeks. Light-induced callus culture produced the highest amount of phenolic and flavonoid compounds amongst the tested plant samples with those treated with Phe producing the highest antioxidants, followed by MJ and SA. After 3 weeks of Phe treatment at 20 mg/l, the highest levels of total phenolic (246.62 µg/10g FW), total

flavonoid (636.26 µg/10g FW), naringenin (12081.05 µg/10g FW), quercetin (134.36 µg/10g FW), kaempferol (11325.13 µg/10g FW) and antioxidant activities (97.35% for DPPH and 5941.66 µg/10g FW for FRAP assay) were detected in light-induced callus cultures of sweet shoot. Since phenylalanine ammonia-lyase (PAL) and chalcone synthase (CHS) were the key enzymes for the biosynthesis of phenolics and flavonoids, both enzymatic activities were also measured in light-induced callus cultures treated with Phe. The highest PAL (101.18 mmol CA/g FW) and CHS (14.49 nkat/mg protein) enzymatic activities were also attained in light-induced callus cultures fed with 20 mg/l of Phe at week 3. Light-induced callus cultures treated with Phe produced the highest amounts of phytochemicals, antioxidant capacity and enzymatic activities, and these results were chosen to undergo Pearson's correlation coefficient analysis, which verified the positive co-relationship seen between all of the above-mentioned parameters. These findings showed that the addition of Phe enhanced the enzymatic activities in the phenylpropanoid pathway and increased the concentrations of phenolic and flavonoid compounds (naringenin, quercetin, kaempferol) which in turn contributed to the increase in antioxidant activities in light-induced callus cultures of sweet shoot. Data from this study showed that sweet shoot has the potential to be developed as a plant-based antioxidant for the pharmaceutical industry. Additionally, this study is the first to report on the complete ontogeny of sweet shoot and the positive effects of elicitation in tissue cultures of sweet shoot.

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ABA	Abscisic acid
ANOVA	Analysis of variance
BA	6-benzyladenine
BAP	6-benzylaminopurine
BIA	Benzylisoquinoline alkaloid
C	Cotyledon
C4H	*Cinnamic acid 4-hydroxylase*
$CaCO_3$	Chalk
CFFRC	Crop for the Future Research Center
CHI	*Chalcone isomerase*
CHIA1	Class I *chitinase*
CHIB1	Class III *chitinase*
CHS	*Chalcone synthase*
cm	Centimeter
cm^2	Centimeter squared
DAHP synthase	*3-Deoxy-D-arabinoheptulosonate 7-phosphate synthase*
DMPD	N,N-dimethyl-p-phenyldiamine
DMRT	Duncan's multiple range test
DNA	Deoxyribonucleic acid
DPPH	2,2-diphenyl-1-picrylhydrazyl
DW	Dry weight
E	Epidermis
ESR	Electron spin resonance
FDF1.2	Defensin
Fe(II)-TPTZ	Ferrous tripyridyltriazine
Fe(III)-TPTZ	Ferric tripyridyltriazine
$FeCl_3.6H_2O$	Iron(III) chloride hexahydrate
FLS	*Flavonol synthase*
FRAP	Ferric reducing antioxidant potential
FRIM	Forest Research Institute Malaysia
Ft	Feet
FW	Fresh weight
g	Gram
GA_3	Gibberellic acid
GC	Gas chromatography
GP	Ground parenchyma
GPC	Glutaraldehyde-Paraformaldehyde-Caffeine
Ha	Hectare
HCl	Hydrochloric acid
HIV	Human immunodeficiency virus
HPLC	High performance liquid chromatography
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
IRPA	Intensification of Research in Priority Areas
K	Potassium
Kg	Kilogram
KH_2PO_4	Potassium phosphate
KJ/g	Kilojoule per gram
m	Meter
mΩ	Milliohm

MARDI	Malaysian Agricultural Research and Development Institute
mg	Milligram
mg/l	Milligram per liter
ml	Milliliter(s)
mM	Millimolar
mm	Millimetre
mm/year	Millimeter per year
mmol CA/0.3g FW	Millimole cinnamic acid equivalent per 0.3 g fresh weight samples
MJ	Methyl jasmonate
MS	Mass spectrometry
MS medium	Murashige and Skoog's medium
N	Nitrogen
N7OMT	*Norreticuline 7-O-methyltransferase*
NAA	α-naphthalene acetic acid
NaOH	Sodium hydroxide
NAP3	3rd Malaysian National Agricultural Policy
NCS	*Norcoclaurine synthase*
nkat/mg protein	Nanokatal per mg protein
nm	Nanometre
NMR	Nuclear magnetic resonance
ORAC	Oxygen radical absorption capacity
P	Phosphorus
PAL	*Phenylalanine ammonia-lyase*
PAR	Photosynthetically active radiation
PC	Procambium
PCV	Packed cell volume
Phe	Phenylalanine
ppm	Parts per million
PR	Pathogenesis-related
RA	Root apical meristem
RIA	Radioimmunoassay
RM	Ringgit Malaysia
ROS	Reactive oxygen species
rpm	Rotations per minute
S	Suspensor
SA	Salicylic acid
SCAR marker	Sequence characterized amplified regions marker
SEP	Subepidermis
SPE	Solid-phase extraction
SPSS	Statistical package for social sciences
TAT	*Tyrosine aminotransferase*
TBARS	Thiobarbituric reactive substances
TBHQ	Tert-butylhydroquinone
TDZ	Thidiazuron
THI2.1	Thionin
TLC	Thin layer chromatography
TPTZ	2,4,6-tri (2-pyridyl)-1,3,5-triazine
Tris	Tris(hydroxymethyl)-aminoethane
UKM	Universiti Kebangsaan Malaysia
UM	University of Malaya
UPM	Universiti Putra Malaysia

USDA	United States Department of Agriculture
UV	Ultraviolet
UV/Vis	Ultraviolet-visible
v/v	Volume/volume
VB	Vascular bundle
VEGF	Vascular endothelial growth factor
WHO	World Health Organization
w/v	Weight/volume
2,4-D	2,4-dichlorophenoxyacetic acid
4CL	*4-coumarate-CoA*
4OMT	*(S)-3'-hydroxy-N-methylcoclaurine 4'-O-methyltransferase*
6OMT	*(S)-norcoclaurine 6-O-methyltransferase*
°C	Degree Celsius
%	Percentage
µg/g	Microgram per gram
µg $FeSO_4$/10g FW	Microgram ferrous sulfate equivalent per 10 g fresh weight samples
µg GAE/10g FW	Microgram gallic acid equivalent per 10 g fresh weight samples
µg RE/10g FW	Microgram rutin equivalent per 10 g fresh weight samples
µl	Microlitre
µm	Micrometer
$\mu molm^{-2}s^{-1}$	Micromole per square millisecond
µM	Micromolar

CHAPTER 1: INTRODUCTION

1.1 Natural products in drug discovery

Today, natural products are the primary source of commercial medicines and drugs. Natural products isolated from plants stimulate wide interest in the industries of pharmaceutical, agrochemical, nutraceutical and cosmetics, due to the vast chemical diversity that plants possess. A survey of pharmacopeias has shown that 20-25% of all medicines are derived from natural sources, such as herbs, flowering and underutilized plants (Lewis, 2001). Alkaloids, tannins, flavonoids, and phenolic compounds are the most common bioactive natural products in underutilized crops, which hold great promises for future application in medical biotechnology.

Underutilized crops have immeasurable ability to synthesize secondary metabolites, of which at least 1,200 species are known to be medicinal, and about a hundred species have been fully investigated phytochemically for their potential benefits. Among the hundred species, only a small percentage of fractions have been submitted to biological and pharmacological screening for their bioactivity (Mahesh and Satish, 2008). Although there have been very few works published on the isolation of natural products from underutilized plants, the huge diversity of Malaysian flora with diverse chemical structures is definitely a plus factor that makes natural products an excellent candidate for phytochemical screening programs (Ismail, 2010).

In the 3rd Malaysian National Agricultural Policy (NAP3, 1998-2010), Malaysia was set to lead the phytomedicine market with its rich biological heritage, cultural background, and trade links. Biotechnology is not a 'silver bullet', but recent advances in plant tissue culture technology provide a feasible alternative for sustainable production of high quality plants with bioactive metabolites (Chrispeels and Sadava, 2002; Tilman *et al*., 2002). Several research institutes and local universities in Malaysia have employed a multidisciplinary approach with which to ultimately increase the yield of desired bioactive metabolites (Anonymous, ASEAN Review of Biodiversity and Environmental Conservation, 2001).

With the aid of biotechnology, new commercial organizations have also switched on to study hitherto unknown medicinal plants, which drive the phytomedicine market in Malaysia (Lin and Yahya, 2008). It has been estimated by the World Health Organization that more than three-quarters of the world's population still relies on phytomedicine for medicinal treatment (Brahmachari, 2012). In the US alone, about 12.1% of the US population was spent on phytomedicine alone amounting to a staggering $5.1 billion. In 2002, the British spent £75 million on phytomedicine products, which was a rise of 57% in five years (Barnes, 2003; De Smet, 2004). From the year 2003 till 2012, there was a gradual rise in global pharmaceutical sales from US$450 billion to US$950 billion (Figure 1.1) (Anonymous, Deloitte, 2014). This corresponded to a rise of 47% in 10 years. In fact, the economic impact of phytomedicine in Malaysia is also huge with RM 1.6 billion in annual sales, making phytomedicinals an attractive business venture (Ismail, 2010). Investment is therefore needed in natural resources to tap onto the global demands where the market and financial forces are ever present.

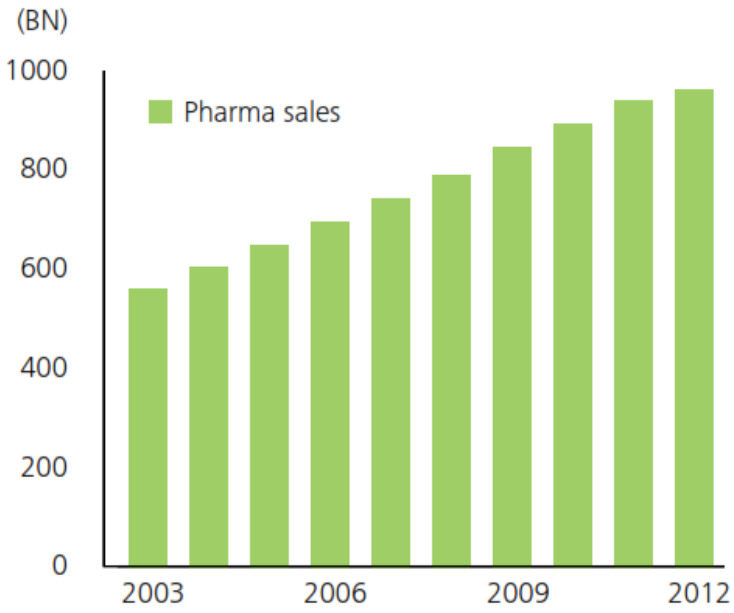


Figure 1.1. An overview of the annual sales values for global pharmaceutical industry (Anonymous, Deloitte, 2014).

1.2 Neglected and underutilized crop species

Underutilized crop species are generally defined as indigenous non-commodity crops with unexploited economic potential, for contributing to food security and eradicating nutrient deficiencies, which affect more than three billion people worldwide (Gruere *et al*., 2006). Underutilized crops are locally abundant in developing countries but are globally rare (Horna and Guillaume, 2003). In Southeast Asia, underutilized crops are part of a larger biodiversity portfolio, where they play a significant role in diversifying and improving rural livelihoods, such as income generation, employment, traditional medicines, local consumption, livestock fodder, construction purposes and the stability of ecosystems. Underutilized crops are also proven to be effective in reducing the environmental degradation and contributing towards environmental sustainability (Gruere *et al*., 2006).

Despite their benefits, underutilized crops are neglected by farmers, consumers, researchers and policy markers, due to the scarcity of scientific information and knowledge about the crop species (Padulosi and Hoeschle-Zeledon, 2004). Farmers are not interested in cultivating underutilized crops because of non profitability and non competitiveness against crops that have come to dominate the world food supply (Schmidt *et al.*, 2008). Hence, a concerted development plan is needed for more environmentally sound agricultural practices and better stewardship of underutilized crops.

1.3 *Sauropus androgynus* (sweet shoot)

Sauropus androgynus L. Merr., commonly known as 'cekur manis' and 'sweet shoot', is a green leafy perennial underutilized shrub belonging to the *Phyllanthaceae* family (Benjapak *et al*., 2008). Sweet shoot has been cultivated commercially in the tropical regions of Southeast Asia, notably for its high yields and palatability (Tejavathi *et al*., 2010). Sweet shoot has been classified as one of the more nutritious tropical vegetables by the United States Department of Agriculture (USDA) due to its high medicinal and nutritive values (Martin and Ruberte, 1980; Padmavathi and Rao, 1990).

The genus *Sauropus* consists of 40 species of plants, but only a few are economically and medicinally important (Wiart, 2006). According to

USDA, sweet shoot provides large amounts of protein (49%), crude fiber (14%-18%), a precursor of vitamins A, B and C; and mineral salts such as potassium (2.77%), calcium (2.77%), phosphorus (0.61%), magnesium (0.55%), iron (199 pmm) and antioxidant (179 µg/g) (Jordan, 2008). It is believed to hold a huge potential for tropical countries where vitamin deficiency is a common problem among pregnant women and malnourished children (Gill, 2009).

Sweet shoot is known to possess antioxidant and febrifuge properties, which help to reduce fever, heal ulcers and prevent urinary disorders (Kanchanapoom *et al*., 2003). A decoction of the root is used traditionally as an antiseptic agent and as an antidote for food poisoning (Kanchanapoom *et al*., 2003). The leaves are usually served as a vegetable to nursing mothers for stimulating breast milk production and for womb recovery after delivery (Chai, 2007). The leaves are also rich in chlorophyll, which is valuable for haematopoiesis and blood circulation, cell rejuvenation, beneficial to intestinal flora, and aids regular bowel elimination (Chai, 2007).

Secondary metabolites, such as quercetin, kaempferol, megastigmane, lignin, corchoionoside, sauroposide, chlorogenic acid, caffeic acid, ferulic acid and papaverine can be extracted from the ethanolic and methanolic leaf extracts of sweet shoot (Kanchanapoom *et al*., 2003). A number of pharmacological studies with quercetin and kaempferol have validated their antimicrobial and antioxidant action in mice, simply by delaying the lipid oxidation and by inhibiting the propagation of oxidizing chain reactions (Cechinel Filho *et al*., 1996). Furthermore, sweet shoot also consists of seven active compounds for the biosynthesis of steroid hormones and eicosanoid compound, which help to control and inhibit the inflammatory responses to injury and infection. For that reason, sweet shoot has been categorized as a good source of dietary nutrients and has earned the name of 'multigreen' for its high medicinal values (Ram, 1994).

In late 1990s, the raw juice from sweet shoot leaves has been widely consumed in Southeast Asia and Taiwan for body weight reduction and vision protection (Yu *et al.*, 2007a). However, its toxicity is a major concern due to

the presence of alkaloid papaverine substances in leaf extract. Papaverine substances of 580 mg can be extracted from 100 g of fresh sweet shoot leaves. Many women with no prior history of medical problems developed respiratory distress, dizziness, drowsiness and constipation after consuming the uncooked leaves of sweet shoot (Lin *et al*., 1996; Ger *et al*., 1997; Yu *et al.*, 2007a).

Obliterative bronchiolitis, an irreversible obstructive lung disease, has been claimed to develope after continuous consumption of uncooked leaves of sweet shoot for more than 3 months (Lai *et al*., 1996). The results of chronic toxicity studies, on the other hand, failed to show any significant detrimental effects in rats receiving 100 mg of papaverine daily, and over 50 % of the LD_{50} daily (Henderson *et al*., 2006). Thus, more research is required to confirm the toxicity of papavarine substances in sweet shoot.

Furthermore, the leaves of sweet shoot have been used as a cattle and poultry feed in India. It can be planted as a fence in home gardens. A green dye obtained from sweet shoot leaves can also be used as food colouring for pastries, rice and preserves (Anonymous, AVRDC World Vegetable Centre, 2008). Despite its local importance, sweet shoot still appears to be underutilized and its usage is diminishing. In order to unlock the true potential of sweet shoot, researchers need to harness their multiple uses and to achieve their full potential (Anonymous, IPGRI, 1999).

1.4 Research development on medicinal plants and sweet shoot

The Malaysian phytomedicine industry has grown rapidly and has the potential to become a leading industry in Malaysian agriculture but many pharmaceutical companies in Malaysia have ignored the development of medicinal plants into therapeutic drugs, due to the low success rate and high capital needed. For the past few years, many novel strategies have been carried out by the research institutes (MARDI, FRIM, MPOB, UKM, UPM and UM) based in Malaysia, in order to search for the bioactive agents from plant-derived natural products. Funds for research were also made available by the Malaysian government via IRPA (Intensification of Research in Priority Areas) program (Sarin, 2005). One of the priority areas of IRPA program is the

commercialization of biotechnology, which involve the development and production of pharmaceuticals from plant genetic resources.

Sweet shoot has been listed as one of the top 40 underutilized crops in Malaysia, as previously reported by International Network of Food Data Systems, Crops for the Future Research Centre (CFFRC) and Education Concerns for Hunger Organization. Malaysian government has been actively involved in the research and development of underutilized crops, in order to support the diversification of agriculture through greater crop diversity. By year 2003, Malaysia has about 1300 medicinal plant products registered by the Ministry of Health and they are available in the market (Kulip, 2003). A number of projects have been carried out by the Malaysian government, such as the production of bioactive metabolites from *Eurycoma longifolia* (tongkat ali), *Labisia pumila* (Kacip Fatimah), *Orthosiphon stamineus* (misai kucing), *Andrographis paniculata* (hempedu bumi) and *Centella asiatica* (Pegaga) (Ismail, 2010). In fact, sweet shoot has also been slated for phytochemical production in the next five years, as stated by Ismail (2010) during the Biotechnology Symposium IV in Sabah, Malaysia. Many scientists believe that plant tissue cultures are the focal point of procedures to produce bioactive metabolites from medicinal plants (Akin-Idowu *et al*., 2009).

Initial findings showed that an array of research investigations have been undertaken in the area of plant cell, tissue and organ cultures to produce valuable medicinal compounds from medicinal plants collected in Malaysia. The applications of plant tissue culture ensure rapid multiplication of medicinal plants on a large perpetual basis with high medicinal values. By doing so, sweet shoot may someday become a commercially viable crop that has huge potential to be exported in the form of crops and bioactive metabolites.

1.5 Aims and objectives

The main objective of this research work was to evaluate the effects of elicitors and precursor on antioxidant capacity and on the yield enhancement of metabolites in tissue cultures of sweet shoot. The influences of plant growth regulators, cultivation systems and culture environment on growth

performance of sweet shoot were examined in this study, in order to overcome the demerits of conventional propagation and to develop a suitable *in vitro* plant regeneration protocol for sustainable production of antioxidant compounds. The optimal concentrations and durations of plant elicitation were also determined in the present study, aiming to improve the antioxidant and metabolite production in elicited cultures of sweet shoot.

This study was also aimed to provide the fundamental scientific insights into antioxidant activity, by identifying the phytochemical constituents responsible for its therapeutic values. The antioxidant capacity of sweet shoot was tested by measuring the ability to scavenge radicals generated by *in vitro* system. Chromatographic detection methods, namely reversed phase high performance liquid chromatography (RP-HPLC) were used to detect and identify antioxidant profiles in sweet shoot, in order to produce a compound of potentially more value for human use. This study was undertaken with the following specific objectives:

1. To develop an effective plant regeneration protocol for *in vitro* multiplication and antioxidant production of sweet shoot.
2. To study the effects of elicitors (methyl jasmonate and salicylic acid) and precursor (phenylalanine) on the biosynthesis of antioxidant compounds in tissue cultures of sweet shoot.
3. To compare the antioxidant capacity (DPPH and FRAP assays), total flavonoid content, total phenolic content, phenylalanine ammonia-lyase (PAL) activity and chalcone synthase (CHS) activity between elicited and non elicited cultures of sweet shoot.
4. To determine the correlation between antioxidant activities (DPPH and FRAP assay), enzymatic antioxidant defense system (PAL and CHS assay) and non-enzymatic antioxidant defense system (total flavonoid content and total phenolic content) present in tissue cultures of sweet shoot.

CHAPTER 2: LITERATURE REVIEW

2.1 Origin and scientific classification of sweet shoot

To date, the exact origin of *Sauropus androgynus* (sweet shoot) remains unknown, as it thrives mainly in the wild and is cultivated extensively in India, Sri Lanka, Southern China, Indochina, Thailand, Malaysia, Australia and throughout Southeast Asia (Figure 2.1) (Van de Bergh, 1994; Tejavathi *et al*., 2010).

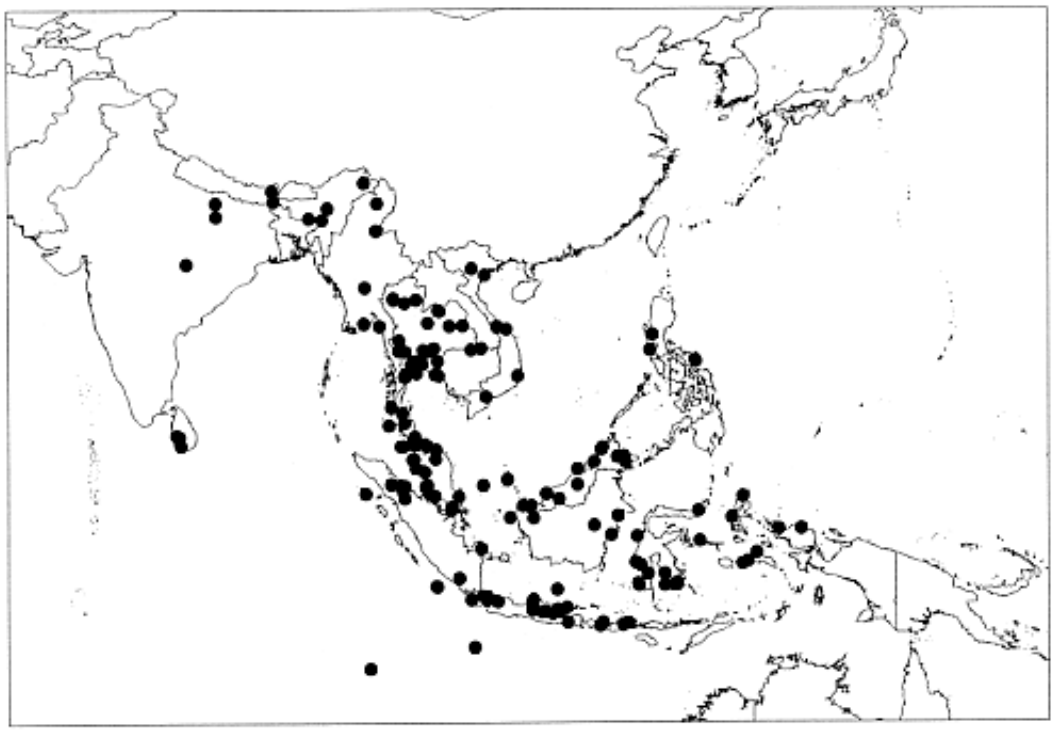


Figure 2.1. Geographical distribution of *Sauropus androgynus* (sweet shoot) in the tropical and subtropical regions of Southeast Asia (originally published by Van Welzen (2003), reprinted with permission from the journal Blumea).

Sweet shoot was formerly classified in the *Euphorbiaceae* subfamily *Phyllanthoideae* (Webster, 1994). The advances in genomic technology helped to construct phylogenetic trees of sweet shoot using maximum parsimony and Bayesian methods as shown in Figure 2.2 (Pruesapan *et al*., 2008). Plastid *matK*, SCAR (sequence characterized amplified regions) markers and nuclear ribosomal ITS DNA sequence data showed that sweet shoot is embedded within the *Phyllanthaceae* family along with its related genera, *Breynia, Glochidion, Reverchonia* and *Phyllanthus* (Table 2.1)

(Theerakulpisut *et al*., 2008). More variable DNA markers are needed to further clarify the species relationship and to revise the generic and infrageneric classification of *Phyllanthaceae* family.

Table 2.1. Scientific classification of *Sauropus androgynus* (sweet shoot) (Anonymous, FAO, 2007; Anonymous, USDA, ARS, 2013).

Scientific Classification of Sweet Shoot	
Kingdom:	Plantae
Division:	Magnoliophyta
Class:	Magnoliopsida
Order:	Malpighiales
Family:	Phyllanthaceae
Subfamily:	Phyllanthoideae
Tribe:	Phyllantheae
Subtribe:	Flueggeinae
Genus:	*Sauropus*
Species:	*androgynus*
Binomial name:	*Sauropus androgynus*
Ecocrop code:	9593

The family *Phyllanthaceae* is the second largest segregated family, which consists of 2000 species in 59 genera, 10 tribes and two subfamilies (Samuel *et al*., 2005). In *Phyllanthaceae* family, the genus *Sauropus* comprises 40 species of monoecious and dioecious woody herbs to small shrubs (Van Welzen, 2003). Since limited studies have been reported for sweet shoot, the resources in this review are solely based on the closest family and subfamily, *Phyllanthaceae* and *Euphorbiaceae*. *Phyllanthus* species, an annual herb belonging to *Phyllanthaceae* family, and *Jatropha curcas*, a perennial shrub belonging to *Euphorbiaceae*, are the preferred crop models for *in vitro* plant regeneration and elicitation of sweet shoot.

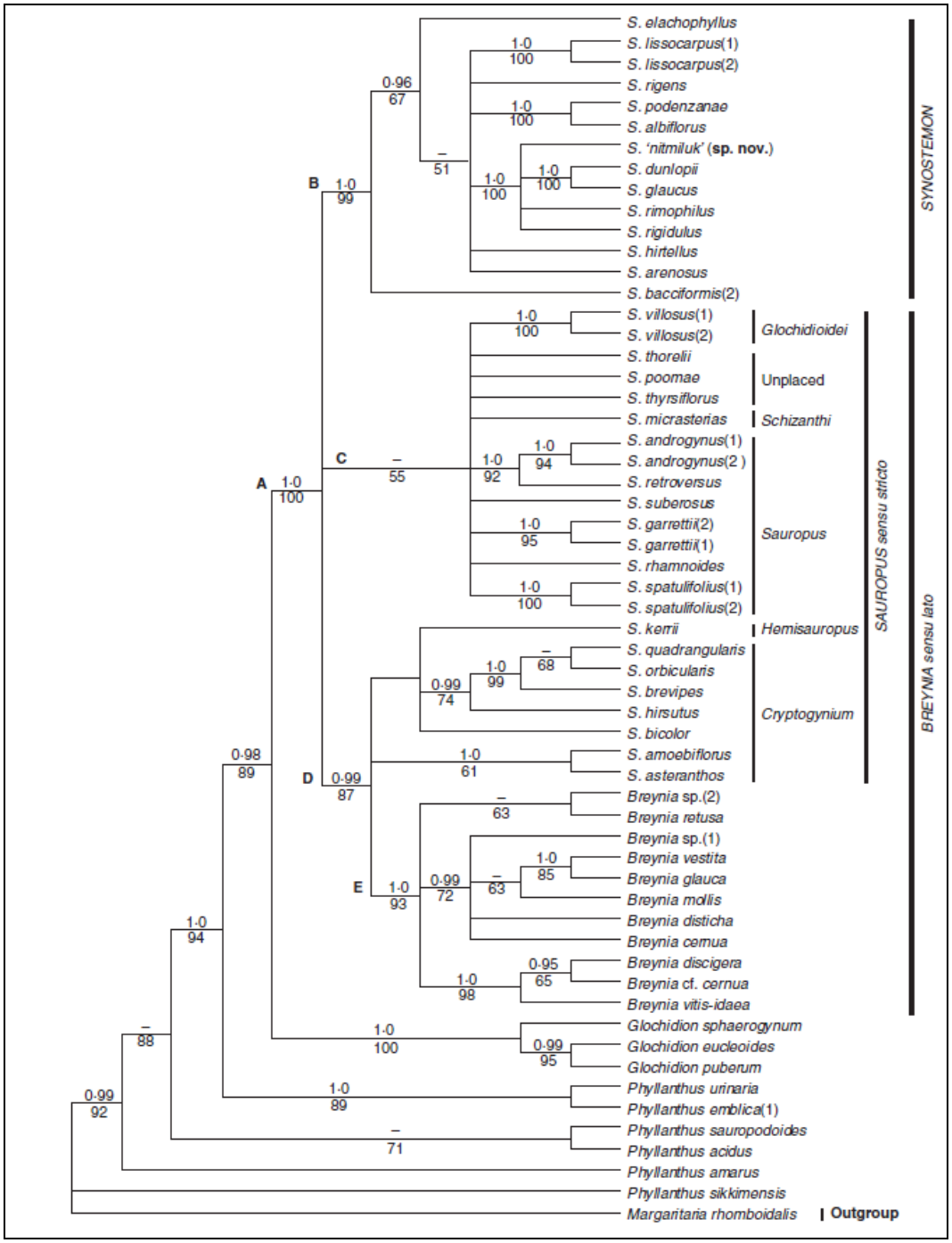


Figure 2.2. Parsimonious trees of *Sauropus androgynus* (sweet shoot). Strict consensus of 8581 most-parsimonious trees (971 steps, CI = 0.57, RI = 0.73) of *Sauropus* and allies based on combined plastid *matK* gene data and nuclear ribosomal ITS data. Bayesian posterior probabilities ≥ 0.95 and bootstrap percentage ≥ 50 are shown and below branches, respectively. '-' indicates Bayesian posterior probabilities (Pruesapan *et al*., 2008).

2.2 Botany and culture of sweet shoot

Sweet shoot is a monoecious, leafy green perennial shrub which can grow up to 3.5 m high (Figure 2.3) (Chai, 2007). It has small, upright main stems measuring upto 2.5 m high with alternate, petiolate and dark green oval-shaped leaves about 5-6 cm long and 1-3 cm wide (Jordan, 2008). The mid region of the leaves is covered with faint greyish spots, though it is not a symptom of microbial infection.

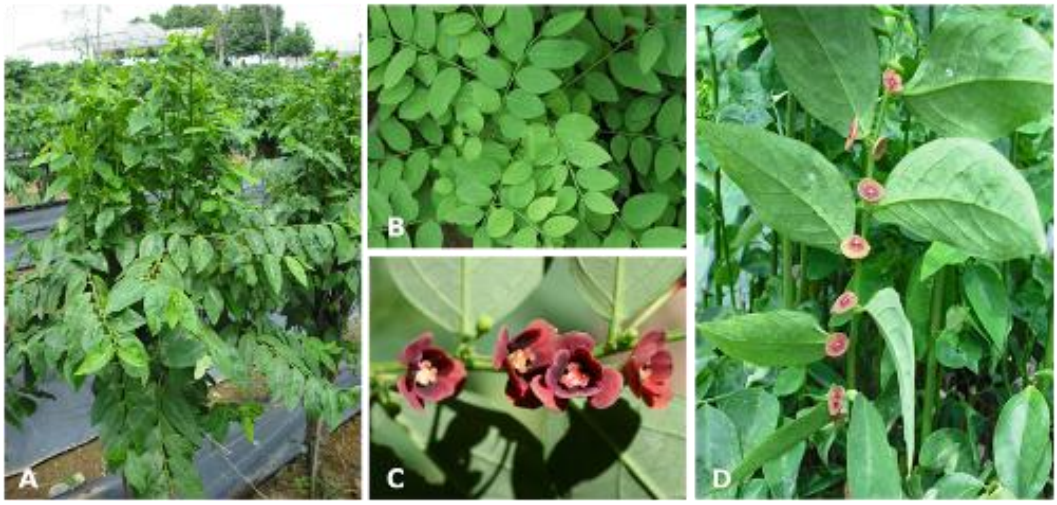


Figure 2.3. Botany of *Sauropus androgynus* (sweet shoot). (A): Shoots measure to 2.5 m high with alternate petiolate. (B): oval to circular dark green leaves 5-6 cm long. (C, D): One to six flowers arise in clusters in the leaf axils.

Flowering and fruiting of sweet shoot typically occur throughout the year. The inflorescence of sweet shoot consists of both staminate (male) and pistillate (female) flowers on the same branch (Chai, 2007). One to six flowers arise in clusters in the leaf axils. In tropical climate, the flat, round, red and orange flowers develop into white and purple round-shaped fruit capsules that consist of 4-6 seeds (Jordan, 2008). The seeds of sweet shoot are small, numerous, black in colour and about 1.5 cm in diameter (Gill, 2009).

Sweet shoot is native to lowland rainforest understory and is grown in Malaysia at altitudes of 1300 m (4000 ft) (Kregor, 1999). It develops rapidly under the full sun or in shade with temperature ranging from 26^{o}C to 35^{o}C, thriving in hot and humid climate with an average annual rainfall of 900 to

1200 mm/year (Anonymous, FAO, 2007; Gill, 2009). However, sweet shoot is not tolerable to drought conditions, which could lead to stunted growth. Sweet shoot is a highly mycorrhizal dependent crop species, thus it has the ability to grow well in acidic soils (pH 5.5), heavy clay soils (pH 7.5) and flooding conditions (Habte *et al*., 1994; Anonymous, FAO, 2007). Previous investigation showed that the root dry matter yield of mycorrhizal *Sauropus* was found to be greater than non mycorrhizal *Sauropus* in all tested soils with an average soil depth of 50 to 150 cm (Habte *et al*., 1994).

Alley cropping systems using nitrogen-fixing trees have been practiced extensively in many commercial farms throughout Southeast Asia, to enhance the production of sweet shoot (Gill, 2009). The ideal spacing between plants of sweet shoot is 60-90 cm (2-3 ft). Under ideal conditions, it can grow up to 1.5 m per month. The stem diameter will not grow at pace with the length, thus it will lead to the falling of stems. For that reason, pruning is essential for effective cultivation, as it encourages the constant production of new tender growth (Kregor, 1999). This method of harvesting has only been observed lately in Sabah, Sarawak, Thailand and Vietnam (Chai, 2007).

2.3 Reproductive characteristics of sweet shoot

The popularity of sweet shoot as a leafy green vegetable is attributed to its prolific growth, abundant fruiting and lack of pest and disease problems (Gill, 2009). Research trials conducted in Malaysia revealed that sweet shoot yields per hectare have surpassed all other green vegetables (Chai, 2007). In Thailand, 739 tonnes of sweet shoot were produced from 148 hectare of harvested area during the year of 2005, which is much higher than other vegetables shown in Table 2.2 (Johnson *et al*., 2008). A recent paper review of Agricultural Mission Conference has also stated that sweet shoot is now being grown and marketed in Hawaii (Gill, 2009). Based on the statistical figures shown in Table 2.2 and 2.3, the demand for sweet shoot has increased tremendously between the year 2003 and 2005. It has been estimated that the annual gross profit of sweet shoot is approximately RM 5000 per hectare (Chai, 2007). With the favourable prices and the increase in demand for sweet shoot both locally and within the region, sweet shoot can be an attractive and profitable crop to invest in for large-scale production.

Table 2.2. Production statistics for vegetables and spices in Thailand for year 2004 and 2005 (Anonymous, DOAE, 2007).

Crops	Harvested area (ha)		Production (tonnes)	
	2004	2005	2004	2005
Parsley	0	120	0	47
Nymphaea	6	6	0	2
Mushroom	0	2438	0	358
Garden pea	383	140	4139	257
Winged bean	0	20	0	194
Muskmelon	0	24	0	377
Solanum torvum	0	5	0	32
Brassica	0	42	0	45
Broccoli	336	8	3246	94
Turmeric	0	20	0	128
Sweet shoot	1	148	8	739
Kitchen mint	0	176	0	652
Chrysanthemum	0	1	0	6
Rosella	3157	970	717	538

Table 2.3. Sweet shoot growing areas in Sarawak, Malaysia (Chai, 2007).

Division	Year			
	2002	2003	2004	2005
Kuching	31.2	18.3	21.4	30.2
Samarahan	21.2	7.5	9.9	10.4
Sri Aman	2.6	7.5	11.4	16.0
Betong	10.4	0.5	6.7	7.3
Sibu	19.1	9.9	17.9	18.2
Sarikei	23.3	12.5	15.0	14.9
Bintulu	1.4	1.8	3.0	5.3
Miri	9.4	4.9	5.4	5.4
Kapit	4.0	3.4	0.1	10.2
State total	129.8	69.5	93.8	121.8

* Crop hectareage equivalent.

Sweet shoot is traditionally propagated via woody stem cuttings and seeds (Li and He, 2006). The seeds are occasionally dispersed by birds and

have been shown to establish in at least one location (Staples and Herbst, 2005). Besides that, vegetative propagation can also be done by stem cuttings under moist and shady conditions (Jordan, 2008). Chinese rose beetles (*Adoretus sinicus*), red spider mite and slugs are the major problems affecting the new cuttings and seedlings.

Propagation of sweet shoot via stem cuttings appears to be limited due to its high susceptibility to fungal and bacterial diseases. A fatal disease, bacterial stem blight disease caused by *Xanthomonas campestris* bacteria, has wiped out many major commercial farms in Sarawak during the late 1990s (Chai, 2007). The symptoms of *Xanthomonas* infection include yellow stem streaks and chlorotic leaves. Several ineffective attempts were made to control this bacterial disease, such as spraying with agrochemicals and applying the antibiotics. Hence, plant tissue culture system and breeding program could serve as an alternative approach to eradicate this highly infectious disease.

Conventional methods of propagation were also hindered by several problems such as being labour intensive, time consuming, low survival, low germination (50% at best) and the possibility of growth and yield retardation (Davidonis and Knorr, 1991). The recalcitrant nature of seeds causes difficulties in generating the plantlets from seeds and the seeds will only retain viability for 3-4 months when kept dry and cool (Gill, 2009). In addition, seed-raised plants exhibit genetic variation (Tejavathi *et al*., 2010). Therefore, *in vitro* propagation of sweet shoot serves as a feasible method to overcome the above-mentioned disadvantages of conventional propagation and to ensure mass multiplication on a sustainable basis.

2.4 Application of plant tissue culture technology in natural product biosynthesis

The evolving commercial importance of natural products created great interest in the alteration of metabolite production by means of plant tissue culture technology. Plant tissue culture technology offers rapid and efficient methods for the production of high value pharmaceutical compounds, which

are not possible with conventional propagation of intact plants (Table 2.4) (Mohan Ram and Agrawal, 1990).

Table 2.4. Comparison of advantages and disadvantages of three different culture regimes for metabolite production from plants (Cseke *et al*., 2006).

Mode of Cultivation	Advantages	Disadvantages
Field cultivation in a nursery	Help to conserve plant biodiversity; able to break seed dormancy; low cost production of a year-old plants.	Short growing season; problems with disease, insect and herbivore attack; no control of weather conditions.
Greenhouse cultivation	Usage of illumination and environmental parameter to regulate plant growth and metabolite production.	Increased energy and labour costs than field cultivation, including automated greenhouse installation and operation.
Plant cell, tissue, organ culture	Able to genetically modify culture to improve metabolite biosynthesis; plant micropropagation can be performed; resulting in genetic reproducibility, elicitation is more convenient; cultivation in bioreactors are possible.	Labour intensive and expensive due to the high cost of culture media constituents and the equipment for sterile culture conditions; stability problems of cell lines; low yield of end products in bioreactors.

Growing plantlets under controlled environments generally provides an opportunity to scrutinize the problems regarding to organization, integration and interaction of plant cells (Akin-Idowu *et al*., 2009). Plant tissue cultures ensure mass proliferation of genetically uniform plantlets that possess desirable traits and bioactive metabolites. The pharmaceutical production can also be upregulated using bioreactors and liquid suspension cultivation system under optimized conditions. These automated controls of tissue growth and rational regulation of metabolite processes would definitely contribute to cost reduction and productivity improvement (Havkin-Frenkel *et al*., 1997).

Besides that, propagation via plant tissue culture helped to reduce the dependence on wild plants and hence increase the number of biogenetic resources in tropical forests. It is also effective in eliminating potential

political boundaries or geographic barriers to the production of sweet shoot (Purohit, 2003). Tissue cultures of sweet shoot can be regenerated in the absence of seeds and pollinators (Sarin, 2005). By doing so, it greatly reduces the chances of transmitting diseases, pests and pathogens.

Bioactive metabolites in cultured tissues can be generated on a continuous year round basis (without seasonal interruptions) and the product yield of pharmaceuticals is shown to be reliable (Oksman-Caldentey and Inze, 2004). In some studies, field-grown sweet shoots have failed to accumulate significant amounts of antioxidant substances. Hence, plant tissue culture is an attractive choice for industrial production of pharmaceuticals like alkaloids, phenolics, flavonoids and essential oils.

2.5 *In vitro* plant regeneration of woody plant species

To date, there are hardly any woody plants that have been successfully propagated *in vitro* compared to herbaceous plants. Woody plants were deduced to be intractable in culture due to (1) the oxidation of polyphenolic compounds from explants, (2) the absence of juvenility in the explants of mature trees, and (3) the difficulty in rooting (Purohit, 2003). Despite these obstacles, significant progress has been made in plant tissue culture in the last 20 years and it is now commonly used in the commercial application of agriculture, horticulture and forestry with more than 800 companies involved in plant tissue culture business worldwide (Sathyanarayana and Varghese, 2007).

Tissue excised from plants can be cultured *in vitro* and regenerated into plantlets via different developmental pathways in response to suitable stimuli, macro and micronutrients, growth regulators and culture environment (Cseke *et al*., 2006). Micropropagation of woody plants includes three different types of vegetative propagation, (1) axillary shoot proliferation, where the axillary buds and apical meristems give rise to more number of shoots; (2) adventitious shoot proliferation, comprising of *de novo* meristem formation from callus tissues or directly from organized tissues; and (3) somatic embryogenesis, in which the root and shoot structures are formed and connected by a closed vascular system (Figure 2.4).

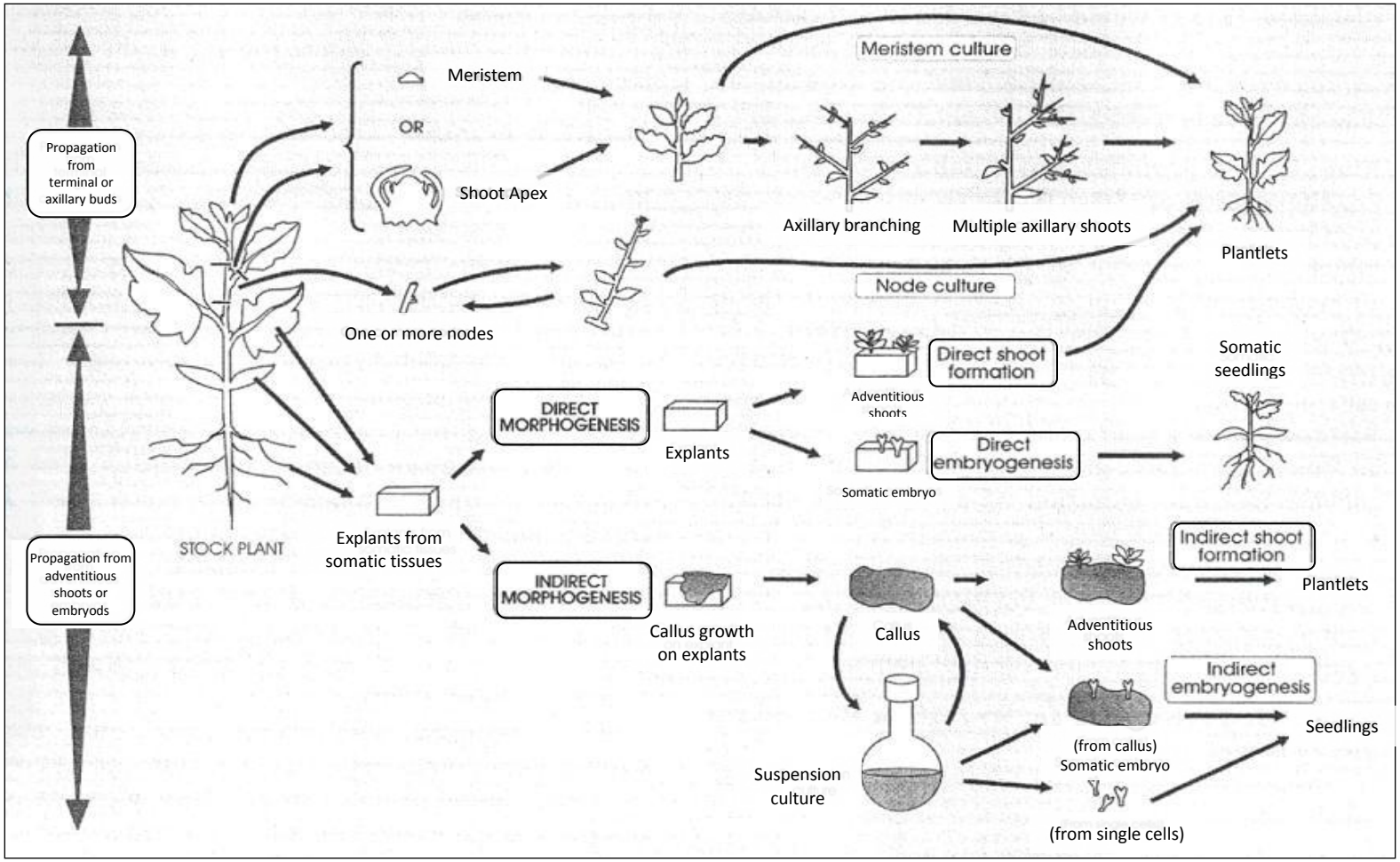


Figure 2.4. The principal methods of micropropagation of plant tissues (George *et al*., 2007).

A successful micropropagation progresses through a series of stages, (1) establishment of aseptic cultures, (2) callus initiation and multiplication, (3) shoot elongation and proliferation, (4) rooting of microshoots, and (5) acclimatization of tissue cultured plantlets. Other successful applications of plant tissue culture technology have also been reported for (1) meristem cultures, (2) organogenesis from undifferentiated tissues, cells or protoplasts, (3) gametic embryogenesis, (4) somatic embryogenesis and (5) zygotic embryo cultures (Bhowmik and Matsuiz, 2001). The applications of plant tissue culture technology are diverse and significant.

2.5.1 Axillary shoot proliferation

Axillary shoot proliferation is an important micropropagation technique, in which the nodal segments harboring an axillary bud are cultured on cytokinin-based media to promote axillary shoot proliferation, without an intervening callus phase (Slater *et al*., 2008). Shoot tip culture is generally used for commercial micropropagation, as shoot tips are easily acquired from the plant materials (Figure 2.5) (Trigiano and Gray, 2000). The merits of shoot tip culture have made a noteworthy success for large-scale clonal multiplication on a sustainable basis.

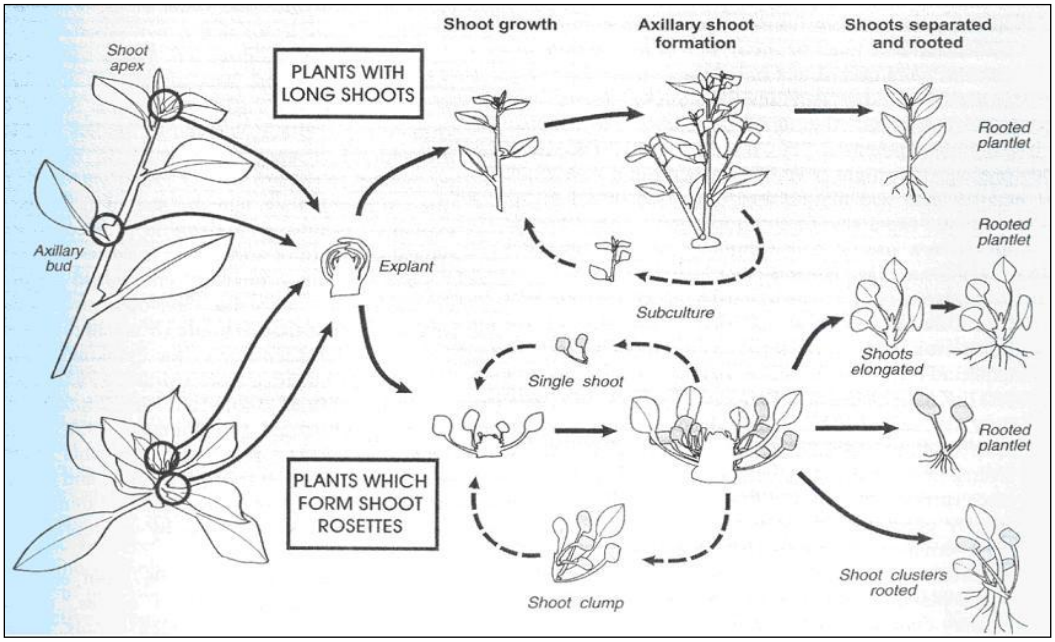


Figure 2.5. A simplified scheme of shoot tip cultures (George *et al*., 2007).

Node culture is a common technique used for shoot production from single or multiple nodes positioned horizontally on the culture medium (Figure 2.6). For instance, the lateral bud initiation of potato (*Solanum tuberosum*) and orchid is difficult to achieve with the availability of cytokinins in the shoot tip culture (Purohit, 2003), whereas single, elongated, unbranched shoots of potato comprising multiple nodes are able to regenerate rapidly into *in vitro* shoot. The beneficial uses of node culture have also been reported in cultured tissues of *Ocimum basilicum* (Shahzad *et al*., 2012), *Eucalyptus camaldulensis* (Girijashankar, 2012) and *Annona squamosa* (Krishna *et al*., 2012). For orchid, the cultured meristems become disorganized and formed spheroid protocorm-like bodies which are similar to non-zygotic embryos (Trigiano and Gray, 2000).

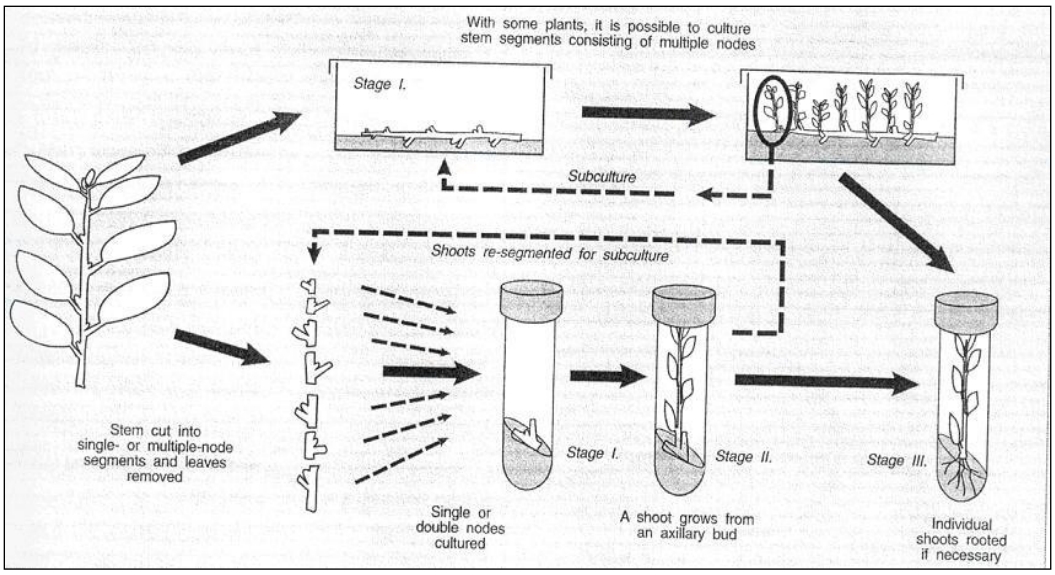


Figure 2.6. The principle of single and multiple node cultures (George *et al*., 2007).

Meristem culture is a micropropagation method, wherein shoot apical meristem is cultured *in vitro* with none or with a few leaf primordia (Purohit, 2003). The cells in shoot apical meristem retain the embryonic capacity for unlimited division and differentiate into whole plants genetically identical to the parent plant (Trigiano and Gray, 2000). This method has been employed to eradicate viral infection in most asexually propagated species and high value genotypes (Sathyanarayana and Varghese, 2007).

Woody plants suffered from slow growth and difficulties in eliminating microbial infections from explants originating from greenhouse plants. The potential of meristem culture for unlimited shoot growth was initially recognized by the successful culture of Nasturtium (*Tropaeolium majus*) (Ball, 1946). Since then meristem culture has attracted much attention for the production of microbe-free plants, such as banana, orchids, strawberry and roses (Styer and Chin, 1984; Chugh *et al*., 2009). The discovery of cytokinins and the improvement of tissue culture media have provided further impetus to meristem culture technique to the extent that it is now viewed as a commercially viable technology for mass multiplication of most woody plants.

2.5.2 Direct and indirect organogenesis

Plant regeneration from callus tissue is usually achieved either by organogenesis or by somatic embryogenesis. Organogenesis generally begins with changes in a single group of parenchyma cells, which divide to produce a globular mass of cells or meristemoids. These cells then give rise to either a shoot or root primordium, whose vascular system is connected to the parent tissues (Slater *et al*., 2008). Organogenesis can occur either directly from an explant or indirectly from a callus culture, through wounded parenchyma. This tissue culture approach increases the number of species and the plant parts from which the *de novo* organized development can arise (Figure 2.7) (Murashige, 1974).

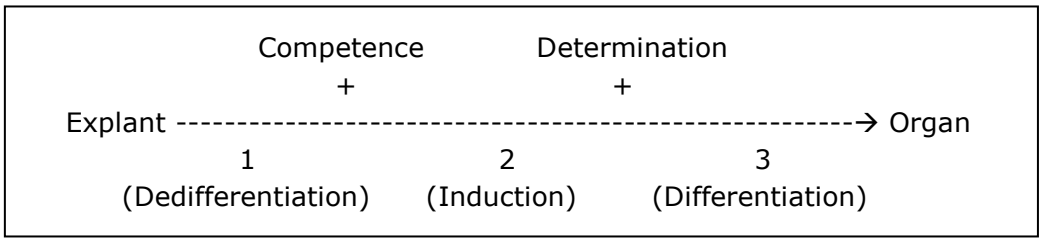


Figure 2.7. The process of organogenesis has been broken into several phases. These phases encompass events that begin with dedifferentiation, which results in the attainment of 'competence', followed by induction, which culminates in the fully 'determined' state. The morphological differentiation and development of the nascent shoot or root then proceed, eventually resulting in a functional organ (Christianson and Warnick, 1985).

Direct and indirect shoot induction on stems, leaves, roots, corms, tubers and rhizomes of intact plants, is an alternative method for propagating woody plant species (Figure 2.8). Bulbs and corms grow adventitiously from leaf meristems and further develop into adventitious shoots. Unlike meristem tips and shoot tips, leaf explants can be easily obtained from the mother plant, without sacrificing it, and their availability is not restricted to any season like inflorescence explants. In other words, organogenesis provides the basis for asexual plant propagation largely from non-meristematic tissue (George *et al*., 2007).

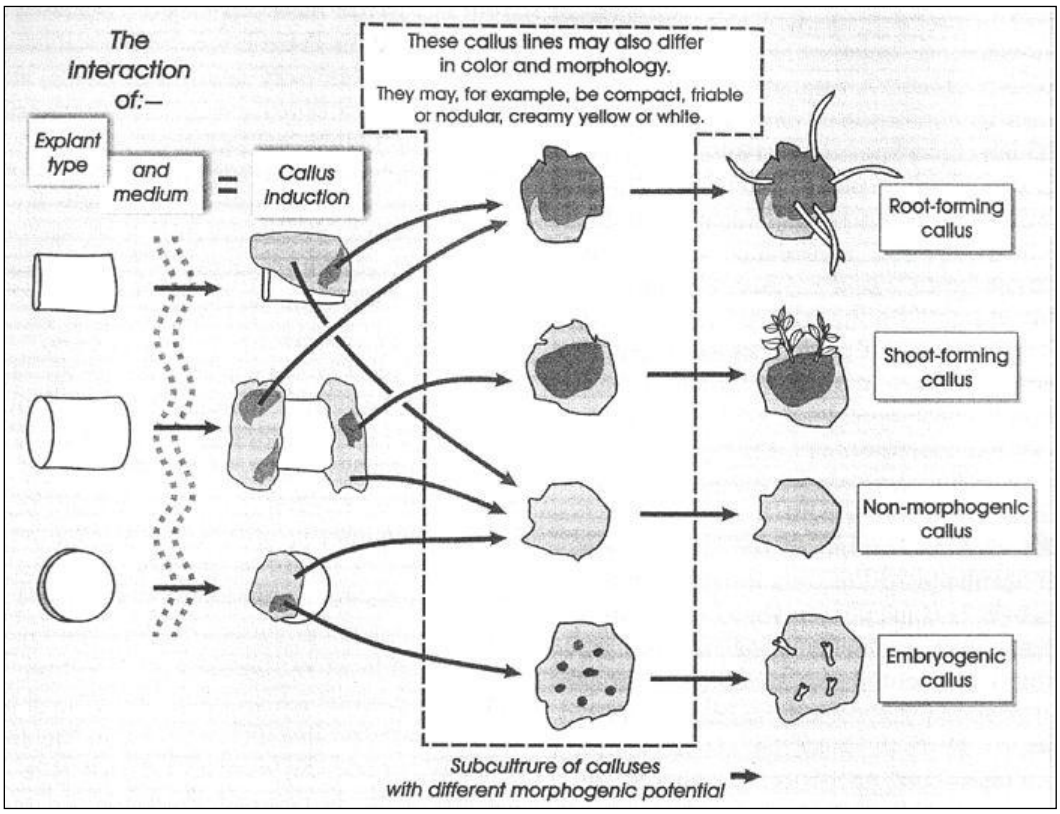


Figure 2.8. Callus with different morphogenic potential isolated from a single explant (George *et al*., 2007).

To date, there are only a few reports indicating the failure of plant organs responding to the treatments designed for callus induction. The earliest report on controlled *in vitro* organogenesis was done by White (1939), who obtained microshoots on callus culture of tobacco hybrid. The addition of auxins is capable of stimulating callus formation (White, 1939). Studies with tobacco callus showed that higher ratio of auxin to cytokinin in the medium

favoured root formation; the reverse ratio promoted shoot formation; and intermediate ratios enhanced callus proliferation. The inhibitory effects of auxins on shoot formation can be reduced by adding inorganic phosphate and adenine.

Callus culture is often incubated in the dark, as light encourages callus differentiation (Trigiano and Gray, 2000). Its propagation can be maintained more or less indefinitely, provided that the callus is subcultured onto fresh medium periodically. During callus formation, there is some degree of dedifferentiation, both in morphology and metabolism (Vasil *et al*., 1990). With the stimulus of plant growth regulators, metabolism of cells changed from being quiescent to metabolically active, thus producing undifferentiated parenchymatous cells. During dedifferentiation, callus culture also loses the ability to photosynthesize, which in turn may create different metabolic profile from the donor plant. Callus cultures can be used to initiate cell suspension, which are used in the studies of elicitation, somatic embryogenesis and plant transformation.

2.5.3 Direct and indirect somatic embryogenesis

An embryo can be defined as the earliest recognizable multicellular stage of a plant that occurs before the organ structures and characteristics have developed. Somatic embryogenesis is a process by which the somatic cells develop into differentiated embryos, and the differentiated embryos further develop into whole plants in a way analogous to zygotic embryos (Thorpe, 1995). The process of somatic embryogenesis can be classified into two types, direct and indirect somatic embryogenesis. Direct somatic embryogenesis is the formation of somatic embryos or embryogenic tissues directly from the explants (from the organized structures such as leaf, hypocotyls, stem, anthers, pollen, nucellus and styles), whereas indirect somatic embryogenesis is a process whereby the somatic embryos arise from callus or isolated single cells in cell suspension culture (Raghavan, 1986; Slater *et al*., 2008). Despite different regeneration pathways, both zygotic and somatic embryos share similar ontogeny passing through globular stage, heart-shaped, torpedo-shaped and cotyledonary stage in the case of dicotyledons; globular, scutellar and coleoptilar stages for monocotyledons.

Somatic embryogenesis has been reported in over 130 species, including cereals, legumes, conifers, *Bambusa nutans* and *Mangifera persiciforma* (Purohit, 2003; Mehta *et al*., 2011; Li *et al*., 2012). Carrot tissue has been a good example for studying plant regeneration via somatic embryogenesis, but most of the physiological studies had only dealt with the development of embryos rather than the whole plants (Reinert, 1959). To date, there are a number of embryogenic carrot cell lines that have been maintained in suspension cultures for more than 10 years. When hormones were removed from the medium, embryogenic cell lines further developed into a plantlet through the morphologically defined stage of globular, heart-shaped and torpedo-shaped embryos (Nomura and Komamine, 1985). As such, somatic embryogenesis has been proven to be effective in resolving the regeneration problem, due to its high multiplication rate, cryopreservation of embryogenic callus, potential for scale up in liquid suspension cultures and bioreactors, somatic synthetic seed technology and served as a target tissue for gene transformation and plant development (Merkle and Dean, 2000).

The embryogenic response is highly genotype and explant dependant (Gray *et al*., 1993). For instance, there were only a limited range of genotypes which were capable of producing embryogenic cultures for corn and, with a few exceptions, only immature embryo can be used. Besides that, the age of immature embryos exerts tremendous influence on the establishment of somatic embryo cultures, as too young and too old somatic embryos do not produce embryogenic callus. More in depth mechanisms underlying somatic embryogenesis needs further clarification in future studies.

Environmental requirements for optimum growth of somatic embryos are quite specific. Cultures are required to grow in either dark or light or a combination of both over time. Culture in dark is necessary in order to suppress unwanted embryogenic cell differentiation in explant tissues (Trigiano and Gray, 2000). Despite the similarities in culture requirements, improvements in somatic embryo development and maturation are increasingly being achieved by the optimization of medium composition. This

approach encouraged a few plant species, such as oil palm, alfalfa and carrot, to propagate on a large-scale via indirect embryogenesis.

2.5.3.1 Difficulties in generating plants via somatic embryogenesis

The difficulties in developing a somatic embryogenesis system for woody plant species are similar to those for herbaceous species. Woody plants exhibit a unique set of tissue culture dependent variability, the genetic variation is higher for woody plant species than the cultivated herbaceous species. Another limitation for woody plant improvement using genetic engineering is that juvenile material has to be collected from the field instead of from a controlled environment.

The problem of ecotypic or physiological variation from individual to individual is also greater due to the overall heterozygosity of most woody plant species. An undesirable difference often exhibited by somatic embryos, where they frequently deviate from the normal pattern of development either by producing callus, undergoing direct secondary embryogenesis, or germinating precociously (Gray and Purohit, 1991). Overall, these additional variables further increase the potential difficulty of developing somatic embryogenesis in woody tree species.

2.6 *In vitro* plant regeneration of sweet shoot

The novelty of this project can be judged by the amount of similar published journals on *in vitro* plant regeneration of sweet shoot. To date, successful micropropagation of sweet shoot has been documented previously using axillary bud (Li and He, 2006), shoot tip (Tejavathi *et al*., 2010), leaf (Wee *et al*., 2010a), nodal (Philomena, 1993; Wee *et al*., 2010b; Zhang *et al*., 2011; Eganathan and Parida, 2012) and internodal tissues (Wee *et al*., 2010c) via direct and indirect shoot regeneration.

Media used in plant tissue culture are composed of salts, vitamins, amino acids, plant growth regulators, sugars and gelling agents. Murashige and Skoog (MS) medium (1962) is the most commonly used medium for tissue growth of sweet shoot (Li and He, 2006; Wee *et al*., 2010a; Zhang *et al*., 2011; Eganathan and Parida, 2012). L2 medium, developed by Phillips

and Collins (1979), also had good responses for shoot induction using shoot tips (Tejavanthi *et al*., 2010), and has benefited cultured shoots of *Trifolium pretense* and *Trifolium rubens* (Carrillo *et al*., 2004).

The use of 3% sucrose is more effective in culturing woody plants when compared to 2% and 4% sucrose (Murashige and Skoog, 1962). MS medium supplemented with 3% sucrose, 0.5 mg/l indole-3-acetic acid (IAA) and 2.0 mg/l 6-benzylaminopurine (BAP) has been used successfully for shoot initiation and multiplication via stem explants (Li and He, 2006). Hence, both studies are consistent in recommending 3% (w/v) sucrose in MS medium as the best carbon source for woody plant tissue culture.

Agar with 0.8% concentration, a gelling agent, was used for culturing sweet shoot due to its desirable characteristics such as clarity, stability and its inertness (Li and He, 2006), however, it contains impurities such as inorganic salts, organic compounds, phenolics and long chain fatty acids, which may interfere with the culture response. Thus, Phytagel™ provides an economical alternative to agar, since it has higher clarity with absence of impurities (Sathyanarayana and Varghese, 2007). In addition, there were no adverse effects of Phytagel™ observed in cultured shoots of *Rosa damascene* (Pati *et al*., 2004) and sweet shoot derived from nodal explants (Wee *et al*., 2010b).

For the establishment of aseptic cultures, a thorough understanding of the physiological status of plants and their susceptibility to pathological contaminants is required, in order to eradicate microbial infections in tissue cultures. Contaminated cultures may exhibit no symptoms, variable growth, reduction in shoot and callus proliferation, poor rooting and poor survival of plantlets (Leifert *et al*., 1989). For sweet shoot, the most commonly adopted procedure involved surface sterilization of explants with 0.1% (w/v) mercuric chloride for 10 minutes followed by three rinses with sterile purified water (Li and He, 2006). Prior to surface sterilization, Tejavathi *et al*. (2010) also treated stem and leaf explants using Tween 20 for 15 minutes followed by 0.1% (w/v) Bavistin for 10 minutes.

However, the use of mercuric chloride, fungicides and antibiotics in explant surface sterilization is not recommended due to potential toxicity to the environment and the possibility of plant growth retardation (Matthew and Duncan, 1993). High concentration and extended exposure of mercuric chloride was shown to be phytotoxic, which resulted in high mortality and tissue necrosis. For that reason, surface sterilization with 70% ethanol for 1 minute followed by 20% (v/v) commercial bleach (Clorox) for 20 minutes was the preferred method in reducing microbial contamination of sweet shoot (Wee *et al*., 2010a; Wee *et al*., 2010b; Wee *et al*., 2010c). Commercial bleach was proven to be one of the most effective bacteriocides, as it killed microbes by oxidizing biological molecules such as proteins and nucleic acids (Bloomfield and Arthur, 1991).

According to Wee *et al.* (2010a), a high percentage of green globular calluses were induced on MS medium supplemented with 2.0 mg/l of IAA and 1.0 mg/l of BAP, which supports the findings from Li and He (2006). Higher concentrations of BAP were used to suppress apical dominance and to regenerate shoots from callus. Li and He (2006) placed nodal explants on MS medium containing 2.0 mg/l BAP and 0.5 mg/l IAA and succeeded in producing plantlets from buds. In 1993, Philomena studied the effects of coconut water on induction and growth of shoots from stem explants of sweet shoot, and the plantlets showed increased shoot formation and growth with NAA, whereas kinetin had no effect. The addition of adenine sulphate and gibberellic acid into L2 medium also facilitated direct regeneration of shoots with roots (Tejavathi *et al*., 2010).

For sweet shoot, several attempts have also been made to enhance the rooting efficacy using different types and concentrations of auxins, such as IAA, IBA, NAA and 2,4-D, ranging from 0.1-2.0 mg/l. Li and He (2006) successfully induced 95% rooting of microshoots on half strength MS medium supplemented with 1.0 mg/l of IAA, whereas Tejavathi *et al.* (2010) reported that sweet shoot can root well in half strength L2 medium containing optimal concentrations of NAA, followed by 2,4-D, IAA and IBA after one month of incubation. Based on the aforementioned literatures, half strength media supplemented with auxin is sufficient for root induction in sweet shoot.

Successful acclimatization of rooted plantlets and their subsequent transplantation to the field is a critical step for commercial exploitation of *in vitro* technology. The acclimatization of rooted sweet shoot was relatively easy compared to other woody species, in which 95% survival was achieved one month after transfer to a shadehouse (Li and He, 2006). Rooted plantlets were initially dipped into the fungicide, carbendazim, for 5 minutes and transplanted to pots containing a mixture of compost and perlite for establishment *ex vitro*. All acclimatized plantlets showed high homogeneity without any visual evidence of somaclonal variation (Li and He, 2006). The experiments described above suggested that sweet shoot can be propagated by both axillary shoot proliferation and organogenesis.

2.7 Factors influencing *in vitro* plant regeneration and biosynthesis of secondary metabolites

Many scientists believed that basic physiological principles of each plant can be studied in detail from the production of secondary metabolites in response to chemical and physical treatments in plant cell cultures (Dicosmo and Misawa, 1995; Purohit, 2003). Up till now, many factors have been investigated for their ability to improve culture responses and metabolite production, including media components, culture environmental conditions, age and choice of explants (Table 2.5). Each factor needs to be optimized either separately or in combination with the other treatments, in order to find a satisfactory equilibrium between cell growth and optimum production of metabolites.

Many research groups have also demonstrated the outstanding metabolic capacities of plant tissue cultures and highlighted the variability of plant tissue biosynthetic capacity (Bisaria and Panda, 1991). The ongoing research are largely focused on the identification of rate-limiting steps in biosynthetic pathways, culture elicitation, cell immobilization, precursor feeding*, in situ* product removal and bioreactor design, which led to the enhancement of secondary metabolite production.

Table 2.5. Different aspects of controlled *in vitro* environment and metabolite biosynthesis factors (Purohit, 2003).

Controlled *in vitro* environment	
Aerial physical environment	**Root zone environment**
(a) Temperature	(a) Physical environment
(b) Composition of culture medium (concentration, proportion, form and pH)	- Temperature - Water potential - Osmotic pressure - Gas and liquid diffusivity in medium - Hardness or compactness of media
(c) Special treatments - Elicitation - Precursor feeding - Biotransformation - Cell immobilization	(b) Chemical environment - Inorganic substance composition - Organic substance composition - Dissolved oxygen
(d) Gaseous composition - Carbon dioxide - Oxygen - Ethylene	(c) Biological environment - Symbiotic environment - Competitive association
(e) Light - Photosynthetic - Photomorphogenic	

In spite of many efforts to increase the yield using various approaches, the concentration of metabolites in cultured cells are relatively low and economically infeasible (Kassem and Jacquin, 2001). The failure of high level metabolite production is mainly due to the insufficient knowledge on how plants regulate metabolite biosynthesis. Thus, future research is needed to elucidate factors controlling metabolite accumulation and to optimize culture environmental conditions for metabolite productivity.

2.7.1 Selection of elite cell lines for an efficient production system

The development of elite cell lines for efficient metabolite production would be an alternative technology to wildcrafting and low economic return practices. High producing plantlets, callus or cell clones can be obtained from single cells, and subsequently be used for screening of high producing strains (Cseke *et al*., 2006). In some cases, stability issues with cell lines are major concerns in metabolite production, as it will create difficulties in connecting to

cell differentiation and morphogenesis. Therefore, issues related to stable elution of metabolite in cell lines need to be resolved in the near future.

The choice of explants is mainly determined by the method adopted for *in vitro* micropropagation (Pati *et al*., 2004). In many species, explants of various organs varied in their growth rate. Explants such as axillary bud, meristem tip and shoot tip were believed to have a higher growth rate, as it produced growth regulating substances like auxins and cytokinins (Akin-Idowu *et al*., 2009). Kirakosyan *et al*. (2003) managed to establish different cell culture systems, such as callus and liquid cell suspension cultures, with higher production of desired metabolites when compared to intact plants of *Pueraria montana*, *Crataegus laevigata* and *Glycyrrhiza glabra.*

2.7.2 Types of plant growth regulators

Hormonal control of metabolite formation has been well established for many years. Growth regulators, such as auxins and cytokinins are crucial for the stimulation of metabolite biosynthetic pathways, and for the growth of many cell lines (Purohit, 2003). As a rule, growth deceleration due to deletion and reduction of auxins prompts the appearance of products such as pigments and alkaloids. Cytokinins have also been used to produce secondary metabolites, such as nicotine in cell cultures of *Nicotiana tabacum*; indole alkaloids in cultured tissues of *Catharanthus roseus*; and anthocyanins, catechins, and lignins in many woody plant species (Purohit, 2003).

A critical balance of endogenous and exogenous plant growth regulators is required to induce desired metabolites and cell responses from culture. Certain combinations of auxins and cytokinins had synergistic effects, whereas others had antagonistic effects on steroidal synthesis (Slater *et al*., 2008). The nicotine synthesis in tobacco cultures is strongly inhibited by 2,4-D, whereas it is promoted by kinetin. A perusal of literature showed that the biosynthesis of shikonin was inhibited by both 2,4-D and NAA, but it was synthesized using IAA in the cell cultures of *Lithospermum erythrorhizon* (Sarin, 2005). A similar phenomenon was also observed in *in vitro* cultures of *Morinda* species and *Cassia tora* in anthraquinone production.

In anthocyanin producing cells of *Glehnia littoralis*, NAA at 1.0 mg/l showed better results compared to IAA and 2,4-D. A study done by Luczkiewicz and Cisowski (2001) showed that 5.0 mg/l of NAA was the optimal concentration for anthocyanin production in cultured tissues of *Rudbeckia hirta.* Meanwhile, *t*he addition of 0.1 mg/l kinetin resulted in better growth and improved the anthocyanin biosynthesis in *Glehnia littoralis* (Miura *et al*., 1998). The use of BAP was found to be suitable for higher anthocyanin production in *Crataegus sinaica* than kinetin (Maharik *et al*., 2009). Besides that, the highest accumulation of anthocyanin can be achieved by adding cysteine, which is one of the constituents of acetyl CoA, a crucial factor in flavonoid biosynthesis. In short, the effects of plant growth regulators on secondary metabolism vary greatly depending upon types of metabolites.

2.7.3 Types of abiotic and biotic elicitors

In many cases, secondary metabolite production can be enhanced by the treatment of undifferentiated or differentiated cells with elicitors, amino acids and precursors such as methyl jasmonate, salicylic acid, chitosan, yeast and fungal extracts, tyrosine and heavy metals (Ebel and Cosio, 1994; Matkowski, 2008). Elicitors generated signals that trigger the formation of phytoalexins. The elicitation of cultured tissues and intact plants increases the amount of natural products, such as taxol in *Taxus chinensis* (Wang *et al.*, 2001), tropane alkaloids in *Datura stramonium* (Zabetakis *et al*., 1999) and indole alkaloids in *Catharanthus roseus* (Rijhwani and Shanks, 1998).

Elicitors are non-specific, as it might increase the levels of certain enzymes and decrease others. In most cases, elicitors mimicked the pathogen defenses or wound responses in plants, and activate the enzyme-encoding genes that are responsible for phytoalexin biosynthesis. Most of the secondary metabolites are associated with these responses, in order to strengthen the plant defense system. Some success in stimulating metabolite production has also been achieved by adding protein synthesis inhibitors and precursors (Hsu, 1981), altering hormone levels (Kutchan *et al*., 1983) and lowering the incubation temperature (Lockwood, 1984).

Elicitor formed inside and outside plant cells are known as endogenous or exogenous elicitors, respectively (Table 2.6). Elicitors can be classified into two groups based on their nature and origin, namely abiotic and biotic elicitors. Abiotic elicitors are physical or chemical in nature, such as ultraviolet radiations, alkalinity, temperature, osmotic pressure and heavy metal ions; whereas biotic elicitors are mostly fungal homogenates or bacterial fractions. Fungal elicitors such as pathogenic (*Phytophthora, Botrytis* and *Verticillium)* and non pathogenic (*Aspergilus*, *Micromucor* and *Rhodotorula*) microbes were used to stimulate the production of gymnemic acid in embryogenic suspension cultures of *Gymnema sylvestre* (Devi and Srinivasan, 2011).

Table 2.6. Origin and nature classification of elicitors (Endress, 1994).

(I) Origin as classification features				(II) Nature as classification features			
(a)	**Exogenous elicitors**	**(b)**	**Endogenous elicitors**	**(a)**	**Abiotic elicitors**	**(b)**	**Biotic elicitors**
1.	Originated outside the cell, inducing reaction or via endogenous mediator.	1.	Formed via secondary reactors, induced by a signal of biotic or abiotic nature in cell.	1.	Physical or chemical nature working via endogenously formed biotic elicitors.	1.	Released by microorganisms and recognized by plant cell (enzymes and cell wall fragments)
2.	Examples: • Polysaccharides • Peptides • Enzymes • Fatty acids • Metallic ions • Ultraviolet light • Chitosan	2.	Examples: • Dodeca-β-1,4-D-galacturonide • Hepta-β-glucosides • Oligocellulose	2.	Examples: • Ultraviolet light • Denatured proteins • Freezing and thawing • Heavy metals • Fungicides • Methyl jasmonate	2.	Formed by action of microorganisms on plant cell walls (pectin fragments)
						3.	Formed by action of plant enzymes on microbial cell walls (chitosan).
						4.	Compounds formed or released by the plant cell in response to various stimuli.

The choice of elicitors has a significant impact on the economics of metabolite production. Biotic elicitors were generally obtained from fermentations, which led to cost ineffectiveness; whereas abiotic elicitors were usually available as off-the-shelf chemicals and at a much cheaper price. However, the optimum employment of an elicitor depended upon factors like (1) elicitor specificity, (2) elicitor concentration, (3) duration of elicitor contact, (4) growth stage of culture, (5) plant growth regulation, and (6) nutrient composition (Purohit, 2003). For instance, the elicitation of field-grown plants has been attempted before, but it was found to be costly, inefficient, and difficult to maintain in the field (Kurtz *et al*., 1998). The reason for this phenomenon may be due to its relative impermeability and hydrophobic shoot surfaces of the field-grown plants leading to poorer elicitor uptake. Thus, it is crucial that the application of elicitor must be performed precisely. One possible method would be the addition of elicitors onto a plant tissue culture medium, in order to enable a better elicitor absorption by the roots of targeted plant.

The use of elicitors as an approach for sustainable metabolite production has been successfully described in the formation of catharanthine in periwinkle (*Catharanthus roseus*) cell cultures, saponin in *Panax* ginseng cultures and sanguinarine formation in *Papaver somniferum* culture. Apart from metabolite induction, methyl jasmonate elicitation provides another example for yield enhancement of chlorogenic acid and reserpine in embryogenic suspension culture of *Eleutherococcus senticosus* and *Rauwolfia serpentine*, respectively (Shohael *et al*., 2007; Harisaranraj *et al*., 2009). This approach has been proven effective in increasing the metabolite production by 10-20 folds in many woody plant species (Samuelsson, 2004).

Despite evidence of success in the above examples, the impact of elicitation on the production of biopharmaceuticals in Malaysian herbs has not been widely investigated, mainly because of the low production of flavonoid and antioxidant compounds in cultured tissues. Furthermore, the production of metabolites greatly depends on the physiological and developmental stage of plant species. Hence, elicitation of tissue cultures has yet to be employed for the production of these natural products. Future research is needed in

order to optimize the culture condition and elicitation condition for optimum production of antioxidant and flavonoid compounds.

2.7.4 Light source

A number of journal articles stated that light source controlled the biosynthesis of many bioactive metabolites *in vitro*. According to Morini *et al*. (2000), light quality was shown to affect the direction of *in vitro* plant morphogenesis, and the switch between gametophytic and sporophytic pathways. Cool white fluorescent lamp with 16-hour photoperiod is usually provided for the culture condition of woody plants (Pati *et al*., 2004). In 1980, Davies observed that 16-hour photoperiod resulted in a better growth compared to 8-hour photoperiod in *in vitro* rose cultures.

Appreciable quantitative changes have been observed in volatile oil content in tissue cultures of *Ruta graveolens* when incubated in the light and dark separately (Purohit, 2003). According to Purohit (2003), there is a slight increase in the accumulation of flavones and flavonol glycosides in ultraviolet illuminated cells. Yield enhancement of polyphenols, flavonoids, carotenoids and plastoquinones were also observed when cultures grew under light (Purohit, 2003). Moreover, ultraviolet radiation not only improved the conditions for metabolite production, the application of plant molecular biology was also affected, such as signal induction, signal transduction, and gene expression in the biosynthesis of metabolites (Purohit, 2003).

In *Passiflora quadrangularis* callus culture, the UV-B irradiation treatment stimulated a large increase in flavonoids, corroborated with the higher radical scavenging activity (Antognomi *et al*., 2007). However, culture irradiation can led to an increase in cost and can generate undesirable temperature gradients in the culture vessels. Moreover, the inhibitory effects of light have also been observed in cell cultures of *Lithospermum erythrorhizon*, especially with the white and blue light on shikonin derivative formation (Purohit, 2003). Therefore, more research needs to be done in order to determine the efficacy of ultraviolet light on the biosynthesis of secondary metabolites.

2.7.5 Cell suspension cultures

Suspension culture of plant cells, tissues or organs under controlled environmental conditions has been developed over the past 50 years, primarily for the production of valuable metabolites such as shikonin and paclitaxel (Hellwig *et al*., 2004). Cell suspension culture technique offers an effective way of incorporating precursors and elicitors, which are often difficult to administer to the field-grown plant, as mentioned earlier in Section 2.7.5.

In general, cell suspension cultures can be classified as homologous and heterologous cell cultures (Kirakosyan *et al.*, 2003). Homologous cultures consist of a fine cell suspension culture of mostly homogenous populations of cells, while heterologous cultures consist of different types of cells made up of cluster and cell aggregates. Both cell cultures either produce secondary metabolites in various amounts or nothing at all (Kirakosyan *et al.*, 2003). An explanation for different biosynthetic abilities of both cell suspension cultures is that cells do not produce certain compounds until it is partially or fully differentiated (Kirakosyan *et al.*, 2003).

A number of physical and chemical factors affecting growth and proliferation of cell suspension cultures have been studied in detail, which include pH medium, inoculum density, agitation speed, the type of culture vessel, light source and temperature conditions. Lee (1997) had studied the precise controlling and monitoring of pH changes during the culture's growth phase, which subsequently helped in improving the plant growth.

There is an example of cell suspension type of cultivation involving globular differentiated structures that have a more practical application in biotechnology. A study done by Vardepetyan *et al*. (2000) reported that suspension cultures of *Hypericum perforatum* with compact globular differentiated structures had a higher amount of secondary metabolites than unorganized cell suspension cultures. This finding is in agreement with the observations made for two other plant systems, *Catharanthus roseus* (Verpoorte, 1998) and *Rhodiola sachalinensis* (Xu *et al*., 1999). Long term cultivation of these cultures showed further accumulation of biomass, due to an increase in the number of globules (Vardepetyan *et al*., 2000).

2.8 Pharmacognosy: phytochemistry of medicinal plants

The word 'pharmacognosy' appears to have been coined in the 18th century by Proferssor Johann Adam Schmidt (1759-1809) (Samuelsson, 2004). It was derived from the two Greek words, pharmakon (drugs) and gnosis (knowledge), thus defined as the knowledge of drugs. Pharmacognosy is a multidisciplinary subject involving the study of the biological and biochemical properties of drug substances derived from natural resources, like plants, animals and microorganisms (Bruneton, 1999). In pharmacognosy, the term 'phytochemicals' refers to the bioactive compounds found naturally in medicinal plants, which provide health benefits for human more than those attributed to micronutrients and macronutrients (Saxena *et al*., 2013). At the same time, Carl Linnaeus (1707-1778) made an important contribution to the development of pharmacognosy by introducting a scientific taxonomy system, in order to facilitate the process of naming, identifying and classifying of medicinal plants (Samuelsson, 2004). This was rapidly followed by the discovery of the first alkaloid, morphine, by Friedrich Serturner (1783-1841) at the start of the 19th century (Brahmachari, 2012), followed by strychnine (1817), quinine and caffeine (1820), nicotine (1828), atropine (1833), cocaine (1855) and the mixture of cardioactive glycosides, digitaline, from foxglove leaves in 1868 (Samuelsson, 2004).

With the development in organic chemistry research, the approach to characterization and isolation of bioactive compounds from medicinal plants started at the late 19th century (Saxena *et al*., 2013). This has led to the isolation of main constituents of all important crude drugs in the 20th century. The diversity of these bioactive compounds and their specific pharmacological effects stimulated researchers to find explanations on the biosynthetic pathway for plant constituents starting with photosynthesis and resulting in more complex structures. Moreover, by the 20th century, there was additional discovery of drugs from the animal kingdom, which were mainly hormones and vitamins (Hamburger and Hostettmann, 1991). During the Second World War, many bioactive compounds were produced using microorganisms, such as tyrothricin (the discovery of first naturally derived antibiotic), penicillin, immunosuppressants and blood-cholesterol lowering drugs (Davies, 1999). Although these bioactive compounds were detected in animals and

microorganisms, they are often present in amounts too small to permit isolation of large quantities for use as drugs (Matkowski, 2008). Therefore, some of these bioactive compounds, such as steroidal hormones, were prepared from plant-derived compounds, the chemical structures of which permit their conversion to the desired drugs.

According to Lawson (2009), the global demand for plant derived drugs is growing exponentially and it is expected to increase from US $19.5 billion in 2008 to US $32.9 billion in 2013, with a compound annual growth rate of 11%. From 1983 to 1994, statistics done by Cragg *et al.* (1997) showed that 39% of the New Approved Drugs were of natural origin, mostly are from original natural products, products derived semi-synthetically from natural products and synthetic products based on natural molecules. However, the chemical potential of medicinal plants is still largely unexplored, as the chemical diversity has only been well analyzed and characterized in about 5-15% of all land plants. Hence, there remains an unprecedented possibility for the discovery of novel chemicals that may find diverse use in pharmaceuticals.

Combinatorial synthesis has been producing synthetic compounds that are similar to natural products in terms of molecular size and property, but these synthetic compounds have not been able to interact well with biomolecules (Piggott and Karuso, 2004). One of the possible reasons may be due to the different chemical space occupied by synthetic and natural products (Feher and Schmidt, 2003). Likewise, analysis between the molecular properties of drugs from synthetic and natural compounds also revealed the distinctiveness of natural compounds, especially concerning the diversity of scaffolds and the large number of chiral centres. As a result, 50% of drugs introduced into the market during the last 20 years have been derived directly or indirectly from natural compounds (Butler, 2008), and it has created more interests amongst scientists to dwell into the isolation and biosynthesis of natural products. In the 21st century, biotechnology has been playing a major role in pharmacognosy, with plant cell and tissue cultures of medicinal plants as the prime research areas (Matkowski, 2008). Many pharmaceutical companies such as Merck, AstraZeneca, and Diversa, have

signed memorandum of understanding with different countries to retrieve new sources of bioactive compounds from flora and fauna, which may have commercially significant entities or may provide lead structures for the development of modified derivatives in the production of newer medications (Samuelsson, 2004).

2.9 Chemical constituents of medicinal plants

Plant metabolism can be classified into two categories, namely primary metabolism and secondary metabolism (Hopkins and Huner, 2004). Primary metabolism refers to the processes for synthesis and consumption of nucleic acids, amino acids, proteins, lipids and carbohydrates that are essential for the survival and well being of a plant. Secondary metabolism is an alternative process that includes non-essential activities for the continuity of the plant lifecycle or for the plant's growth and development. Most of the plant constituents that are used medicinally are secondary metabolites (Narasinga, 2003).

There were over 100,000 secondary metabolites isolated from medicinal plants, with nearly half of them distributed in three major groups, alkaloid (12,000), flavonoid and phenolic (10,000) and terpenoid (25,000) (Bowsher *et al*., 2008). Most of these secondary metabolites occurred in relatively low quantities and their production may be widespread or restriced to particular families, genera, or species. The main focus in this study was on the secondary metabolites isolated from sweet shoot, since it has been documented as a rich source of bioactive phytochemicals. Many efforts have been made in the last few years to quantify the presence of secondary metabolites in sweet shoot using HPLC and GC-MS analysis (Table 2.7).

Table 2.7. Chemical constituents of *Sauropus androgynus* (sweet shoot) isolated by Asian scientists in the period from 1997-2012.

No.	References	Plant materials	Chemical constituents
1	Wang and Lee, 1997	Stems and leaves	• 3-*O*-β-D-glucosyl-7-*O*-α-L-rhamnosyl-kaempferol • 3-*O*-β-D-glucosyl-(1_6)-β-D-glucosyl-7-*O*-α-L-rhamnosyl-kaempferol • 3-*O*-β-D-glucosyl-(1_6)-β-D-glucosyl-kaempferol
2	Koo and Mohamed, 2001	Leaves	• Quercetin • Kaempferol
3	Kanchanapoom *et al*., 2003	Stems and leaves	• Megastigmane glycosides • Lignan glycosides • Sauroposides
4	Lee *et al*., 2011	Stems	• Octadecatrienoic acid • Glycerin • Acetic acid • Acetylpyrrolidine • Oleic acid • Benzofuran • L-phenylalanine • Pentaethylene glycol • B-sitosterol
5	Andarwulan *et al*., 2012	Leaves	• Chlorogenic acid • Caffeic acid • Ferulic acid
6	Selvi and Basker, 2012	Leaves	• 1,14-Tetradecanediol • 1-Octadecyne • Decanoic acid • Phytol • Pyrene • Squalene • Azulene

2.9.1 Plant alkaloids

Alkaloids are derived from amino acids and contain heterocyclic nitrogen atoms in their molecular structure. Alkaloids are widely distributed in 7231 species of higher plants in 1730 genera within 186 plant families (Hopkins and Huner, 2004). According to the review written by Cordell *et al*. (2001), a total number of 21120 alkaloids were successfully isolated from monocotyledons, dicotyledons and gymnosperms. Among the

monocotyledons, the families *Amaryllidaceae*, *Poaceae* and *Liliaceae* are found to be rich in alkaloids. Meanwhile, most alkaloids detected in dicotyledons are from families of *Apocynaceae* (2844 alkaloids), *Rutaceae* (1730 alkaloids), *Ranunculaceae* (1559 alkaloids), *Fabaceae* (1452 alkaloids) and *Papaveraceae* (1309 alkaloids) (Cordell *et al*., 2001).

In most cases, alkaloids can be isolated from almost all parts of the plant. Different tissues from the same plants may produce different alkaloids. The highest concentration of alkaloids was observed in seeds, fruits, barks, roots and leaves of a plant, depending on the type of plant species (Cordell *et al*., 2001). For instance, opium alkaloids were encountered specifically in the latex vessels of an opium poppy, and the tropane alkaloids of *Datura* species accumulated in the leaf petiole and in the tissue adjacent to the midrib of a leaf (Samuelsson, 2004). The alkaloids in an invidual cell usually present in the vacuoles instead of protoplasm and cell wall (Samuelsson, 2004).

Besides that, the organ with high alkaloid content is not necessarily the place where the alkaloids are formed (Bowsher *et al*., 2008). An active transport of alkaloids from one organ to another has been detected in many plant species. Such processes were observed in *Atropa belladonna* and tobacco plant, in which the nicotine and tropane alkaloids were formed in the roots and then transported to the leaves for storage (Samuelsson, 2004). Furthermore, lupin alkaloids were also found to be synthesized in the stem and then transferred to the roots. The migration of alkaloids from one organ to another seems to occur mainly via the xylem vessel, as many alkaloids have been found both in the vessels and the fluid dripping from leaf tips (Samuelsson, 2004).

Most alkaloids derived from plants have many pharmacological effects, simply by displaying a wide range of biological responses and having an extremely high potency (Wink *et al.*, 1998). Many alkaloids are in current use as drugs, such as the painkiller morphine, the antimalarial drug quinine and the cancer remedies vincristine and vinblastine (Rao *et al.*, 1978). Alkaloids also represent a vast reservoir for drug development but there has been very little cooperation between pharmacologists and chemists; the latter being

mainly interested in the isolation and structure determination of alkaloids, but those alkaloids have never been subjected to investigation for their biological and pharmacological properties.

Despite the extensive investigations on alkaloidal constituents of wild plants, no study has been carried out on the alkaloid biosynthesized from shoot cultures, callus cultures and somatic embryos of sweet shoot. The extraction and quantification of secondary metabolites in this research project are solely based on the genus *Papaver*. The genus *Papaver* includes 25 major alkaloids such as morphine, codeine, noscapine, thebaine, papaverine and narcotine, which are of pharmaceutical importance (Sarin, 2005; Oluk, 2006). Hence, *Papaver somniferum*, a flowering plant belonging to *Papaveraceae* is selected as a crop model for metabolite extraction and quantification.

The production of six major opium alkaloids has been reported in somatic embryos and callus cultures of *P. somniferum* (Khanna and Khanna, 1976; Kassem and Jacquin, 2001), *P. bracteatum* (Zito and Staba, 1982), *P. rhoeas* (Sarin and Khanna, 1989), and four other *Papaver* species. Ikuta *et al.* (1974) and Furuya *et al*. (1972) have also reported the presence of nine alkaloids in callus and differentiated plantlets of eleven *Papaveraceae* species. This statement has been further strengthened by Laurain-Matter *et al*. (1999), where three-month-old differentiated tissues of *P. somniferum* were able to produce morphine, codeine, baine and papaverine substances. The suspension culture of *Papaver bracteatrum* was also found to be effective in producing the above alkaloid substances (Lockwood, 1984).

The interest in studying the accumulation of alkaloids in *Papaver* species has followed the premise that these metabolites may be stress compounds formed in response to extremes in culture conditions. Alkaloids such as sanguinarine and berberine have been produced at relatively high concentration in cell cultures of *P. somniferum* and *Coptis japonica* (Shohael *et al*., 2006). The enhancement of alkaloid production by cell suspension cultures has also been achieved through the use of biotic elicitor preparation from fungal plant pathogens (Cline and Coscia, 1987).

2.9.2 Plant flavonoids and phenolics

Phenolic phytochemicals play a significant role in the area of natural products due to their ubiquitous distribution in plant kingdom. All phenolics share a common component, which is an aromatic hydrocarbon ring (phenyl or benzyl ring) that is usually attached to at least one hydroxyl group. Phenolic compounds are well known to provide protection against a wide range of diseases such as coronary heart disease, stroke and cancers. It can be categorized into three broad groups, (1) simple phenols (phenolic acids), (2) polyphenols (tannins and flavonoids) and (3) a miscellaneous group (Figure 2.9) (Ferreira *et al*., 2010).

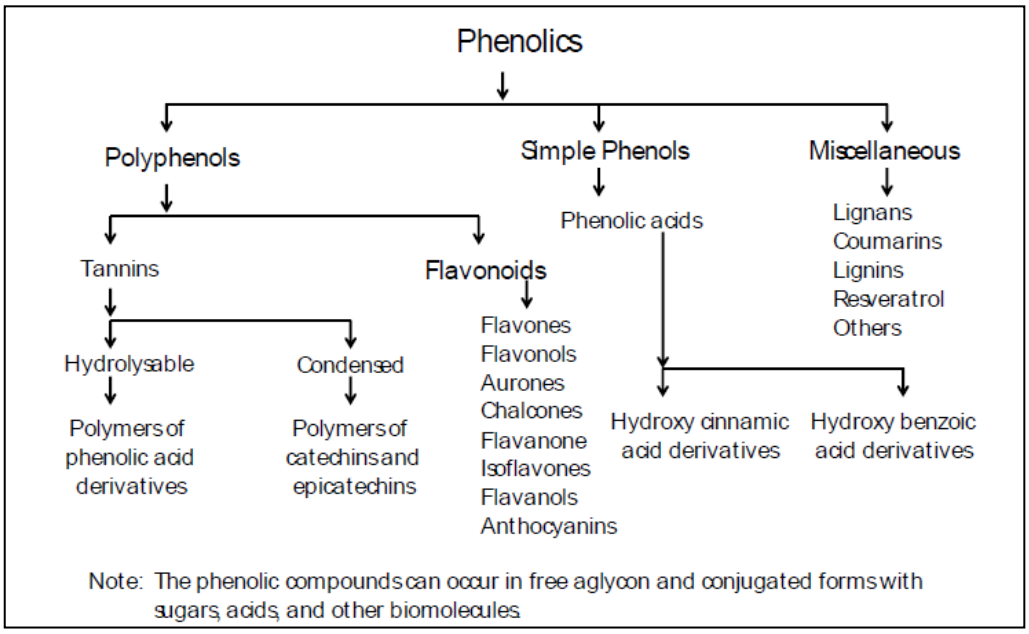


Figure 2.9. General classification of plant phenolics (Ferreira *et al*., 2010).

Polyphenols usually serve as antioxidants for the prevention and neutralization of the damaging effects of free radicals (Barros *et al*., 2007). Polyphenols can be categorized into two broad classes, namely tannins and flavonoids. To date, there are over 4,500 different flavonoids isolated and identified from plants, herbs, vegetables and fruits (Ferreira *et al*., 2010). The chemical structures of flavonoid are derived from the combination of shikimate/phenylpropanoid pathway and acylpolymalonate pathway (Matkowski, 2008). Flavonoids were documented to occur both as aglycones (without sugar residues) and as glycosides (with sugar residues in conjugated form). They differ in their substituents, mostly hydroxyl or methoxyl groups,

and in the nature and position of the sugar residues bound to aglycones (Pretorius, 2003). Flavonoids can be further subdivided into multiple groups such as anthocyanins, flavones, flavonols, flavanones, chalcones, aurones, isoflavonoids, bioflavonoids and others (Figure 2.10) (Bravo, 1988).

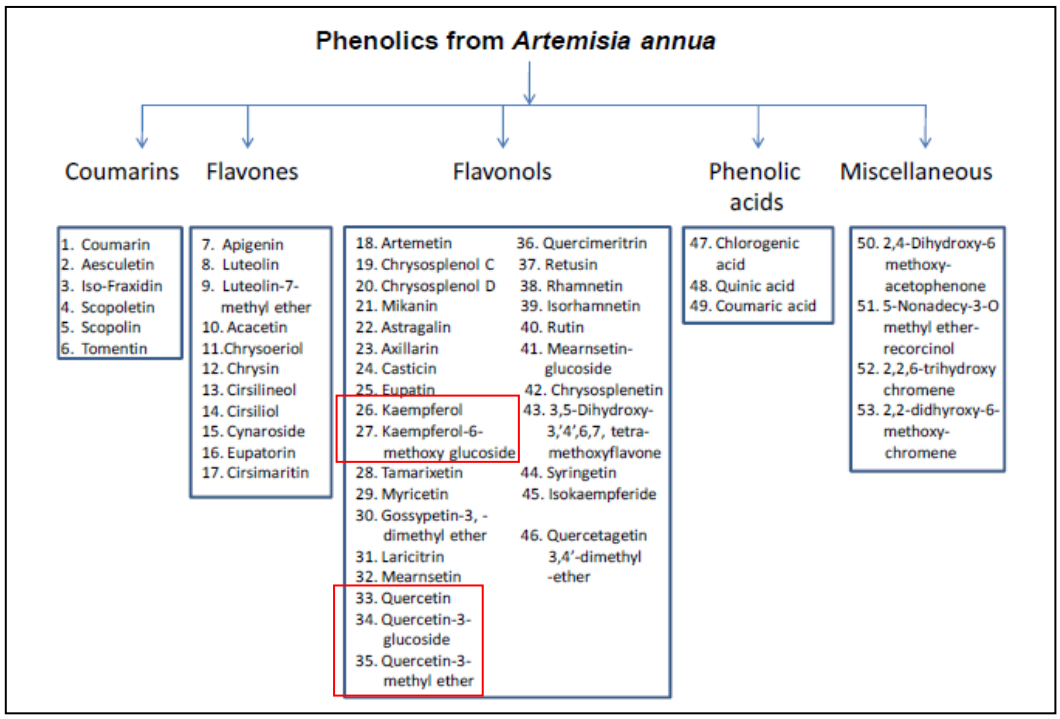


Figure 2.10. Major phenolics from *Artemisia annua*, with the great majority being flavones or flavonols (Ferreira *et al*., 2010). Kaempferol and quercetin, which responsible for antioxidant activities, were successfully extracted from *Artemisia annua*.

Flavonoids are the red, blue and purple pigments in plants that are important in the recruitment of pollinators and seed dispersers (Grotewold, 2006). Besides providing beautiful pigmentation in plants, flavonoid also played a major role in signaling between plants and microbial symbionts, and in plant defence as feeding deterrents and antimicrobial agents (Bowsher *et al*., 2008). For example, daidzein and genistein were released from legume roots into the soil, in order to induce the expression of nodule-inducing genes in *Rhizobium* bacteria (Bowsher *et al*., 2008). This symbiotic response appeared to be highly species-specific, both in terms of the type of flavonoid released from the plants, and the effect that it has on different species and individual strain of *Rhizobium*. Quercetin and kaempferol were also found to

be effective in protecting the plants from free radicals generated by ultraviolet B (Harborne, 2001).

Flavonoids are ubiquitous among vascular plants, and have been studied extensively for its biological and pharmacological activities including antioxidative activity, free radical scavenging capacity, antimicrobial activity, anticancer activity and anti-human immunodeficiency virus activity (Yao *et al*., 2004). The foremost proposal of pharmacological activity of flavonoids was presented by Szent-Gyorgyi in 1938, who reported that citrus peel flavonoids were effective in preventing capillary bleeding and fragility associated with scurvy (Gordon, 1996). Since then a great number of pharmacological effects have been ascribed to flavonoids, and certain individual members of the flavonoid group have been found to exert a multiplicity of actions.

Most of the flavonoids in vegetables and fruits acted as powerful antioxidants which can protect the human body from coronary heart disease and cancers (Gordon, 1996; Tapas *et al*., 2008). The capacity of flavonoids to act as antioxidants depends upon their molecular structure. For instance, flavonoids such as quercetin and kaempferol have been shown to inhibit the cell proliferation of breast cancer, colon cancer, human ovarian cancer, colorectal cancer, pancreatic cancer, prostate cancer and to induce cell death in squamous cell carcinoma and promyelocytic leukemia cells (So *et al*., 1997; Wenzel *et al*., 2004; Bandyopadhyay *et al*., 2008; Zhang *et al.*, 2008). In addition, quercetin was also documented to reduce the cytotoxic effects of oxidized LDL (low density lipoproteins) cholesterol (Egert *et al*., 2009).

There are considerable difficulties involved in the study of the importance of dietary flavonoids, (1) the possible effects of flavonoids may be dependent on the interaction with other dietary component, as many foods contain other active substances such as vitamin C; (2) the effect seems to be mainly preventive and it is difficult to study prevention than treatment; (3) flavonoids have a broad spectrum of effects and there seems to be no effect for which the flavonoids are solely responsible; (4) the metabolism and pharmacokinetics of flavonoids in humans are not very well understood, and

(5) flavonoids undergo structural changes in the gastrointestinal tract, as adsorbed metabolites may differ in structure from those ingested (Samuelsson, 2004). It is therefore necessary to sharply distinguish between experiments where flavonoids are given orally and those where administration is performed via other routes.

2.9.3 Antioxidant compounds

Plants are potential sources of natural antioxidants. Antioxidant compounds isolated from plants were found to delay the oxidation of lipid, inhibit the propagation of oxidizing chain reactions and neutralize the toxic effects of reactive oxygen species (ROS) in cells (Matkowski, 2008). Most living organisms have a complex system of natural enzymatic and non-enzymatic antioxidant defenses which act against the excessive production of reactive oxygen species (Leutner *et al.*, 2001). The homeostatic imbalance between reactive oxygen species (ROS) and natural antioxidants disturbed the redox equilibrium established under healthy conditions, causing the breakdown of extracellular matrix component hyaluronan into lower molecular weight fragments, which in turn activate innate immune responses and perpetuate tissue injury (Eberlein *et al*., 2008). Thus, antioxidants that scavenge reactive oxygen species may be of great value in preventing the onset and propagation of oxidative diseases like autoimmune diseases, neurovascular diseases and cardiovascular diseases.

In most cases, flavonoids are well known for their antioxidant capacity due to the redox properties, and it has been assumed that a diet rich in flavonoids is inversely correlated with the incidence of cancer, cell aging, cardiovascular disease and mortality. However, it is not well established on how flavonoids exert their beneficial actions and it seems that the best antioxidant flavonoids, such as quercetin, kaempferol and naringenin, are the ones with the best bioavailability, stability and biological effects (Ferreira *et al*., 2010). A list of research conducted on the biological activity of secondary metabolites, such as quercetin, kaempferol, naringenin and papaverine are summarized in Table 2.8.

Table 2.8. The medical benefits of quercetin, naringenin, papaverine and kaempferol isolated from medicinal plants.

Secondary metabolites	Medical benefits	References
Quercetin	• Price: RM1037.58/100g. • Anti-histamine to treat allergies. • Anti-hepatitis B activity. • Defending human colonocytes from oxidative attack (20%-25%). • Prevent cataract formation and diabetes. • Inhibit the growth of breast cancer cell. • Inhibition of HIV-1 reverse transcriptase, with IC_{50} values of 60μM. • Anti-inflammatory activity to alleviate pollinosis and fribomyalgia. • Analgesic properties. • Reducing the incidence of prostatitis, lung, stomach and pancreatic cancer. • Effective against dengue virus type II.	• Lucas *et al*., 2006. • Wu *et al*., 2007. • Yu *et al*., 2007b. • Jung *et al*., 2010. • Kumar and Pandey, 2013.
Naringenin	• Price: RM805.06/100g. • Act as an inhibitor of vascular endothelial growth factor (VEGF) release, which causes angiogenesis. • Reducing diabetes-induced neuropathy. • Protective effects on cognition and oxidative damage. • Effective against respiratory syncytial virus.	• Schindler and Mentlein, 2006. • Kumar *et al*., 2010. • Kandhare *et al*., 2012.
Papaverine	• Price: RM1052.57/100g. • Treat spasms of the gastrointestinal tract, bile ducts and ureter. • Use as a vasodilator in coronary artery bypass surgery. • Use as a smooth muscle relaxant in microsurgery. • Use as an erectile dysfunction drug.	• Bella and Brock, 2004. • Takeuchi *et al*., 2004. • Liu and Couldwell, 2005.
Kaempferol	• Price: RM1622.61/100mg. • Anti-proliferative activity to decrease ovarian and breast cancer cell number. • Anti-inflammatory activity to reduce the risk of lung and pancreatic cancer. • Cardioprotective properties to prevent cardiovascular diseases. • Neuroprotective properties against NOX-mediated neurodegeneration. • Medicinal values such as anti-microbial, anti-diabetic, anti-osteoporotic, anti-estrogenic and anti-allergic activities. • Positive effect against herpes simplex virus.	• Thors *et al*., 2008. • Calderon-Montano *et al*., 2011. • Jang *et al*., 2011. • Kumar and Pandey, 2013.

Many reports showed that the protective effect of flavonoids against oxidative stress is not only mediated by direct radical scavenging. Low concentration of flavonoids was proven to be effective in increasing the concentration of intracellular glutathione via transcriptional induction of *gamma-glutamylcysteine synthetase* (Myhrstad *et al*., 2002). This activity was selective for quercetin, kaempferol and apigenin, whereas the more potent antioxidant flavonoid myricetin failed to induce the transcriptional activation.

In 2009, the antioxidant potential and nutritive values of sweet shoot were evaluated by measuring the ability to scavenge radicals generated by *in vitro* systems and it showed that the metabolite contents of sweet shoot has potential to prevent free radical induced diseases (Subhasree *et al*., 2009). A similar result was reported by Benjapak *et al.* (2008), where the antioxidant activity correlated with the presence of flavonoids in sweet shoot. Flavonoids such as lignin, quercetin, kaempferol and megastigmane glycosides have also been obtained from the field-grown plants of sweet shoot in low concentration (Wang and Lee, 1997; Koo and Mohamed, 2001; Kanchanapoom *et al*., 2003). Hence, elicited plant tissue culture is used in this study to enhance the metabolites productivity (Koo and Mohamed, 2001; Yu *et al*., 2007b).

2.10 Traditional, analytical and preparative separations of secondary metabolites

2.10.1 Extraction methods of secondary metabolites

Sample preparation and extraction are of paramount importance to isolate bioactive compounds from plant materials, before subjecting to any qualitative and quantitative analysis. Sample preparation procedures for the analysis of phenolic acids and flavonoids generally varied from simple urine and beverage filtration to more complicated routines, such as hydrolysis of glycosides and solid-phase extraction (SPE) prior to analysis (Samuelsson, 2004). There is no coherence in the choice of pretreatment procedure because of the great variety of phenolic acids and flavonoids with regards to polarity, acidity, number of hydroxyl groups and aromatic rings, concentration levels, and complexity of plant matrix (Stalikas, 2007). Hence, it is essential to optimize the protocol for sample preparation and sample extraction of

sweet shoot, according to the chemical structures and properties of analyzed compounds.

In most cases, plant samples are usually subjected to milling, grinding and homogenization, which may be preceded by air drying, freeze drying or oven drying (Rijke *et al*., 2006). In contrast, liquid samples such as fruit juices and wines are often centrifuged and filtered using Whatman filter paper, followed by injecting the liquid into the separation system (Rijke *et al*., 2006). Plant sample preparation via grinding mill is an important step to ensure all the particles are of as uniform a size as possible, since larger particles take a longer duration for complete extraction thus slowing down the extraction process (Lopez *et al*., 2001). Although grinding mill is very useful for the production of fine plant powders, a certain amount of heat will be generated by the grinding mill which may lead to degradation of heat sensitive compounds (Lopez *et al*., 2001). Therefore, a thorough sample preparation procedure needs to be established for sweet shoot, in order to increase the extraction efficiency by having a maximum recovery of pure bioactive compounds, which can be incorporated into tablets and other ready-made drugs.

For sample extraction process, different polarity of solvents can be used to isolate different types of bioactive compounds from plant materials. The solvents used for extraction will diffuse into the cell to dissolve the desired compounds whereupon the solution will pass the cell wall in the opposite direction and mix with the surrounding liquid (Claeson and Bohlin, 1997). A satisfactory equilibrium needs to be established between the solute inside the cells and the solvent surrounding the fragmented plant tissues. According to Sultana *et al.* (2009), the ideal solvent for bioactive compounds has to be highly selective for the compound to be extracted. For instance, solvents with low polarity (diethyl ether and chloroform) are suitable for the extraction of nonpolar extraneous compounds such as oils, sterols and chlorophyll; whereas polar solvents (ethanol and methanol) are used to extract polar compounds such as flavonoid and phenolic compounds (Sermakkani and Thangapandian, 2010).

Phenolic acids with very high polarity (benzoic and cinnamic acids), however, could not be extracted entirely using a pure solvent, hence the mixtures of methanol/ethanol and water are highly recommended (Samuelsson, 2004). Many scientists believed that the mixture of ethanol and water helped to induce distension of the plant particles and to increase porosity of the cell walls, which in turn facilitate the diffusion of extracted substances from inside the cells to the surrounding solvent (Claeson and Bohlin, 1997). Besides that, there are also a number of factors influencing the optimum extraction conditions, such as pH, temperature, particle size, movement of the solvent and sample-to-solvent volume ratio (Hadjmohammadi and Sharifi, 2009). The manipulation of pH in extraction solvent is of great importance for charged constituents (Cseke *et al.*, 2006). Likewise, movement of the solvent relative to the particles facilitates diffusion of the solvent and the solution through the cell walls (Cseke *et al.*, 2006).

According to Stalikas (2007), flavonoid extraction from plant materials is usually one of the fastest and less time consuming tasks, since it only uses a relatively small amount of samples for extraction. In this research project, simple maceration and sequential solvent extraction were carried out in room temperature by mixing the tissue culture samples of sweet shoot with solvents (diethyl ether, chloroform, ethyl acetate, ethanol and methanol with sample/solvent ratio of 1:8) and left for 2 days with occasional shaking. However, the HPLC chromatogram of sweet shoot revealed the absence of flavonoid (kaempferol and quercetin) analytical peak in cultured tissues extracted using this method (data not shown). The absence of an analytical peak for the flavonoids may be caused by the deficiency of a specific enzyme to facilitate the degradation of glycosidic bond, which eventually lead to the failure in releasing the intact aglycone (Lopez *et al*., 2001).

For medicinal plants, one of the most important aspects of flavonoid analysis is to determine the chemical structures of targeted analytes; as the targeted analytes could be present in the form of either intact conjugates or aglycones (Pretorius, 2003). However, most reference compounds of these flavonoid glycosides are not commercially available (Stevens *et al*., 1999). When the plant flavonoids were to be examined in their glycosylated form,

hydrolysis-digestion step was by-passed (Stevens *et al*., 1999). Many researchers have lately diverted their attentions onto the importance of aglycone in medicinal plants, therefore an additional hydrolysis-digestion step is needed to disrupt the glycoside linkages and to remove the sugar moieties from glycosides using either enzyme, acid or base catalysts (Stalikas, 2007). The rate of acidic hydrolysis of flavonoid glycosides relies on the acidic strength of extraction solvent and type of glycosides connected to flavonoids (Hadjmohammadi and Sharifi, 2009). The isolated analytes can be further isolated with additional steps using SPE and liquid-liquid chromatography to eliminate the impurities and interfering materials from plant extracts (Liu *et al*., 2008).

In this research project, basic reflux extraction system was used to isolate flavonoids from fresh samples of sweet shoot at 90^{o}C for 2 hours. This was based on studies performed on *Coriandrum sativum* (Hadjmohammadi and Sharifi, 2009) and sweet shoot (Koo and Mohamed, 2001; Andarwulan *et al*., 2010), which showed successful extraction of quercetin and kaempferol. As quercetin, kaempferol and naringenin are flavonoid aglycones, acid hydrolysis of flavonoid was performed using basic reflux system with 1.2 M hydrochloric acid (HCl) and 2.0 g/l tert-butylhydroquinone (TBHQ) in the presence of 62.5% (v/v) aqueous methanol, in order to liberate the flavonoid glycosides into aglycones. Plant extracts were subsequently filtered through 0.45 µm membrane filter and injected into reversed-phase high performance liquid chromatography (HPLC) system.

Based on the study done by Hadjmohammadi and Sharifi (2009), the recovery of flavonoids increased as the extraction duration increased to 2 hours. However, prolonged extraction time of *Coriandrum sativum* resulted in a negative impact on the recovery of flavonoids, since some of the flavonoids may have degraded during the extraction process (Hadjmohammadi and Sharifi, 2009). The extension of extraction period alternatively increased the amount of flavonoids and phenolic acids present in *Tilia europea*, *Urtica dioica* and *Mentha spicata* after 12 hours soxhlet extraction with methanol (Fiamegos *et al*., 2004). Therefore, it is necessary to optimize the extraction conditions to maximize recovery of flavonoids in sweet shoot.

2.10.2 Qualitative metabolite identification

Up till now, the antioxidant activities of cultured tissues have not been determined in a majority of the medicinal plants. Despite an increase of 340% of manuscripts mentioning the word "antioxidant" in the Medline database between year 1995 and 2005, actual manuscripts highlighting the use of antioxidants from plant and animal origin only rose a meagre 39% (Prior *et al*., 2005). The understanding of antioxidant properties from cultured tissues were usually obtained from the studies involving dietary antioxidants and compounds extracted from the ethnomedicinal plants (Matkowski, 2008). It has been reported that a great majority of secondary metabolites exhibited high antioxidant capacity, but real time monitoring of antioxidant activity in plant tissue culture seem to be redundant (Sanchez-Moreno, 2002). Thus, the huge variability among antioxidants and their complex structure-activity relationships allowed scientists to conduct more research on the antioxidant action and other biological activities in tissue culture of medicinal plants. In view of this, simple assays such as free radical scavenging assays (DPPH radical scavenging activity and FRAP assay) were carried out for the determination of antioxidant capacity in sweet shoot (Halliwell, 1995; Matkowski, 2008).

The number of methods to measure antioxidant activities in plants has increased significantly during the past 10 years. There are several criteria to be fulfilled before standardizing a method for antioxidant capacity of plants, (1) measures chemistry occurring in potential application, (2) utilizes a biologically relevant radical source, (3) simple, (4) uses a method with defined endpoint and chemical mechanism, (5) instrumentation is readily accessible, (6) good within-run and between-day reproducibility, and (7) adaptable to high-throughput analysis (Prior *et al*., 2005). Performance characteristics is also needed to be taken into consideration for the standardization of an assay, such as (1) analytical range, (2) recovery, (3) repeatability, (4) reproducibility, and (5) recognition of interfering substances (Matkowski, 2008).

The antioxidant assays generally demonstrated a huge variety of defense mechanisms in plants, depending on the features of a particular

assay. Simple investigative methods include free radical scavenging with the use of coloured and artificial stable free radicals such as DPPH (2,2-diphenyl-1-picrylhydrazyl free radical) (Molyneux, 2004), DMPD (N,N-dimethyl-p-phenyldiamine) (Fogliano *et al.*, 1999) and ABTS (2,2'-azinobis(3-ethylbenzothiazoline-6-suslfonic acid) (Re *et al*., 1991). The metal based methods including FRAP (ferric reducing antioxidant power), ferric thiocyanate assay (Halliwell, 1995) and molybdenum ion-phosphomolybdenum (P-Mo) assay (Prieto *et al*., 1999) can be used to monitor the antioxidant capacity of plants using colourimetry. All of these tests are fairly easy to be conducted in the lab and it can be used in high throughput screening (Table 2.9).

Table 2.9. Selected examples of antiodixant production from the cultured tissues of medicinal plants.

Plant species	**Secondary metabolites**	**Culture system**	**Antioxidant assays**	**References**
Artemisia judaica	Flavonoids	Shoot cultures	DPPH	Liu *et al*., 2004
Ipomoea batatas	Anthocyanins	Callus, cell suspension	DPPH	Konczak-Islam *et al*., 2000
Passiflora quadrangularis	Flavone-C-glycosides	UV irradiated callus	DPPH	Antognomi *et al*., 2007
Salvia officinalis	Rosmarinic acid, abietane diterpenoids	Callus, shoot culture, hairy roots	DPPH, P-Mo, lipid peroxidation	Grzegorczyk *et al*., 2007
Stevia rebaudiana	Flavonoids	Callus	FRAP, DPPH	Tadhani *et al.*, 2007

Several antioxidant assays, based on their substrate degradation inhibition effects, are also available to be tested on their protective effect on biomolecular entities from oxidative damage. These degradation products can be monitored by spectrophotometry (TBARS, thiobarbituric reactive substances, carotene bleaching), fluorescence (ORAC assay, oxygen radical absorption capacity) and chromatography (Halliwell, 1995; Matkowski, 2008; Sanchez-Moreno, 2002).

However, the main drawback from these antioxidant assays were the unrealistic conditions they were produced in the lab due to the usage of artificial compounds and may not necessarily reflect those produced in true environmental conditions (Aruoma, 2003). This problem can be overcome by observing the free radical either indirectly with chromophore reagents or with electron spin resonance technique (Matkowski, 2008). The scavenging of superoxide radical anion, hydroxyl radical and nitric oxide can also be observed using the above proposed method (Sreejayan and Rao, 1997).

2.10.3 Identification and quantification of metabolites

Natural products continue to play a crucial role in the industrial drug discovery process. Drug discovery is a difficult, lengthy, expensive and often inefficient process with low success rates (Blake *et al*., 2009). In the pharmaceutical industry, the current trend in strategies for drug discovery is combinatorial chemistry with high-throughput screening techniques, wherein large amount of bioactive compounds are tested for the therapeutic ability (Samuelsson, 2004). The high-throughput screening techniques are proven to be effective in screening millions of samples per year (Liu *et al*., 2008). Therefore, many research centers and pharmaceutical companies started establishing units for research on natural products, by applying their resources for high-throughput screening to extract hitherto unknown medicinal plants, organisms, fermentation broths and cultured tissues.

Chromatography is an exceptionally powerful technique in separating a mixture of bioactive compounds and in identifying the presence of bioactive compounds in plant extracts. In chromatography, the plant extracts are separated by differential distribution of the components between a stationary phase and a mobile phase (Plazonic *et al*., 2009). There are many detection methods in monitoring the desired metabolites such as thin layer chromatography (TLC), high performance liquid chromatography (HPLC), gas chromatography (GC), mass spectrometry (MS), infrared spectroscopy and nuclear magnetic resonance (NMR) with the appropriate detection (Rijke *et al*., 2006). Elucidation of the structure of active principles definitely paved the way for synthesis and derivatization for compounds with higher efficacy and lower adverse effects. All of these equipment are the best means for

structure determination, when novel compounds are present *in vitro,* that are yet known in intact plants of sweet shoot.

2.10.3.1 Thin layer chromatography

Thin layer chromatography (TLC) has been commonly used in phenolic and flavonoid analysis of plant extracts with eighty papers published between year 2001 and 2005, regarding isolation of flavonoids from medicinal plants using this technique (Rijke *et al*., 2006). TLC is extremely useful for the rapid screenings of bioactive substances in plant extracts prior to detailed analysis by instrumental techniques, mainly because of its capacity for high sample throughput (Cseke *et al*., 2006). In most cases, TLC entails using silica as a stationary phase and the TLC plates are developed with either a combination of 2-(diphenylboryloxyl)-ethylamine and polyethylene glycol or with aluminium chloride . The detection of phenolic acids and flavonoids is mainly observed under UV light at 250-260 nm (short wave irradiance), 350-365 nm (long wave irradiance) or with densitometry at the same wavelengths (Stalikas, 2007). The detection of bioactive compounds may also be accomplished by spraying the TLC plates with a suitable reagent, such as iodine reagent, chromic acid solution and 2,4-dinitrophenylhydrazine reagent (Rijke *et al*., 2006).

One of the first steps in TLC separation is the selection of a suitable mobile phase, whereby the mobile phase carries the bioactive compounds and ascends the TLC plates by capillary action (Cseke *et al*., 2006). The diverse bioactive compounds are separated based upon their interaction with the adsorbent coating. Commercially available TLC plates, 60 Å silica gel, 250 µm layer thickness on aluminum backing, are suitable for rapid anlaysis of crude plant extracts and for following the progress of preparative separation (Cseke *et al*., 2006). For instance, Soczewinski *et al.* (2004) used TLC to separate a flavonoid mixture containing nine glucosides and seven aglycones. The more polar glycosides were separated using an eluent with high solvent strength, whereas the aglycones were separated in a second step using another relatively weak eluent. Similar result was observed by Hawryl *et al*. (2002) in the *Flos sambuci* extracts, whereby eight flavonoids and three phenolic acids were identified using TLC plates with a suitable mobile phase composition.

According to Lewis *et al*. (1998), the presence of phenolic acids, anthocyanins and flavonoids were detected in potatoes using TLC method with the following developing solvents, (i) 15% (v/v) acetic acid in water; (ii) n-butanol, acetic acid, water (4:1:2); and (iii) acetic acid, HCl, water (30:3:10). Besides that, TLC technique was also adopted by Jamshidi *et al*. (2000) to isolate kaempferol and quercetin from *Ginkgo biloba* leaf extracts in the reflectance mode of 254 nm. The recovery rate of 94% was obtained for both quercetin and kaempferol using a standard spiking procedure. In year 2004, a similar method was also used by Medic-Saric *et al*. (2004) to quantify the presence of flavonones and phenolic acids in propolis at ultraviolet wavelength of 254 nm and 366 nm.

2.10.3.2 High performance liquid chromatography

Classical liquid chromatography (LC) was first discovered by Mikhail Tswett, a Russian botanist who separated the plant pigments using a glass column packed with chalk ($CaCO_3$) in year 1903 (Ettre, 2002). Since the 1930s, chemists used gravity-fed silica columns to purify organic materials and ion-exchange resin columns to separate ionic compounds and radionuclides (Dong, 2006). In the late 1960s, LC turned 'high performance' with the utilization of small-particle columns that required high pressure pumps (Stalikas, 2007). In-line detectors and reliable injectors were subsequently developed for commercial high performance liquid chromatography (HPLC) machine, in order to create a more sensitive and quantitative technique for identification of bioactive compounds (Cseke *et al*., 2006). Since then, HPLC analytical technique has dominated the separation and characterization of flavonoid compounds for the next 50 years.

Today, the number of papers dealing with flavonoid assay in HPLC is increasing in accordance with the growing interest in the investigation of pharmacological and biological effects of flavonoids. HPLC continues to evolve rapidly toward higher speed, efficiency and sensitivity, driven by the emerging needs of life sciences and pharmaceutical applications (Dong, 2006). HPLC techniques offer many advantages in the analysis of flavonoid compounds in medicinal plants, such as (1) the ability to execute quick and precise quantitative analysis, (2) programmed operation with high sensitivity

detection, (3) wide range of commercially available column, (4) the possibility of combining two columns in a switching mode, (5) the ability to separate the analyzed components from the derivatives and degradation products, and (6) the ability to determine low concentrations of analytes in the presence of interfering and co-eluting components (Stalikas, 2007).

The introduction of reversed-phase (RP) columns helped to enhance the HPLC separation of phenolic and flavonoid compounds of pertinent parameters, namely (1) HPLC column, (2) mobile phase, (3) flowrate, and (4) detector (Dong, 2006). For reversed-phase chromatography, the separation of flavonoids was based on analytes partition coefficients between a polar mobile phase and a hydrophobic (non polar) stationary phase (Cseke *et al*., 2006). The stationary phases are usually solid particles coated with hydrophobic groups, such as octadecyl (C18) bonded groups on a silica support (Miller, 2004). The polar analytes are the first to elute from the column, followed by non polar analytes, as non polar analytes interact more strongly with the hydrophobic C18 group. This elution order of 'polar first and non polar last' is the reverse of that observed in normal-phase chromatography, thus the term 'reversed-phase chromatography'.

HPLC column is the heart of HPLC system, as it holds the fine support media for the stationary phase that provides differential retention of flavonoid compounds. Basic HPLC column parameters such as column types (silica, polymer), dimensions (preparative, analytical) and packing characteristics (particle and pore size) are presented with key column development trends (Dong, 2006). With either reversed-phase or normal phase chromatography, C18 columns ranging from 100 to 250 mm in length and usually with an internal diameter of 3.9 to 4.6 mm were employed for flavonoid analysis of medicinal plants (Stalikas, 2007). Likewise, particle sizes generally fall in the range of 3 to 10 µm. The silica sorbents with reduced metallic residue contents and minimum residual silanol groups on the surface were documented to positively influence the peak symmetry as chromatographic resolution (Ryan *et al*., 1999). The efficiency of HPLC column is also better for column with good free silanol group covering, end-capping or embedding. An end-capped column was preferred for flavonoid analysis as the residual

silanol group appeared to impair the separation (Cuyckens and Claeys, 2004). Besides that, most HPLC analyses of flavonoid compounds are performed at ambient column temperature between 30-40^{o}C (Roggero *et al*., 1997). All of these factors need to be taken into consideration for the analysis of phenolic and flavonoid compounds characterized by a wide spectrum of physicochemical properties.

In HPLC, the mobile phase is the solvent that shifts the analytes through the column. Both isocratic and gradient elution can be applied for the analysis of flavonoid compounds. The choice between isocratic and gradient elution usually depends on the type of analytes and on the nature of plant matrix (Ahuja and Dong, 2005). Ideally, solvents used for HPLC mobile phases should have the following characteristics, (1) high solubility for sample components, (2) non-corrosive to HPLC system, (3) high purity, (4) low cost and (5) UV transparency (Stalikas, 2007). Acetonitrile and methanol are the most commonly used organic modifiers. In some cases, the use of acetonitrile as a mobile phase gives a better resolution than methanol, by providing a sharper peak shape and resulting in a higher HPLC plate number (Dong, 2006). However, methanol is always the preferred solvent than acetonitrile, due to its non-hazardous properties and the possibility of higher percentage used in mobile phase which can help in the protection of HPLC column (Dalluge *et al*., 1998). For instance, Casteele *et al*. (1982) have established the separation of 141 flavonoids from polar triglycosides to nonpolar polymethoxylated aglycones belonging to the classes of flavones, flavonols and flavanones, by using a LiChrosorb RP C18 column with 5% aqueous formic acid and methanol. According to Zgorka and Dawka (2001), phenolic acids (chlorogenic acid, caffeic acid, vanillic acid and ferulic acid) can also be separated from medicinal plants using a simple isocratic mobile phase (methanol-water-acetic acid).

The pH of the solvent in mobile phase was noted to create a dramatic effect on the retention time of ionizable (acidic or basic) analytes (Dalluge *et al*., 1998). A marked alteration observed in the mobile phase was the type of acid used as a modifier to minimize the peak tailing (Dong, 2006). Most phenolic acids have pKa values of 4, while flavonoids with several ionizable

hydroxyl groups have pKa values greater than 4 (Stalikas, 2007). One of the major concerns in improving the resolution and reproducibility of retention characteristics is to evade the ionization of analytes during flavonoid analysis. Therefore, the pH range for HPLC assay can be modified using acid modifiers such as acetic acid, formic acid, phosphoric acid and trifluoroacetic acid (TFA) (Katz *et al*., 1998). There are no generally applicable mobile phase additives for flavonoid glycosides separation, hence optimization of mobile phase composition is needed for sweet shoot.

In general, the HPLC system is equipped with an ultraviolet/visible (UV/Vis) detector to measure the concentration of eluting analytes. Many scientists showed that flavonoids can be detected using a photodiode array (PDA) detector, UV-fluorescence detector, electrochemical coulometric array detector, electroarray detector, mass spectrometric and NMR detector (Dong, 2006). Most of the flavonoid aglycones consist of at least one aromatic ring, which helps to absorb UV light efficiently (Mabry *et al*., 1970). The A-ring of flavonoid compounds can be detected in the range of 240-285 nm UV wavelengths (first absorption maximum), while the C-ring can be detected within the 300-550 nm range (second absorption maximum) (Mabry *et al*., 1970). Simple substituents such as methyl, methoxy, and non dissociated hydroxyl groups only have a minor effect in the position of absorption maxima (Cseke *et al*., 2006). No single wavelength is sufficient for simultaneous monitoring of various natural plant extracts.

The rapid progress in phytochemical instrumentation development and the trend towards a metabolomic approach and high throughput screening is likely to influence the monitoring of *in vitro* secondary metabolites production. In recent publications, the chromatographic determination of antioxidants predominates and is frequently accompanied by bioactivity guided fractionation, spectral structure elucidation of isolated chemicals by MS or NMR (Tian *et al*., 2005). The use of hyphenated techniques such as LC-MS, LC-NMR, 2D-NMR or ESI-MS are considered to be the best means for structure determination when novel compounds are present *in vitro* (Farag *et al*., 2007).

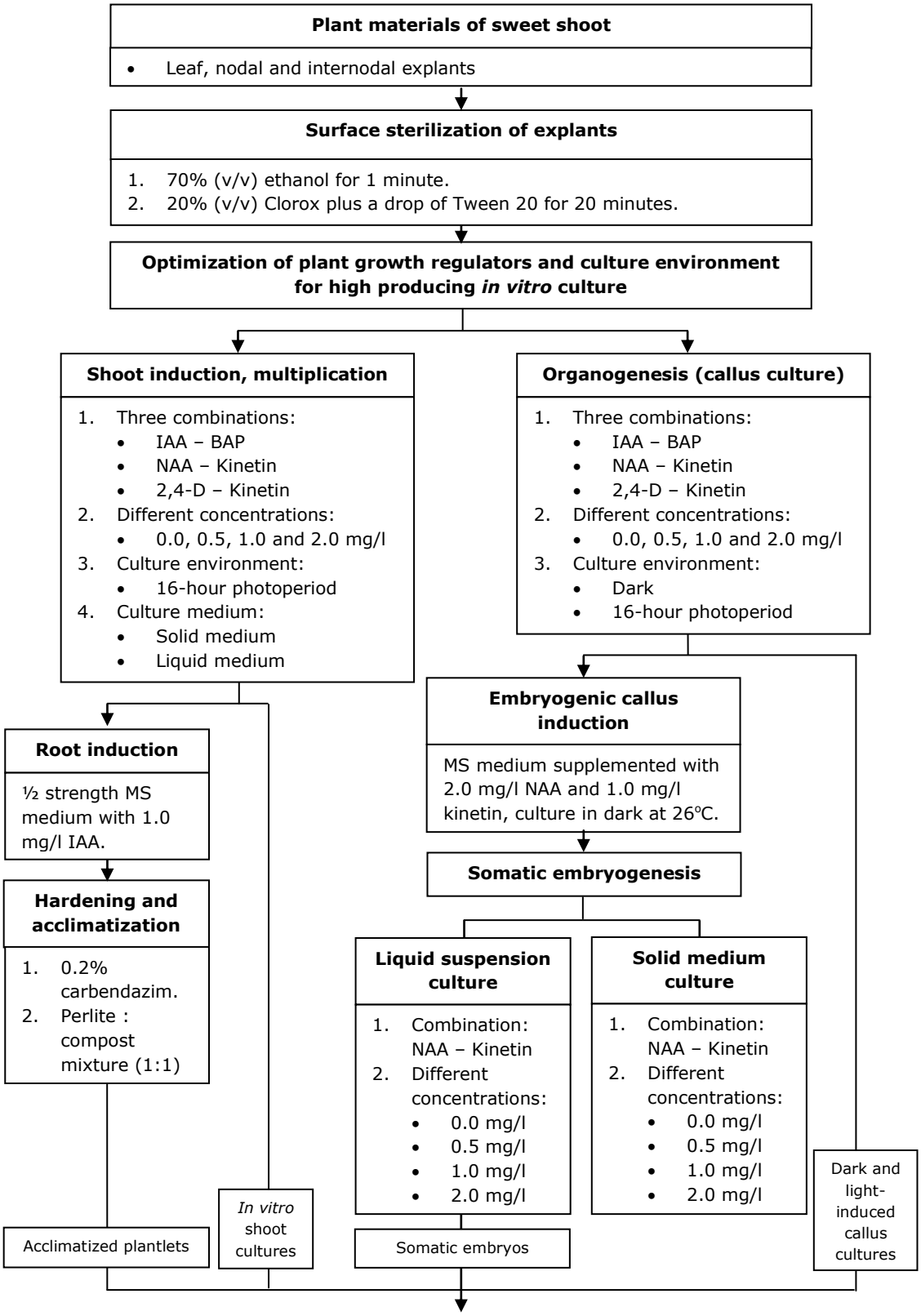
2.11 Significant contributions to research and development

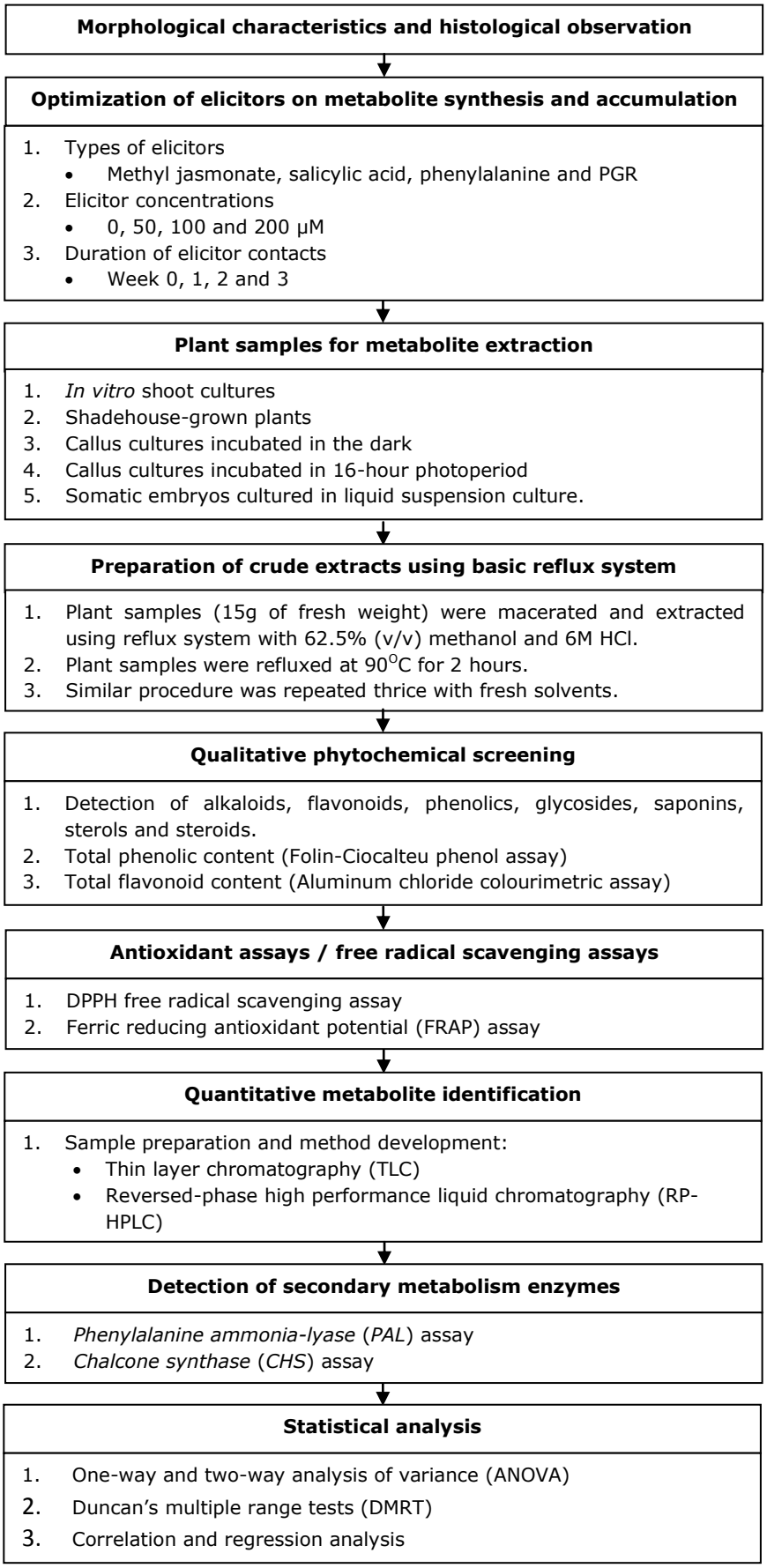
Overall, this project provides an overview of current and developing methods available for sweet shoot micropropagation. The established method from this study may provide instructive information for the development of *in vitro* regeneration protocols for other *Phyllanthaceae* species and medicinal plants. The developed methods have huge potential for rapid and mass multiplication of sweet shoot, for commercialization, conservation purposes, genetic improvement and the production of antioxidant compounds on a sustainable basis.

To date, only a few synthetic antioxidant compounds are approved for the use in healthcare industry due to potential toxicity and carcinogenic impact on human cells. Natural plant-based antioxidants are well known to have a higher effect in preventing several types of cancer as compared to the synthetic types. With the help of plant tissue culture technology, the amount of natural plant-based antioxidants can be improved remarkably in the near future. This should help satisfy the growing demand for natural antioxidants. The conclusion, if established by *in vivo* studies on biological systems, can surely open up a new avenue in the search for natural antioxidants, and help discover a cost effective and safe plant-made antioxidant product for the diagnosis and treatment of inflammatory cancer diseases.

This study also allows scientists to determine the possible association between the antioxidant activity and the content of phenolic and flavonoid compounds present in sweet shoot. The outcome of this study provides a helpful tool to explore the extraordinary complexity of plant biochemical capacity and the mechanism of biosynthesis, which involve multiple levels of products, enzymatic genes and the aspects of compartmentation. The observations may be used to substantiate the scientific reasoning that free radical scavenging is certainly the mode of operation in the prevention of deadly disorders like arthritis, cancer and atherosclerosis. The burgeoning metabolomic and metabolic engineering approaches will definitely add a new fascinating dimension to the possibilities of flavonoid-making plant cell factories.

2.12 Research project outline




Morphological characteristics and histological observation
Optimization of elicitors on metabolite synthesis and accumulation
1. Types of elicitors
• Methyl jasmonate, salicylic acid, phenylalanine and PGR
2. Elicitor concentrations
• 0, 50, 100 and 200 µM
3. Duration of elicitor contacts
• Week 0, 1, 2 and 3
Plant samples for metabolite extraction
1. In vitro shoot cultures
2. Shadehouse-grown plants
3. Callus cultures incubated in the dark
4. Callus cultures incubated in 16-hour photoperiod
5. Somatic embryos cultured in liquid suspension culture.
Preparation of crude extracts using basic reflux system
1. Plant samples (15g of fresh weight) were macerated and extracted using reflux system with 62.5% (v/v) methanol and 6M HCl.
2. Plant samples were refluxed at 90°C for 2 hours.
3. Similar procedure was repeated thrice with fresh solvents.
Qualitative phytochemical screening
1. Detection of alkaloids, flavonoids, phenolics, glycosides, saponins, sterols and steroids.
2. Total phenolic content (Folin-Ciocalteu phenol assay)
3. Total flavonoid content (Aluminum chloride colourimetric assay)
Antioxidant assays / free radical scavenging assays
1. DPPH free radical scavenging assay
2. Ferric reducing antioxidant potential (FRAP) assay
Quantitative metabolite identification
1. Sample preparation and method development:
• Thin layer chromatography (TLC)
• Reversed-phase high performance liquid chromatography (RP-HPLC)
Detection of secondary metabolism enzymes
1. Phenylalanine ammonia-lyase (PAL) assay
2. Chalcone synthase (CHS) assay
Statistical analysis
1. One-way and two-way analysis of variance (ANOVA)
2. Duncan's multiple range tests (DMRT)
3. Correlation and regression analysis

CHAPTER 3: AXILLARY AND ADVENTITIOUS SHOOT PROLIFERATION OF *Sauropus androgynus*

3.1 INTRODUCTION

To date, considerable attention has been devoted to underutilized crops as a source of food and pharmaceutical commodity. Traditionally, sweet shoot is propagated via woody stem cuttings and seeds (Philomena, 1993). Unfortunately, propagation by stem cuttings is unfavourable due to the spread of fungal diseases, labour-intensiveness and time required, additionally, there is some evidence of growth and yield retardation in sweet shoot via this method (Li and He, 2006; Tejavathi *et al*., 2010). The problems listed above have been proven to be true after comparison with the preliminary results obtained in this study, in which propagation of sweet shoot via stem cuttings failed to enhance the rooting responses from shoot tips, internodal and nodal cuttings. An average rooting percentage of 61% was observed in sweet shoot cuttings, after 2 months of cultivation (Appendix Table 2). Thirty nine percent of cuttings showed no signs of rooting, and some did not even root after three months. The low success rate of root induction caused great difficulty in meeting the market demand for propagules via this method. Hence, plant tissue culture technique served as a viable method for rapid and mass multiplication of sweet shoot.

In order to mass propagate sweet shoot on a sustainable basis, 'true-to-type' plants need to be rapidly generated within a short span of time and with limited space. The most commonly used micropropagation methods for commercial production utilizes enhanced axillary and adventitious shoot proliferation from leaf and stem explants, as it has been documented to produce more than 250 million plants yearly for worldwide industry (Trigiano and Gray, 2000). This micropropagation method provides genetic stability and is easily attainable for many woody plant species (Trigiano and Gray, 2000). The major constraints in plant tissue culture of sweet shoot are the slow growth and low percentage of culture responses under controlled environment. That is why the effects of plant growth regulators and cultivation systems on shooting behavior were investigated in this chapter.

Endogenous plant growth regulators normally play a crucial role in regulating the formation of axillary shoot, adventitious shoot, callus and somatic embryo in *in vitro* cultures (Sathyanarayana and Varghese, 2007). It is widely accepted that auxin, like NAA plays a significant role in the regulatory process of callus and somatic embryo induction of woody plants such as *Euphorbia pulcherrima* (Wilkins, 1988) and *Codiaeum variegatum* (Orlikowska *et al*., 2000). Meanwhile, BAP is frequently used in axillary and adventitious shoot proliferation of *Paeonia suffruticosa* (Yulong *et al*., 1984), *Fraxinus americana* (Heutteman and Preece, 1993) and *Hydrangea quercifolia* (Ledbetter and Preece, 2004). The combination of these hormones in the optimum concentration increased the multiplication rate of shoot and allowed shoot elongation in woody plant species cultured *in vitro*.

Even though many reports are available for woody plant species, the protocols are relatively complicated for *in vitro* shoot regeneration, as many factors were documented to influence the ability of explant to proliferate into adventitious shoot (Kalimuthu *et al*., 2006). It is definitely imperative to investigate the related factors on shoot regeneration, in order to effectively propagate and supply planting materials for large-scale plantation. As the success of a culture is affected by the type and concentration of plant growth regulators, the present study aimed to induce adventitious shoots from leaf and stem explant of sweet shoot using various combinations and concentrations of plant growth regulators in both semi-solid and liquid media cultivation systems. This experiment helped to reveal the importance of cytokinin in combination with auxin in bringing about complete morphogenesis in sweet shoot under the present experimental conditions.

3.2 MATERIALS AND METHODS

3.2.1 Plant materials and growth conditions

Containerized plants of *Sauropus androgynus* (sweet shoot) were obtained from the University Putra Malaysia (UPM), Selangor, Malaysia and maintained in ambient conditions in a shadehouse at the University of Nottingham Malaysia Campus (Figure 3.1). Sweet shoot grown in pots was identified by post-harvest horticulturist, Dr. Mahmud bin Tengku Muda Mohamed, Faculty of Agriculture, UPM, Selangor, Malaysia. Plant materials

were watered by direct application to the soil in pots, to moisten the soil and to reduce water stress. Overhead sprinkler was not used for irrigation, as it may cause the entry of microbial spores into cracks and crevices on the plant surface (Purohit, 2003).

Figure 3.1. *Sauropus androgynus* (sweet shoot). (A and B): Plant materials brought from UPM to shadehouse (School of Biosciences, the University of Nottingham Malaysia Campus) for multiplication and development.

Plant materials were sprayed as required with the insecticide Furatox 3G (0.2% v/v) and fungicide carbendazim (0.2% v/v), in order to prevent the infestation of insect and fungal pathogens, such as red spider mites, slugs, black garden ants, black mould and yellow spots on leaves. Commercial fertilizer (Neo Growth Green 45 Garden Fertilizer) containing 15% nitrogen (N), 15% phosphorus (P) and 15% potassium (K) was applied to compost every three weeks (Ong *et al*., 2009).

WatchDog data logger from Spectrum Technologies Incorporation was used to record the environmental conditions over the entire experimental duration, such as temperature (^{o}C), relative humidity (%), and photosynthetically active radiation (PAR). The seasonal mean of maximum

and minimum temperature ranged between 23.5°C and 33.5°C. The seasonal mean of relative humidity was at a range of 52.3 % to 96.4 %. Last but not least, the seasonal mean of photosynthetically active radiation was from 40.85 to 104.61 μmolm^{-2}s^{-1}.

3.2.2 Sterilization techniques for tissue culture procedures

3.2.2.1 Preparation of glassware and instruments

All procedures for *in vitro* culture of sweet shoot were performed in a laminar flow cabinet under strict aseptic conditions (Figure 3.2). All surgical instruments, glassware, and other necessary tools were initially sterilized and soaked with liquid disinfectants (Cleaning Agent Decon® 90, Decon Laboratories Limited, United Kingdom) overnight, followed by rinsing under running tap water, to completely remove all traces of disinfectants. All items were subsequently autoclaved at 121°C with 15 psi for 15 minutes, and dried in an oven at 70°C.

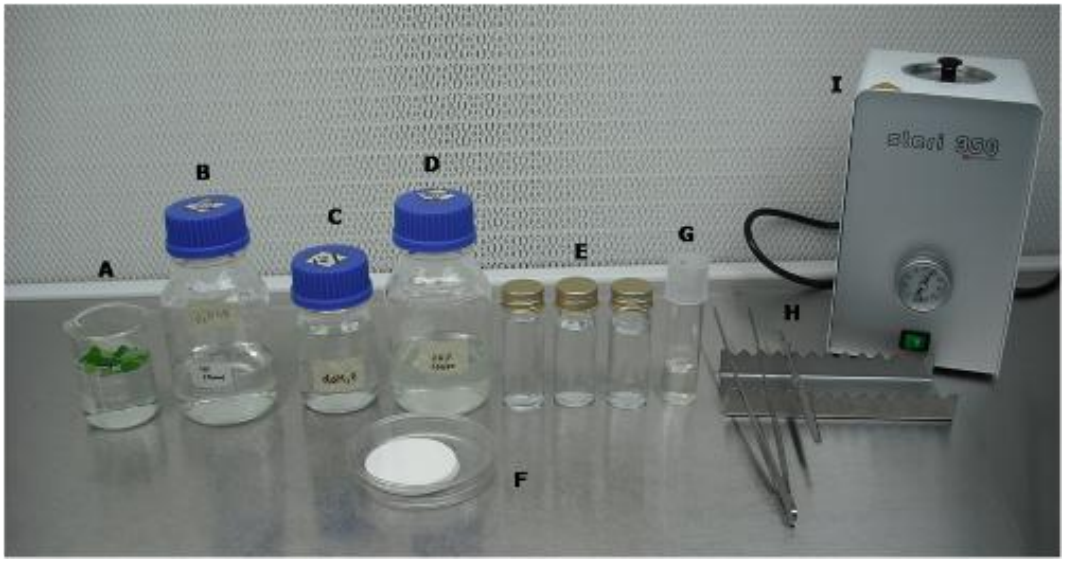


Figure 3.2. Materials needed for surface sterilization of explants. (A): Plant materials. (B): Schott bottle with 70% (v/v) ethanol. (C): Schott bottle with sterile purified water for rinsing purposes. (D): Schott bottle with 20% (v/v) Clorox. (E): Universal bottles with sterile purified water. (F): Sterile Petri plate and sterile filter paper for trimming purposes. (G): MS medium supplemented with 3% (v/v) sucrose, 0.25% (v/v) Phytagel™, 2.0 mg/l of BAP and 0.5 mg/l of IAA. (H): Sterile scalpel and forcep. (I): Glass bead sterilizer.

An Esco Airstream Horizontal Laminar Flow Cabinet (ESCO®, USA) with Ultra Low Penetration Air (ULPA) filter was used, to ensure all sterilized materials remained free from contamination. The transfer area in laminar flow cabinet was cleaned with 70% (v/v) ethanol, then sterilized by exposing to germicidal ultraviolet (UV) light for 30 minutes prior to usage (Sathyanarayana and Varghese, 2007). All necessary tools such as forceps, scalpels and disposable blades were also sterilized regularly using glass bead sterilizers (Inotech Biosystems International, USA) for 30 seconds, where the temperature reaches 200°C to 250°C (Figure 3.2).

3.2.2.2 Preparation of plant growth regulators

Auxins and cytokinins were the two major plant growth regulators used in different concentrations and combinations along with MS medium for the induction and growth of shoots, calluses, somatic embryos and roots. The requirement for these substances varied considerably with the tissues and their endogenous levels. The ratio of auxins and cytokinins was important with respect to morphogenesis in the culture system (Purohit, 2003).

A) Auxins

The auxins used in this study were IAA, NAA and 2,4-D (Sigma-Aldrich, USA), to promote cell enlargement, callus and root induction. One hundred milligrams of auxin were dissolved in diluted sodium hydroxide (NaOH) (2-5 ml of 0.5 M NaOH in a total volume of 100 ml), and made up to 100 ml with purified water. Gentle heating was required to completely dissolve the crystals. Auxin solution was then sterilized using 0.2 µm syringe filter (Minisart, Sartorius) and stored at 4°C as a stock solution for further use.

B) Cytokinins

In order to promote cell division, axillary bud proliferation and shoot multiplication by means of micropropagation, the cytokinins used in this research project were BAP (Sigma-Aldrich, USA) and kinetin (Duchefa, Netherlands). The preparation of BAP and kinetin was similar to auxin preparation, as mentioned above in Section A.

3.2.2.3 Preparation of media

MS medium supplemented with vitamins, organic salts and inorganic salts (shown in Appendix A) was used for the establishment of aseptic cultures, axillary shoot, callus and somatic embryo induction, adventitious shoot multiplication and root induction. MS medium was supplemented with 3.0% (w/v) sucrose (Duchefa, Netherlands), 0.25% (w/v) Phytagel™ (Sigma-Aldrich, USA) and different concentrations of plant growth regulators.

MS media preparation was done by dissolving 4.42 g of MS powder (Duchefa, Netherlands), followed by 30 g of sucrose in 800 ml of purified water. The solution was mixed thoroughly and heated gently on a hot plate until fully dissolved. The pH of media was adjusted to 5.8±0.1 with diluted NaOH or HCl prior to autoclaving. A total of 2.5 g Phytagel™ were added and completely dissolved in the microwave oven. Purified water was added to bring the volume to 1000 ml. The culture medium was then autoclaved at 121^{o}C with 15 psi for 15 minutes.

Filtered auxins and cytokinins were added aseptically to autoclaved culture medium depending on the experimental objectives, and allowed it to cool to almost 35^{o}C to 45^{o}C. Thirty microliters of MS medium supplemented with plant growth regulators were then dispensed aseptically into sterile culture jars in the laminar flow cabinet before the solidification of Phytagel™.

3.2.3 Establishment of aseptic cultures

Leaf, internodal and nodal segments of sweet shoot were used as explants for the establishment of aseptic cultures. Juvenile explants, 1.5 cm to 2.0 cm in length, were washed thoroughly under running tap water for five minutes. Explants were prewashed with 70% (v/v) ethanol for one minute, followed by three rinses with sterile purified water to remove all traces of ethanol. All explants were then surface sterilized in 20% (v/v) Clorox with a drop of Tween 20 emulsifier for 20 minutes (Wee *et al*., 2010a). Explants were then agitated for a few minutes while disinfecting, in order to allow complete exposure of explants to disinfectants. Disinfected explants were rinsed three times with sterile purified water and transferred onto sterile filter paper to remove excess moisture present on the explants surface.

After surface sterilization of explants, explants were subjected to final trimming using sterile scalpels and forceps to remove bleach affected areas. The trimmed explant was no larger than 1 cm cubed and cultured on MS medium supplemented with 3% (w/v) sucrose and 0.25% (w/v) Phytagel™. Each culture jar was sealed with parafilm before incubating at 26±2^{o}C under 16-hour light photoperiod, provided by cool white fluorescent light at a photosynthetic flux of 40 to 50 μmolm^{-2}s^{-1}.

The axenic status of cultured tissues was confirmed by using nutrient broth culture assay. Samples of tissues were cultured in nutrient broth for five to seven days to detect the presence of microbial contaminants (Wee *et al*., 2010b). Data on contamination, mortality and survival percentage were recorded after four weeks from culture initiation.

3.2.4 Effects of plant growth regulators and cultivation systems on shoot proliferation and multiplication

3.2.4.1 Semi-solid medium cultivation system

Juvenile leaf, internodal and nodal explants were cultured on MS medium supplemented with IAA, NAA or 2,4-D (0.0, 0.5, 1.0 and 2.0 mg/l) in combination with BAP or kinetin (0.0, 0.5, 1.0 and 2.0 mg/l) (Table 3.1) (Purohit, 2003). Three different combinations of plant growth regulators (IAA-BAP, NAA-kinetin, 2,4-D-kinetin) and a total of 48 growth regulator treatments were tested in this experiment. The culture medium was solidified with 0.25% (w/v) Phytagel™ and supplemented with 0.3% (w/v) sucrose.

All cultures were incubated at 26±2^{o}C under cool white fluorescent light with 16-hour light and 8-hour dark cycle. Adventitious shoots formed were subcultured onto fresh MS medium at the intervals of four weeks for shoot multiplication. The percentage of responded explants, shoot length and number of shoots formed per explant were recorded after eight weeks of culture.

Table 3.1. Combinations and concentrations of plant growth regulators used for shoot, callus and somatic embryo induction and proliferation.

Treatment	Plant growth regulators (mg/l)	
	Auxin (IAA, NAA or 2,4-D)	Cytokinin (BAP or Kinetin)
1	0.0	0.0
2	0.0	0.5
3	0.0	1.0
4	0.0	2.0
5	0.5	0.0
6	0.5	0.5
7	0.5	1.0
8	0.5	2.0
9	1.0	0.0
10	1.0	0.5
11	1.0	1.0
12	1.0	2.0
13	2.0	0.0
14	2.0	0.5
15	2.0	1.0
16	2.0	2.0

* Three different combinations of plant growth regulators were tested in this experiment, (1) IAA – BAP, (2) NAA – kinetin, and (3) 2,4-D – kinetin.

3.2.4.2 Liquid medium cultivation system

Nodal explants harboring an axillary bud were inoculated on liquid MS medium containing 3% (w/v) sucrose and various concentrations of auxin (IAA or NAA) and cytokinin (BAP or kinetin) ranging from 0.0-2.0 mg/l in 100 ml Erlenmeyer flasks (Table 3.1). Liquid cultures were maintained by adding 30 ml of fresh medium to old suspension at three week intervals. Cultures were agitated at 100 rpm on an orbital shaker (Tech-Lab, Malaysia) under cool white fluorescent light with a 16-hour light photoperiod. After 60 days, the percentage of responded explants, shoot length and number of shoots formed per explant was recorded.

3.2.5 Morphological characteristics

Observations were made every three weeks and the morphogenetic of shoot regeneration in cultures were observed using a Nikon® ECLIPSE 80i stereomicroscope (Nikon, USA) with 100x total magnification. Cultures with any potential physiological abnormalities were also recorded every four weeks.

3.2.6 *In vitro* root induction

For *in vitro* rooting, 3-5 cm of well proliferated shoots were implanted on half strength MS medium supplemented with 1.0 mg/l of IAA (Wee *et al*., 2010b). All media were supplemented with 3% (w/v) sucrose and solidified with 0.25% (w/v) Phytagel™. Cultures were maintained in the dark and the data on rooting were evaluated after 10 days.

3.2.7 Hardening and acclimatization of plantlets

Rooted shoots (5-6 cm in height) were harvested from the rooting media and washed thoroughly in running tap water to remove any residual agar. Rooted plantlets were soaked in a 0.2% (v/v) solution of the fungicide carbendazim for 5 minutes (Wee *et al*., 2010c). For hardening-off, the rooted plantlets were transferred to culture jars containing purified water and maintained at ambient conditions for one month. The plantlets were then transplanted to pots containing perlite:compost mixture (1:1) for establishment *ex vitro*. High humidity was maintained in the shadehouse with 70% shading and intermittent mist during the first four weeks of acclimatization. The survival percentage of plantlets was recorded after one month of acclimatization.

3.2.8 Statistical analysis

The experiments were conducted in a completely randomized block design with 12 replicates of five cultures for each treatment. Each experiment was repeated twice. Data in percentage were transformed using logistic regression analysis, in order to satisfy the assumptions of general linear models. All data were also subjected to analysis of variance (ANOVA) using Statistical Package for Social Sciences (SPSS) software version 16. Duncan's multiple range tests (DMRT) with significant difference level at $p<0.05$ was then used to analyze the differences between the treatments.

3.3 RESULTS AND DISCUSSION

3.3.1 Establishment of aseptic cultures

The initial stage of micropropagation is to establish aseptic cultures of sweet shoot using a surface sterilization method developed by Wee *et al.* (2010a). A two step surface sterilization approach using 70% (v/v) ethanol

for 1 minute, followed by a final treatment with 20% (v/v) Clorox for 20 minutes was used to reduce the incidence of microbial contamination to around 17% in all tested explants (Table 3.2). The contamination percentage of internodal (17.09%) and nodal (16.57%) explants was greater than leaf (6.25%) explants. One possible reason could be due to the poor contact of nodal explants with disinfecting agents. During trimming of stem explants prior to surface sterilization, there is transient exposure of intercellular spaces and vessel cavities of the cut ends to contaminants (Thakur and Sood, 2006). These contaminants remained inside the tissues and escaped from the surface sterilization treatments, which resulted in the occurrence of contamination in *in vitro* cultures. Thus, the use of juvenile explants is one of the alternative methods in reducing the risk of contamination, since it has a shorter exposure period to the environment.

Table 3.2. Effects of established surface sterilization method on the incidence of contamination, mortality (browning and bleaching) and survival (aseptic) of three different types of explants, one month after surface sterilization.

Types of explants	**Contamination percentage (%)**	**Mortality percentage (%)**	**Survival percentage (%)**
Leaf explant	6.25 ± 3.41	2.92 ± 3.11	90.83 ± 2.88
Internodal explant	17.09 ± 3.77	2.50 ± 2.61	80.41 ± 3.96
Nodal explant	16.57 ± 3.26	1.25 ± 2.26	82.08 ± 2.57

* Values expressed as means with standard deviations.
* Each mean was based on 12 replicates, each of which consists of 5 cultures.

In addition, low mortality (<2.92%) and high survival (>80%) percentage of cultured explants were obtained after one month of surface sterilization (Table 4.3). Prolonged treatment duration of powerful disinfectants such as ethanol and mercuric chloride has been proven to be phytotoxic, which resulted in necrosis and death of tissues (Oyebanji *et al*., 2009). In this experiment, juvenile leaf explants (2.92%) showed a higher percentage of phytotoxicity compared to stem explants. The thin cuticle layer of leaf explant may not be able to withstand the strong concentration of disinfectant and those trimmed edges may also serve as a site for the deep penetration of disinfectant into tissues, causing toxic effects and resulting in reduced growth responses (Thakur and Sood, 2006). This phenomenon has been observed in sugarcane (Moutia and Dookun, 1999), *Carissa carandas*

(Rai and Misra, 2005) and *Andrographis paniculata* (Haripriya and Kannan, 2008).

Qualitative nutrient broth assay was carried out to detect the presence of microbial contamination in tissue cultures of sweet shoot. Microbial contamination was observed in the nutrient broth assay and on culture medium 3-21 days after surface sterilization of explants. Bacterial and fungal pathogens generally produced visible growth on the culture media and can be easily identified based on the morphological characteristic such as colony formation, shape, colour and growth (Figure 3.3). Fungal pathogens grown in this study rapidly colonized the culture media and resulted in increased tissue mortality. The results obtained were in line with those obtained by Obuekwe and Osagie (1989), Omamor *et al.* (2007) and Wee *et al*. (2010a).

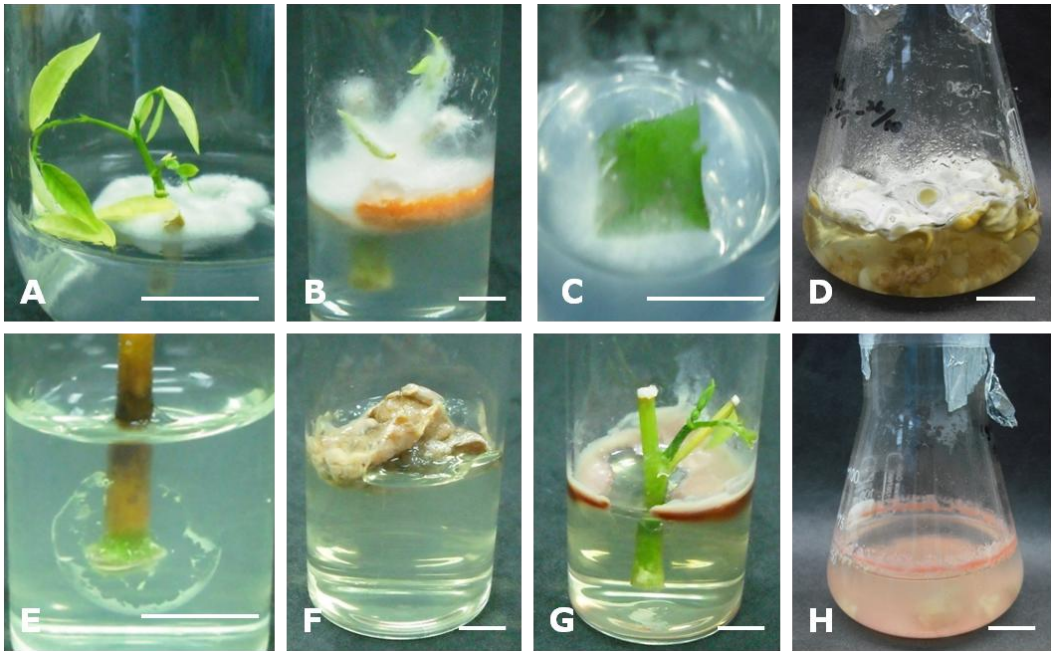


Figure 3.3. Fungal and bacterial contamination of sweet shoot explants. (A): Fungal contamination of propagated plantlets. (B): Fungal contamination of nodal explants after two weeks of culture initiation. (C): Leaf explants contaminated by fungal after 3 days of culture. (D): Fungal contamination of cell suspension cultures, 14 days after callus inoculation. (E): Bacterial contamination observed at the base of internodal explants after 2 weeks of culture. (F): Bacterial infection caused browning of leaf explants. (G): Bacterial contamination caused poor plant regeneration ability of nodal explants. (H): Bacterial contamination was observed in cell suspension cultures, after 14 weeks of callus inoculation. (Bar = 1 cm).

Explant taken from *in vitro* cultures could also be a source of contamination (Omamor *et al*., 2007) (Figure 3.3). Occasionally, some bacteria were not detected by visual examination at the initial or later stage of cultures. This may be due to the lateral infection of pathogens from root to shoot system via xylem vessels, thus *in vitro* cultures are likely to be contaminated after several subcultures. A prudent selection of explant materials coupled with an effective surface sterilization method will definitely help to reduce the incidence of contamination to a minimum level and to maintain a high survival percentage of explant.

3.3.2 Effects of plant growth regulators and semi-solid medium cultivation system on shoot proliferation and multiplication

To maximize the shoot proliferation capacity of sweet shoot, three different types of explants were cultured onto MS basal medium supplemented with different combinations (BAP-IAA, NAA-kinetin and 2,4-D-kinetin) and concentrations (0, 0.5, 1.0 and 2.0 mg/l) of plant growth regulators. Of the different treatments tested, shoot induction using MS medium enriched with BAP and IAA (Table 3.3) was superior than the usage of other hormone combinations (Appendix Table 3 and 4), as it significantly increased shooting response, had more number and longer shoots from the leaf and stem explants. Results obtained in this study support the findings from Meszaros *et al*. (1999) in *Melissa officinalis,* Tang *et al*. (2002) in sweet cherry and Nagesh (2008) in *Curculigo orchioides*, where the usage of BAP is more effective than kinetin in enhancing the multiplication of microshoots.

Table 3.3. The influence of IAA and BAP hormone treatments on shoot regeneration capacity, shoot length and number of shoot for leaf, internodal and nodal explants cultured on semi-solid medium, after 60 days of culture.

Treatment code	Growth regulators (mg/l)		Types of explants								
			Leaf explants			Internodal explants			Nodal explants		
	BAP	IAA	% of responded explant	Number of shoot per explant	Longest shoot length (cm)	% of responded explant	Number of shoot per explant	Longest shoot length (cm)	% of responded explant	Number of shoot per explant	Longest shoot length (cm)
T0	0.0	0.0	0.00 $_{e}$	0.00 $_{g}$	0.00 $_{g}$	0.00 $_{e}$	0.00 $_{h}$	0.00 $_{h}$	0.00 $_{d}$	0.00 $_{i}$	0.00 $_{k}$
T1	0.0	0.5	88.33 $_{b}$	3.49 $_{ef}$	2.67 $_{d}$	68.33 $_{bc}$	2.57 $_{ef}$	1.50 $_{f}$	81.67 $_{c}$	4.53 $_{ef}$	3.32 $_{fg}$
T2	0.0	1.0	90.00 $_{ab}$	3.40 $_{ef}$	2.47 $_{e}$	63.33 $_{cd}$	2.45 $_{f}$	1.42 $_{fg}$	85.00 $_{b}$	3.64 $_{g}$	2.48 $_{ij}$
T3	0.0	2.0	83.33 $_{d}$	2.70 $_{f}$	2.04 $_{f}$	56.67 $_{d}$	1.68 $_{g}$	1.06 $_{g}$	81.67 $_{c}$	2.66 $_{h}$	2.12 $_{j}$
T4	0.5	0.0	86.67 $_{bc}$	4.52 $_{cd}$	2.76 $_{d}$	73.33 $_{ab}$	3.53 $_{de}$	1.77 $_{ef}$	75.00 $_{c}$	5.17 $_{cd}$	3.00 $_{hi}$
T5	0.5	0.5	90.00 $_{ab}$	5.33 $_{b}$	3.57 $_{bc}$	73.33 $_{ab}$	4.43 $_{bc}$	2.59 $_{d}$	85.00 $_{b}$	5.43 $_{bcd}$	3.72 $_{ef}$
T6	0.5	1.0	91.67 $_{ab}$	3.90 $_{e}$	3.08 $_{cd}$	68.33 $_{bc}$	2.92 $_{ef}$	2.04 $_{e}$	88.33 $_{b}$	4.75 $_{cde}$	3.09 $_{gh}$
T7	0.5	2.0	83.33 $_{cd}$	4.00 $_{de}$	2.55 $_{e}$	58.33 $_{d}$	3.08 $_{ef}$	1.51 $_{f}$	83.33 $_{bc}$	4.43 $_{ef}$	2.67 $_{i}$
T8	1.0	0.0	91.67 $_{ab}$	5.37 $_{b}$	3.64 $_{b}$	76.67 $_{a}$	4.55 $_{ab}$	2.61 $_{cd}$	85.00 $_{b}$	5.68 $_{abc}$	4.08 $_{cd}$
T9	1.0	0.5	95.00 $_{ab}$	5.69 $_{a}$	3.20 $_{c}$	78.33 $_{ab}$	4.70 $_{ab}$	3.00 $_{bc}$	91.67 $_{ab}$	5.86 $_{a}$	4.39 $_{bc}$
T10	1.0	1.0	96.67 $_{a}$	4.88 $_{c}$	4.57 $_{a}$	75.00 $_{a}$	3.92 $_{c}$	3.31 $_{b}$	91.67 $_{ab}$	5.59 $_{ab}$	4.58 $_{b}$
T11	1.0	2.0	83.33 $_{cd}$	4.64 $_{cd}$	2.68 $_{d}$	63.33 $_{cd}$	3.63 $_{c}$	1.67 $_{ef}$	83.33 $_{bc}$	4.79 $_{e}$	2.71 $_{i}$
T12	2.0	0.0	88.33 $_{b}$	5.86 $_{a}$	3.13 $_{cd}$	71.67 $_{b}$	4.97 $_{a}$	2.10 $_{e}$	81.67 $_{c}$	5.72 $_{a}$	3.54 $_{fg}$
T13	2.0	0.5	93.33 $_{a}$	5.67 $_{a}$	4.94 $_{a}$	83.33 $_{a}$	4.71 $_{ab}$	3.90 $_{a}$	98.33 $_{a}$	6.74 $_{a}$	5.74 $_{a}$
T14	2.0	1.0	93.33 $_{a}$	4.60 $_{cd}$	4.56 $_{a}$	71.67 $_{b}$	3.70 $_{c}$	3.69 $_{b}$	91.67 $_{ab}$	4.43 $_{ef}$	3.97 $_{de}$
T15	2.0	2.0	81.67 $_{cd}$	4.10 $_{d}$	2.63 $_{de}$	58.33 $_{d}$	3.14 $_{ef}$	1.55 $_{f}$	81.67 $_{c}$	4.43 $_{ef}$	2.71 $_{i}$

* Values expressed as means of 12 replicated experiments, each replicate consisted of 5 cultures.

* Different letters within column (lowercase) indicate a significant difference ($p<0.05$) according to Duncan's multiple range test.

The analysis of variance showed significant effects of hormonal treatments (combination of BAP and IAA) on the production of axillary and adventitious shoots generated from leaf and stem explants ($p<0.05$) (Table 3.3). In the presence of auxin, axillary and adventitious shoot formation were strongly influenced by both increased cytokinin concentration and the types of explants ($p<0.05$). All explants were found to be responsive towards the tested hormonal treatments regardless of different combinations, except for the control treatment, whereby no response was recorded in the control explants (Table 3.3). MS medium supplemented with 2.0 mg/l BAP and 0.5 mg/l IAA was noted to be effective in inducing shoot proliferation of sweet shoot, where 98.33% of nodal explants were able to produce axillary and adventitious shoots, followed by leaf and internodal explants (Table 3.3). Overall, the percentage of responsive explants was greater in nodal explants (75.00% to 98.33%), followed by leaf (83.00% to 93.33%) and internodal (56.67% to 83.33%) explants.

In this study, the number of shoots responded well in a linear manner to increasing level of cytokinin. As the BAP concentration increased from 0.5 mg/l to 2.0 mg/l, the number of shoots increased significantly in all the tested explants ($p<0.05$) (Table 3.3). The maximum number of shoots (6.74 shoots per explant) was obtained from nodal explants cultured on MS medium supplemented with 2.0 mg/l BAP and 0.5 mg/l IAA, followed by leaf (5.67 shoots per explant) and internodal (4.71 shoots per explant) explants (Table 3.3). At lower concentrations of BAP (0.5 mg/l), the leaf and stem explants produced significantly fewer number of shoots ($p<0.05$). These results were comparable with previous reports for *Gardenia jasminoides* (George *et al*., 1993), *Plumbago* species (Das and Rout, 2002) and *Spilanthes acmella* (Saritha and Naidu, 2008), where high concentrations of BAP enhanced the number of shoots.

The effect of hormonal treatments on the length of adventitious shoot was found to be dependent on both the cytokinin concentration and the types of explants ($p<0.05$). The average shoot length in high BAP treatments (2.0 mg/l BAP) exhibited smaller shoots, which was in the range of 1.55 cm to 2.71 cm (Table 3.3). The combination of 2.0 mg/l BAP and 0.5 mg/l IAA

produced the longest shoots with a mean greater than 5.74 cm in nodal explants, followed by a mean shoot length of 4.94 cm in leaf explants and 3.90 cm in internodal explants. In other words, the length of shoot decreased as the concentration of cytokinin increased, which supports the findings from *in vitro* culture of *Prunus avium* (Purohit, 2003) and *Hoslundia opposite* (Prakash and Staden, 2007).

Malik *et al*. (2005), Naik and Chand (2011) suggested the advantage of BAP for shoot induction may be due to the ability of plant tissues to metabolise BAP more readily, and also the ability of BAP to induce the production of natural hormones, such as zeatin within the tissue. Moreoever, BAP is not easily metabolized and thus, persists in the medium in free or ionised forms (Buah *et al*., 2010). Kadota and Niimi (2003) also observed the importance of BAP in stimulating shoot formation whereby the addition of 0.25 mg/l BAP in MS medium significantly enhanced the shoot multiplication in pear cultivars.

3.3.2.1 Morphological observation of adventitious shoot cultivated on semi-solid medium cultivation system

The combination of 2.0 mg/l BAP and 0.5 mg/l IAA has successfully produced callus and adventitious shoots from the leaf and stem explant within 60 days of culture (Figure 4.4). Adventitious shoot regeneration from leaf and stem explants occurred via direct and indirect shoot regenerative pathway. After three days of culture initiation, the axillary buds on nodal explants had enlarged and subsequently proliferated into axillary shoots. Hard globular callus was formed at the basal end of nodal explants within 30 days of culture. The formation of shoot buds on callus which developed into adventitious shoots was visible after 60 days from culture initiation. Subculture of these shoots resulted in the development of more shoots without any apparent decline in vigour or reduction in proliferation over a period of 3 months. When a complete organ was cut into pieces, the various segments differed in their morphogenic capability. This might be due to the presence of endogenous and exogenous plant growth regulators responsible for the regeneration ability of different types of explants (Tang *et al*., 2002).

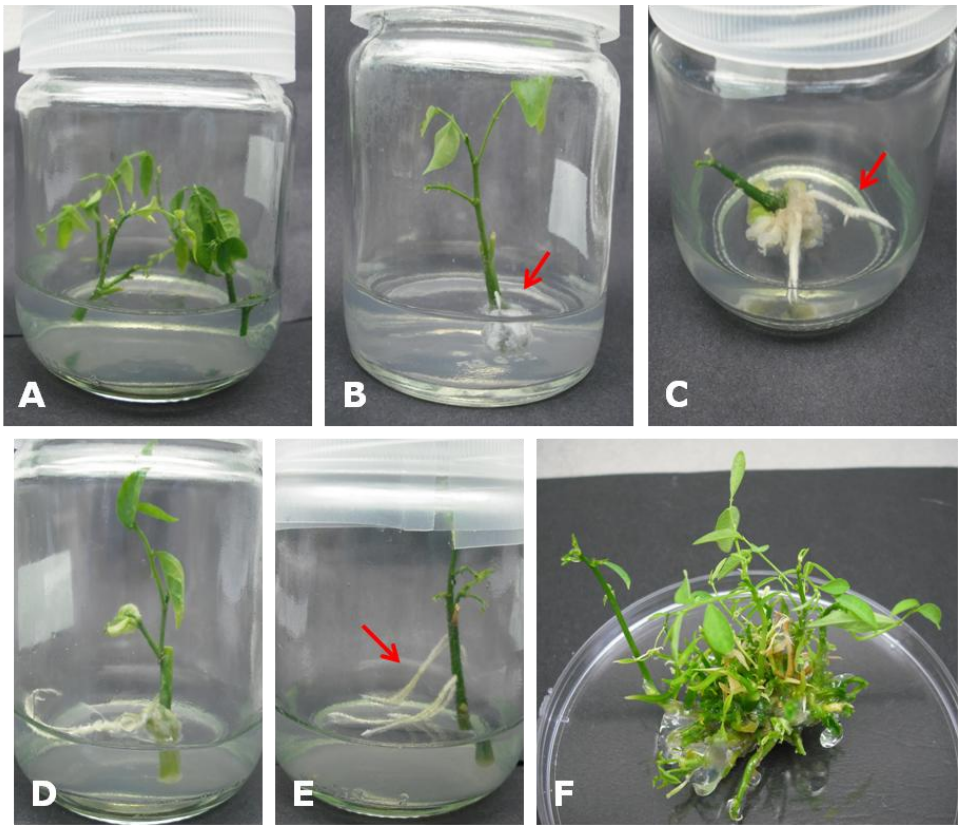


Figure 3.4. *In vitro* shoot regeneration of sweet shoot from nodal explants. (A): Shoot regeneration from nodal explants after 21 days of culture on MS medium supplemented with 2.0 mg/l BAP and 0.5 mg/l IAA. (B): Regeneration of callus observed at the base of nodal explants cultured on MS medium with high concentration of auxins. (C, D): Callus mass accompanied by a few roots obtained on MS medium containing 1.0 mg/l of auxins, after 45 days of culture. (E): Stunted growth of nodal explants with long roots observed. (F): Development of adventitious shoots (6.7 shoots per explant) after 60 days of culture on MS medium enriched with 2.0 mg/l BAP and 0.5 mg/l IAA (Bar = 1 cm).

3.3.3 Effects of plant growth regulators and liquid medium cultivation system on shoot proliferation and multiplication

In the present study, nodal segments were used as an explant to determine the shoot regeneration capacity via liquid cultures, since nodal explants (98.33% shoot formation, 6.74 shoots per explant and 5.74 cm shoot length) exhibited higher shooting responses in semi-solid MS medium as compared to leaf (93.33% shoot formation, 5.67 shoots per explant and 4.94 cm shoot length) and internodal (83.33% shoot formation, 4.71 shoots per explant and 3.74 cm shoot length) explants. Lincy and Sasikumar (2010) reported that the rate of shoot bud regeneration varied with different

genotypes of ginger, where nodal explants produced the highest percentage of shoot development in contrast to conventional explants like rhizome buds.

In all the tested hormonal treatments, the data on shoot regeneration capacity, shoot length and number of shoot formed per explant revealed a minor increment in the liquid cultures, but it was not statistically significant when compared with the semi-solid medium cultivation system ($p<0.05$). Liquid MS medium containing IAA and BAP was more effective than NAA and kinetin, since it induced the highest shoot regeneration capacity in nodal explants, and all these differences were statistically significant at 0.05 level (Appendix Table 5). After 60 days of culture initiation, an average 98.33% of nodal explants exhibited high shoot regeneration efficiency after the application of 2.0 mg/l BAP and 0.5 mg/l IAA (Table 3.4). The enhancement of shoot proliferation may be due to whole explants being submerged in the liquid medium, thereby facilitating better uptake of nutrients and growth regulators compared to semi-solid medium (Piatczak *et al*., 2005). Liquid culture has also been reported to provide a more economically viable method for mass propagation of woody plant species (Hung *et al*., 2006).

Table 3.4. Effect of IAA and BAP hormonal treatment on shoot regeneration in liquid medium using nodal explants of sweet shoot cultured for 60 days.

Treatment code	Growth regulators (mg/l)		% of responded explant	Number of shoot per explant	Longest shoot length (cm)	% of root formation	Number of root per explant
	BAP	IAA					
T0	0.0	0.0	0.00 $_{c}$	0.00 $_{f}$	0.00 $_{g}$	0.00 $_{d}$	0.00 $_{d}$
T1	0.0	0.5	88.33 $_{b}$	5.46 $_{cd}$	3.90 $_{ef}$	13.33 $_{ab}$	5.33 $_{ab}$
T2	0.0	1.0	88.33 $_{b}$	4.17 $_{e}$	3.09 $_{f}$	18.33 $_{a}$	7.33 $_{a}$
T3	0.5	0.0	86.67 $_{b}$	6.01 $_{bc}$	3.52 $_{ef}$	0.00 $_{d}$	0.00 $_{d}$
T4	0.5	0.5	90.00 $_{ab}$	6.64 $_{b}$	4.38 $_{cd}$	10.00 $_{ab}$	3.92 $_{b}$
T5	0.5	1.0	91.67 $_{ab}$	5.39 $_{cd}$	4.32 $_{cd}$	13.33 $_{ab}$	5.25 $_{ab}$
T6	1.0	0.0	91.67 $_{ab}$	6.77 $_{b}$	4.53 $_{c}$	0.00 $_{d}$	0.00 $_{d}$
T7	1.0	0.5	93.33 $_{ab}$	7.55 $_{a}$	5.20 $_{bc}$	6.67 $_{bc}$	2.33 $_{c}$
T8	1.0	1.0	95.00 $_{a}$	6.08 $_{bc}$	5.48 $_{b}$	8.33 $_{bc}$	3.33 $_{bc}$
T9	2.0	0.0	86.67 $_{b}$	7.55 $_{a}$	4.00 $_{de}$	0.00 $_{d}$	0.00 $_{d}$
T10	2.0	0.5	98.33 $_{a}$	7.43 $_{ab}$	6.94 $_{a}$	5.00 $_{c}$	1.75 $_{c}$
T11	2.0	1.0	93.33 $_{ab}$	5.09 $_{d}$	4.16 $_{de}$	5.00 $_{c}$	2.00 $_{c}$

* Values expressed as means of 12 replicated experiments, each replicate consisted of 5 cultures.
* Different letters within column (lowercase) indicate a significant difference ($p<0.05$) according to Duncan's multiple range test.

The highest concentration of BAP at 2.0 mg/l was more stimulatory to shoot development than the lower concentrations (Table 3.4). According to Firoozabady and Gutterson (2003), the addition of BAP in MS medium was essential for the regeneration of plantlets from shoot apices of *Ananas comosus*. Nodal explants inoculated on hormone-free liquid MS medium showed no sign of shoot regeneration (Table 3.4). The successful application of liquid cultures has been described for several plant species such as *Swertia chirate* (Wawrosch *et al*., 1999)*, Curcuma longa* (Prathanturarug *et al.*, 2005) and *Anacardium occidentale* (Aliyu, 2005).

The hormonal composition of regenerative medium greatly influenced the number of shoot formed per explant in liquid medium. It was observed that liquid culture produced more number of shoots with longer shoot length than those obtained on semi-solid medium, as previously described by Zuraida *et al*. (2011) in *in vitro* culture of *Ananas comosus*. An average of seven shoots were formed per explant on the MS medium supplemented with 2.0 mg/l of BAP and 0.5 mg/l of IAA, whereas the lowest number of shoot

was observed (4.17 shoots per explant) on MS medium supplemented with 1.0 mg/l of IAA, after 60 days of culture. Higher numbers of adventitious shoots were induced with the increment in BAP concentration. These results were consistent with those obtained by Piatczak *et al.* (2005), Be and Debergh (2006), who studied the effectiveness of IAA and BAP on shoot multiplication of *Centaurium erythraes* and *Ananas comosus.*

The nature of growth regulators affects not only the number of shoots, but also the morphology of microshoots. Hormonal treatment of IAA and BAP (Table 3.4) resulted in significantly longer shoots than the combination of NAA and kinetin in liquid medium (Appendix Table 5). A marked increase in shoot length (6.94 cm) was also observed in liquid MS medium containing 2.0 mg/l of BAP and 0.5 mg/l of IAA, as compared with those obtained on semi-solid medium (5.74 cm). Medium supplemented with kinetin was found to inhibit shoot elongation, resulting in the production of compact shoot masses. The ineffectiveness of kinetin suggests that axillary and adventitious shoot production is limited by endogenous cytokinins. Therefore, liquid medium containing 2.0 mg/l of BAP and 0.5 mg/l of IAA could serve as an alternative to semi-solid medium cultivation for adventitious shoot regeneration.

3.3.3.1 Morphological observation of adventitious shoot cultivated on Liquid medium cultivation system

This study showed that sweet shoot can be propagated via liquid medium, with symptoms of hyperhydricity (<30.00%) (Figure 3.5). The higher growth rate in liquid culture may be due to the enhancement of nutrient uptake as there is greater surface area for absorption (Alvard *et al.*, 1993). Liquid medium was also documented to disperse the phenolic exudates from explants, consequently resulting in faster growth rate (Zuraida *et al*., 2011). However, Yoshida (2002) claimed that prolonged contact of explants into the medium may suppress adventitious shoot formation in soybean. This suggests that there are substantial variations that existed between different types of explants in their ability to form adventitious shoots.

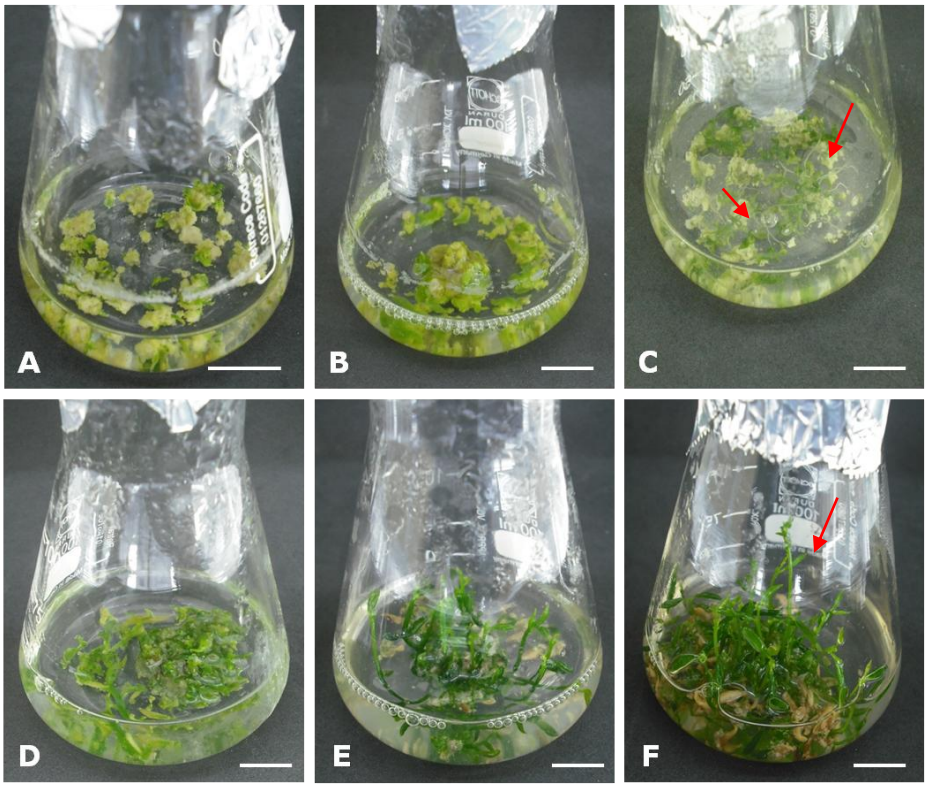


Figure 3.5. Various stages of *in vitro* plant regenera tion using nodal explants of sweet shoot. (A, B): Callus formation from nodal explants after 21 days of explant inoculation. (C): multiple root development from shoot and callus on MS medium containing 2.0 mg/l of BAP and 0.5 mg/l of IAA. (D, E. F): Proliferation of multiple shoots from node derived callus on MS medium supplemented with 2.0 mg/l of BAP and 0.5 mg/l IAA after 30 days of explant inoculation, with symptoms of hyperhydricity. (Bar = 1 cm).

In this study, nodal explants exhibited extraordinary high capacity to form densely branched and rooted shoot when cultured in liquid medium for 60 days. The shoot bud initiation from nodal explants was observed after 3 days of culture. Axillary shoot proliferation started after 7 days of culture on the MS medium supplemented with 2.0 mg/l of BAP and 0.5 mg/l of IAA. After 30 to 35 days, the shoot multiplied and the shoot length increased considerably. A relatively low percentage of callus formation was observed at the base of nodal explants used in liquid cultures. The presence of thin and fragile roots was obtained from the culture medium containing IAA and BAP after 40-50 days of culture initiation. Bhagyalakshmi and Sing (1988) have also reported that the usage of IAA was more effective than NAA for root regeneration in meristem culture of *Zingiber officinale*.

In liquid medium, a decrease of the coefficient of micropropagation was observed with the increasing number of subcultures. Four weeks of hormonal treatment led to the reduction of shoot size and percentage of responding explants. Thus, subculture at 3 week intervals is recommended to improve the regeneration efficiency with minimal malformed appearance, as previously reported in *Centaurium erythrace* (Piatczak *et al*., 2005) and *Phaseolus vulgaris* (Velcheva and Svetleva, 2005). In other words, the adoption of liquid media helped to reduce labour input and potentiates bioreactor dimensions, as it is easier to exchange media and inoculate.

3.3.4 Abnormalities caused by unsuitable hormonal treatment

3.3.4.1 Vitrification

In liquid medium, high concentration of BAP (2.0 mg/l) has also been found to induce vitrification in shoot cultures of sweet shoot (<30.00%). Anatomical, morphological and physiological anomalies in tissue cultured plants with vitrification have been described as translucency, hyperhydration, succulency and glassiness (Purohit, 2003). In this study, hyperhydric shoots produced short internodes due to the reduction of apical dominance (data not shown). Affected shoots were swollen, pale green and the leaves were translucent, watery or glass-like in appearance (Figure 3.6). The causes of this disorder were suspected to be the inappropriate osmotic potential of culture medium, high humidity, superfluous nutritional factors, high levels of growth regulators, low light intensity and ethylene accumulation in the air space of culture vessel (Debergh *et al*., 1981; Purohit, 2003; Sudhersan *et al*., 2003). This phenomenon has been frequently reported in many plant species such as *Picea abies* (Bornman and Vogelmann, 1984) and *Paeonia suffruticosa* (Bouza *et al*., 1994).

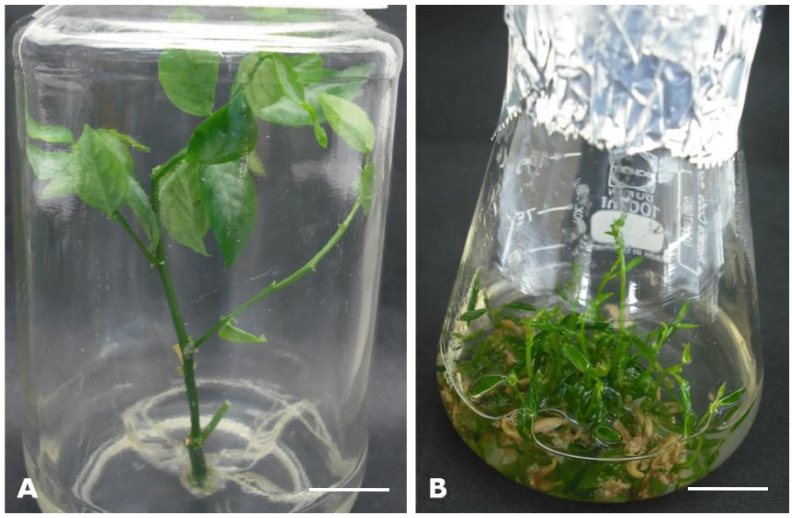


Figure 3.6. Comparison between shoot cultures generated in semi-solid MS medium and those generated in liquid MS medium. (A): Shoot cultures generated in semi-solid medium showed no visual evidence of abnormal morphological characteristics in sweet shoot. (B): Hyperhydricity shoots were obtained in liquid culture medium, with watery or glass-like in appearance.

A few methods can be proposed to reduce vitrification such as, 1) addition of gibberellic acid (Pereira *et al.*, 2000; Saritha and Naidu, 2008), 2) maximizing air exchange through the container closure, 3) adding phloroglucinol to the culture media (Sathyanarayana and Varghese, 2007), 4) addition of sucrose to help lower the water potential by increasing the agar concentration to 1% (Debergh *et al*., 1981), and 5) inoculating silver nitrate into culture media, as silver ions are known to act as ethylene synthesis inhibitors in plants (Sudhersan *et al*., 2003). In year 2003, Sudhersan *et al.* also published reports on *Nitraria retusa,* showing enhancement of plant growth via partial desiccation of organogenic callus with shoot buds, frequent subculturing, reduction of plant growth regulator concentration and addition of activated charcoal to the media.

3.3.4.2 Shoot abnormalities

Long term benefits of micropropagation lies in the production of clonally uniform plants. Somaclonal variation needs to be eliminated, in order to reduce any abnormalities in woody plant culture. Many scientists believe that high concentration of plant growth regulators and longer cultivation periods are the sources of variation in plantlets cultured *in vitro* (Chen *et al*., 1999; Chugh *et al*., 2009). Although the addition of BAP significantly

improved both multiplication rate and shoot elongation, it induced certain undesirable features such as axillary bud deformation, falling of leaves and leaf necrosis (Figure 3.7). In the present study, low percentage (<15.00%) of abnormal growth was observed in the stem explants cultured in MS medium supplemented with high level of cytokinins (data not shown).

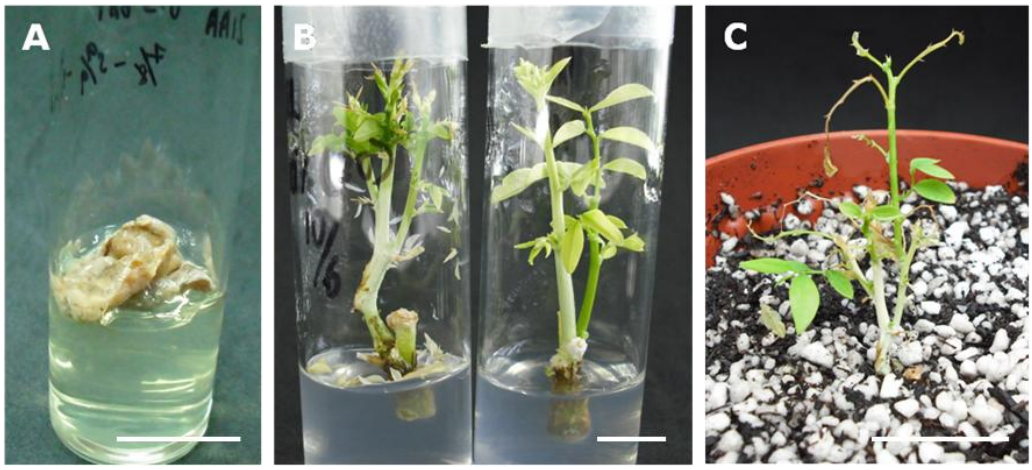


Figure 3.7. Morphological and physiological disorder of explants. (A): leaf necrosis. (B, C): Abnormal growth of axillary shoots from nodal explants. (Bar = 1 cm).

At equal concentrations of BAP (1.0 mg/l) and IAA (1.0 mg/l), it induced some morphological and physiological disorders (<15.00%). The new buds and stems were pale and white in colour (Figure 3.7). The presence of IAA together with BAP allowed the development of normal leaves in central shoots, but newly formed leaves of axillary shoots turned brown, became deformed and wilted. Furthermore, high concentration of BAP caused shoot apical and leaf necrosis at the end of the subculture. The remaining shoots usually die if subcultured onto the medium with same hormonal composition. This could be due to their mode of metabolism and site of action in the explants (Prakash and Staden, 2007).

3.3.5 *In vitro* root induction

For root induction of sweet shoot, 3-5 cm long shoots were transferred to half strength MS medium supplemented with 1.0 mg/l of IAA for root induction, as previously described by Wee *et al.* (2010a) in cultured shoots of sweet shoot. After 10 days of culture, roots were formed on 90% of shoots

on half strength MS rooting medium, with an average of 6.59 roots per shoot and an average root length of 6.13 cm. Similar result was observed for cultured shoots of *Vanilla planifolia* (Giridhar *et al*., 2001; Gopi *et al.,* 2006) and sweet shoot derived from leaf (Wee *et al*., 2010a), nodal (Wee *et al*., 2010b) and internodal (Wee *et al*., 2010c) explants. Strong root systems were obtained on the IAA rooting medium, where primary and lateral roots were produced from the root meristems, and a few shoot buds were formed on the root tips (data not shown).

In this study, a relatively low percentage (10%) of microshoots showed no sign of rooting. The possibility for this observation could be due to the carry-over effects of cytokinin treatments during the previous multiplication stage, as auxin and not cytokinin is required for rooting. This finding was supported by Purohit (2003) in root induction of woody plants species. Although IAA has been documented as a weak auxin for adventitious root formation as compared to NAA and 2,4-D, the superior effects of IAA in root induction of sweet shoot might be due to several factors, such as its preferential uptake, transport, and stability over IBA and NAA and subsequent gene activation. The beneficial effects of IAA on root development *in vitro* have also been noted previously for *Azadirachta indica* (Venkateswarlu *et al*., 1998), *Vitis thunbergii* (Lu, 2002) and *Hoslundia opposite* (Prakash and Staden, 2007). Therefore, not all auxins are equally effective for rooting purpose, and their response varies significantly with the plant used (Swamy *et al*., 2002).

3.3.6 Hardening and acclimatization of plantlets

Out of 60 rooted shoots transplanted *ex vitro*, survival of 85% was achieved after one month of transfer to the shadehouse (Figure 3.8). All acclimatized plantlets grown vigorously in compost. A development period of 4 months was required for the micropropagation of sweet shoot starting from surface sterilization stage to acclimatization stage. Similar result was reported by Saxena *et al.* (1997), in which 95% rooted plantlets of *Psoralea corylioflia* were successfully transferred to a 1:1 mixture of soil and sand. This high survival rate also concurred with studies performed by Fouche and

Jouve (1999) and Janarthanam and Seshadri (2008), whereby a survival rate of more than 80% was observed in tissue cultures of *Vanilla planifolia*.

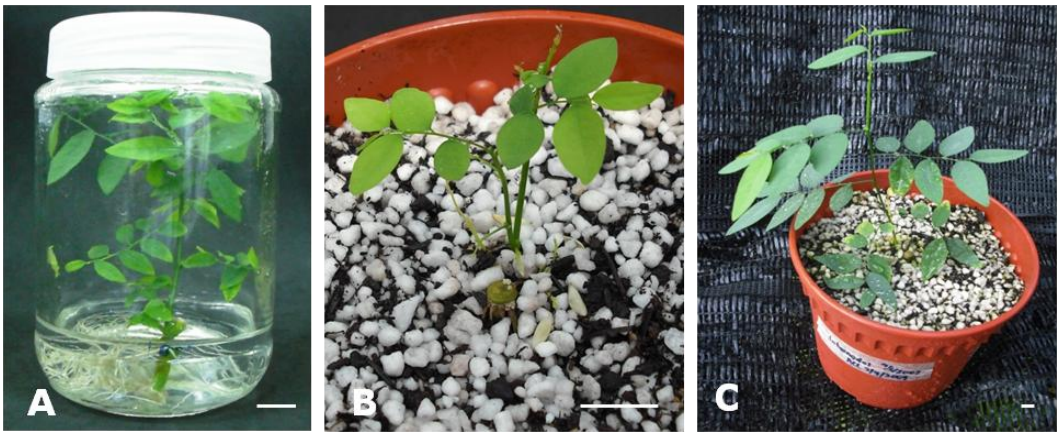


Figure 3.8. Hardening and acclimatization of sweet shoot. (A): Hardening of sweet shoot plantlets was done in the culture jar at ambient conditions. (B): Rooted plantlet of sweet shoot transferred to pot containing a mixture of perlite and compost (1:1) during acclimatization in the shade house. (C): Establishment of *in vitro* grown sweet shoot plantlets in pot after one month and two months, respectively. (Bar = 1 cm).

3.4 CONCLUSION

A high frequency of *in vitro* multiplication system for adventitious shoot was successfully established for sweet shoot via direct shoot regenerative pathway. Nodal explants of sweet shoot produced the highest number of shoots (6.74 shoots per explant) with a mean length of 5.74 cm when cultured on semi-solid MS medium containing 2.0 mg/l BAP and 0.5 mg/l IAA. Higher concentrations of BAP enhanced the number of shoots, whereas shoot length decreased as the concentration of cytokinin increased. Rooting of 90% of the microshoots was achieved with 1.0 mg/l IAA after 10 days of incubation with an average of 6.59 roots per shoot and average root length of 6.13 cm. Survival of 85% was achieved during the acclimatization of plantlets. Even though liquid MS medium with the same hormone composition was able to produce more shoots (7.43 shoots per explant) and longer shoots (6.94 cm), the microshoots cannot be used as a plant material for the antioxidant assays, as they have undergone vitrification which shorten their life span. Hence, the adventitious shoot obtained from semi-solid MS medium were adopted for usage in the subsequent experiments of antioxidant assays.

CHAPTER 4: INDIRECT ORGANOGENESIS OF *Sauropus androgynus*

4.1 INTRODUCTION

Organogenesis is an indispensable tool for plant regeneration using tissue culture technologies for plant transformation and for the extraction of bioactive metabolites, which is involved in *de novo* organ formation from differentiated plant tissues. Organogensis is a process whereby differentiated cells dedifferentiate to acquire pluripotency (Fan *et al*., 2012). Because callus has an unorganised structure and high regeneration capacity, it serves as an alternative method to seed propagation in agricultural, horticultural and forestry production system (Hartmann *et al*., 2002). One of the most successful examples were observed in aspen (*Populus tremuloides*) populations that can have over 1000 clonal offspring from callus cultures covering over 30 acres (Kemperman and Barnes, 1976). Plant regeneration via an intermediate callus phase was also reported in *Arabidopsis* (Valvekens *et al*., 1988), tobacco (Dhaliwal *et al*., 2003) and *Ulex europaeus* (Ramirez *et al*., 2012). The recent use of *Arabidopsis* as a model system for organogenesis has dramatically advanced our insight into the cell activity during the redifferentiation and dedifferentiation phases of organogenesis (Zhao *et al*., 2008b).

Since the classic experiments done by Skoog and Miller (1957), it has been recognized that the ratio of auxin to cytokinin drives morphogenesis toward either root or shoot organogenesis. The balance between auxin and cytokinin need to be established for organogenesis of sweet shoot, as it is a major driving force in initiating the differentiation pathway of explants (Ramirez-Parra *et al*., 2005). The movement and distribution of auxin has been shown to be important for cell attainment of organogenic competency (Vieten *et al*., 2007). The establishment of an auxin gradient within a tissue is critical for organ formation at the whole-plant level during callus, embryo and shoot formation (Trigiano and Gray, 2000). Impeding auxin distribution on culture medium was documented to disrupt callus formation (Benkova *et al*., 2003). For callus induction, the addition of cytokinin in culture medium is needed during the early stage of dedifferentiation, as reported by Gordon *et al.* (2007) in *Arabidopsis*, since it is the time of cell determination for shoot

formation. Thus, it is essential to determine the type and concentration of plant growth regulators, in order to induce the desired callusing responses from sweet shoot.

Light source is a very important physical factor for callus induction, cell growth and production of plant secondary metabolites. Light and dark conditions influence callus induction of woody plant species, as it affects the dedifferentiation process in callus morphology and metabolism. The slow growth of callus grown under light condition might be due to the depletion of auxin in culture medium, since the auxins are prone to breakdown under light condition (Dunlap and Robacker, 1988; Leuba *et al*., 1989). Light, in addition to its effect on *in vitro* growth and development, is also an important factor affecting the production of plant metabolites, including primary products such as enzymes, lipids and amino acids, and secondary products such as flavonoids, anthocyanins, terpenes and phenolics (How and Smith, 2003). Hence, the aim of the present study was to optimise the *in vitro* conditions for callus production from leaf and stem explants of sweet shoot.

4.2 MATERIALS AND METHODS

4.2.1 Plant materials

Juvenile leaf, internodal and nodal segments of sweet shoot, 1.5 cm to 2.0 cm in length, were surface sterilized according to the optimized protocol described in Section 3.2.4.

4.2.2 Effects of plant growth regulators and culture environment on callus induction and multiplication

The aseptic leaf, internodal and nodal explants were transplanted onto petri plates containing callus induction medium. This medium consisted of full strength MS medium supplemented with different concentration of IAA, NAA or 2,4-D (0.0, 0.5, 1.0 and 2.0 mg/l) in combination with BAP or kinetin (0.0, 0.5, 1.0 and 2.0 mg/l) (Table 3.1) (Purohit, 2003). Three different combinations of plant growth regulators (IAA-BAP, NAA-kinetin, 2,4-D-kinetin) and a total of 48 growth regulator treatments were tested for 60 days. The culture medium was supplemented with 3.0% (w/v) sucrose and 0.25% (w/v) Phytagel™.

Two batches of cultures were prepared in this experiment, in order to study the effect of culture environment on callus induction. First batch of cultures was maintained in culture room at 26±2°C under 16-hour light photoperiod, whereas another batch of cultures was incubated in the dark. Callus formed were subcultured onto same fresh MS medium every four weeks for mass multiplication of callus. Data on the percentage of callus formation, estimated callus fresh weight, callus expansion rate, percentage of browning tissue were recorded, 60 days after culture initiation.

After 60 days, callus from *in vitro* cultures was transferred onto a pre-weighed sterile petri plate and weighed using a weighing machine in the laminar flow cabinet. The fresh weight was recorded to two decimal points. Meanwhile, callus expansion rate was recorded every four weeks to check on the capacity of self expansion *in vitro*. Grid paper with cell size 0.5 cm x 0.5 cm was used to measure the expansion rate of callus. After placing the callus on the grid paper, the number of grid squares is calculated. Area of the callus is calculated according to the following formula:

$$\text{Callus expansion rate } (\text{cm}^2) = \text{Number of grid squares x Area of one grid square}$$

4.2.3 Shoot proliferation and multiplication

The 60-days-old light-induced callus was separated from the callus induction medium and transferred to MS medium containing 2.0 mg/l of BAP and 0.5 mg/l of IAA for shoot initiation and elongation, as previously mentioned in Sections 3.2.5.1 and 3.5. The elongated shoots were subcultured onto the same fresh medium every four weeks and maintained as stated in Section 3.2.4.1. The percentage of callus cultures forming shoots, number of shoots per callus, and shoot length were recorded after eight weeks of shoot induction, where the shoots achieved 3-5 cm in length.

4.2.4 Morphological characteristics

The morphogenesis of shoot and callus regeneration in cultures were determined using a Nikon® ECLIPSE 80i stereomicroscope (Nikon, USA) with 100x total magnification. Observations were made every three weeks on actively growing callus cultures and on callus cultures with potential physiological abnormalities.

4.2.5 *In vitro* root induction

Proliferated shoots, around 3-5 cm in height were transferred for root induction on half strength MS medium supplemented with 1.0 mg/l of IAA. The cultures were incubated as described in Section 3.2.6. The percentage of shoots with roots, number of roots per shoot and root length were recorded after 10 days of root induction, where the length of roots was 3.0 to 4.0 cm long.

4.2.6 Hardening and acclimatization of plantlets

Rooted shoots (5-6 cm in height) were harvested from the rooting medium and acclimatized in the shadehouse with optimal environmental conditions, as stated earlier in Section 3.2.7. The survival percentage of plantlets was recorded after one month of acclimatization in the shadehouse.

4.2.7 Statistical analysis

The experiments were conducted in a completely randomized block design with 12 replicates of 5 cultures for each treatment. Each experiment was repeated twice. Significance of the treatment effects was determined by analysis of variance (ANOVA) using Statistical Package for Social Sciences (SPSS) software version 16. Least significant differences among treatment means were estimated using Duncan's multiple range tests (DMRT) at the 5% level of significance.

4.3 RESULTS AND DISCUSSION

4.3.1 Effects of plant growth regulators on the multiplication of light-induced callus

The effects of photoperiod conditions and different combinations (IAA-BAP, NAA-kinetin and 2,4-D-kinetin) and concentrations (0.0, 0.5, 1.0 and 2.0 mg/l) of plant growth regulators on callus induction was optimized for a better understanding of callus performance and callus morphology. After 30 days of culture initiation, callus was formed on the cut surfaces of leaves and basal end of stem explants with high induction capacity. A significant interaction between auxins and cytokinins for callus growth was observed in this study ($p<0.05$). The interaction of NAA with kinetin was found to be effective in obtaining callus from all the tested explants (Table 4.1). Many

reports showed that NAA are more effective than 2,4-D in inducing callus for woody plant species, since it was the only auxin which easily penetrated into the plasma membrane without the need for active uptake (Nordstrom *et al*., 2004; Zhao *et al*., 2008a). The rapid increase of NAA level inside the cells stimulated cell expansion or elongation (Yin *et al*., 2008). In the presence of high auxin (1.0 and 2.0 mg/l) and low cytokinin (0.5 and 1.0 mg/l), root induction was also observed in the leaf and stem explants (Table 4.1). It is reasonable to assume that the level of endogenous auxins may be increased during the primary shoot development and contributed towards root initiation.

The analysis of variance showed significant effects of hormonal treatments on the incidence of callus formation from leaf and stem explants ($p<0.05$). Among the combinations tested, NAA was significantly more effective in enhancing callus regeneration capacity than 2,4-D and IAA (Appendix Table 6 and 7). MS medium supplemented with 2.0 mg/l NAA and 1.0 mg/l kinetin was the best medium for callus initiation, in which 71.67% of leaf explants produced callus, followed by internodal (30.00%) and nodal explants (25.00%), after 2 months of incubation under light condition (Table 4.1, Figure 4.1). The control explants did not promote callus formation due to the absence of growth regulators. No sign of callus formation was observed on the leaf and stem explants when cultured on MS medium containing kinetin alone, as it was too weak to facilitate the callus initiation, even after 90 days of cultivation. Thus, these findings suggest that the frequency of callus production increased with respect to different concentrations of plant growth regulators, and this increment was significant ($p<0.05$).

Table 4.1. Effect of NAA and kinetin on the incidence (%) of callus formation, callus fresh weight and callus expansion of the leaf and stem explants incubated under light condition, two months after culture initiation.

Treatment code	Growth regulators (mg/l)		Types of explants											
			Leaf explants				Internodal explants				Nodal explants			
	NAA	KIN	% of callus formation	Estimated callus fresh weight (g)	Callus expansion factor (cm^2)	% of Browning	% of callus formation	Estimated callus fresh weight (g)	Callus expansion factor (cm^2)	% of Browning	% of callus formation	Estimated callus fresh weight (g)	Callus expansion factor (cm^2)	% of Browning
T0	0.0	0.0	0.00 $_{e}$	0.00 $_{g}$	0.00 $_{g}$	100.00 $_{a}$	0.00 $_{d}$	0.00 $_{c}$	0.00 $_{e}$	0.00 $_{a}$	0.00 $_{e}$	0.00 $_{e}$	0.00 $_{f}$	0.00 $_{a}$
T1	0.0	0.5	0.00 $_{e}$	0.00 $_{g}$	0.00 $_{g}$	100.00 $_{a}$	0.00 $_{d}$	0.00 $_{c}$	0.00 $_{e}$	0.00 $_{a}$	0.00 $_{e}$	0.00 $_{e}$	0.00 $_{f}$	0.00 $_{a}$
T2	0.0	1.0	0.00 $_{e}$	0.00 $_{g}$	0.00 $_{g}$	100.00 $_{a}$	0.00 $_{d}$	0.00 $_{c}$	0.00 $_{e}$	0.00 $_{a}$	0.00 $_{e}$	0.00 $_{e}$	0.00 $_{f}$	0.00 $_{a}$
T3	0.0	2.0	0.00 $_{e}$	0.00 $_{g}$	0.00 $_{g}$	100.00 $_{a}$	0.00 $_{d}$	0.00 $_{c}$	0.00 $_{e}$	0.00 $_{a}$	0.00 $_{e}$	0.00 $_{e}$	0.00 $_{f}$	0.00 $_{a}$
T4	0.5	0.0	0.00 $_{e}$	0.00 $_{g}$	0.00 $_{g}$	0.00 $_{d}$	0.00 $_{d}$	0.00 $_{c}$	0.00 $_{e}$	0.00 $_{a}$	0.00 $_{e}$	0.00 $_{e}$	0.00 $_{f}$	0.00 $_{a}$
T5	0.5	0.5	50.00 $_{d}$	1.28 $_{f}$	5.09 $_{f}$	16.67 $_{bc}$	11.67 $_{c}$	0.35 $_{bc}$	1.90 $_{d}$	0.00 $_{a}$	6.67 $_{d}$	0.16 $_{de}$	0.83 $_{e}$	0.00 $_{a}$
T6	0.5	1.0	58.33 $_{c}$	2.26 $_{d}$	7.99 $_{d}$	13.33 $_{c}$	15.00 $_{c}$	0.45 $_{bc}$	2.28 $_{d}$	0.00 $_{a}$	10.00 $_{cd}$	0.30 $_{bcd}$	1.58 $_{c}$	0.00 $_{a}$
T7	0.5	2.0	56.67 $_{cd}$	2.03 $_{d}$	7.64 $_{d}$	13.33 $_{c}$	15.00 $_{c}$	0.38 $_{bc}$	2.12 $_{d}$	0.00 $_{a}$	10.00 $_{cd}$	0.26 $_{cde}$	1.42 $_{cd}$	0.00 $_{a}$
T8	1.0	0.0	53.33 $_{d}$	1.59 $_{e}$	5.87 $_{e}$	21.67 $_{b}$	13.33 $_{c}$	0.40 $_{bc}$	2.40 $_{d}$	0.00 $_{a}$	8.33 $_{d}$	0.22 $_{cde}$	1.17 $_{de}$	0.00 $_{a}$
T9	1.0	0.5	60.00 $_{bc}$	3.65 $_{c}$	11.60 $_{c}$	18.33 $_{b}$	18.33 $_{c}$	0.55 $_{b}$	2.97 $_{c}$	0.00 $_{a}$	15.00 $_{bc}$	0.46 $_{bcd}$	2.42 $_{bc}$	0.00 $_{a}$
T10	1.0	1.0	66.67 $_{ab}$	4.14 $_{ab}$	16.34 $_{ab}$	16.67 $_{bc}$	23.33 $_{b}$	0.70 $_{b}$	3.69 $_{bc}$	0.00 $_{a}$	18.33 $_{b}$	0.60 $_{bc}$	3.00 $_{b}$	0.00 $_{a}$
T11	1.0	2.0	63.33 $_{abc}$	3.99 $_{b}$	14.55 $_{bc}$	13.33 $_{c}$	20.00 $_{bc}$	0.58 $_{b}$	3.07 $_{c}$	0.00 $_{a}$	16.67 $_{bc}$	0.54 $_{bcd}$	2.75 $_{bc}$	0.00 $_{a}$
T12	2.0	0.0	56.67 $_{cd}$	1.98 $_{e}$	6.37 $_{de}$	18.33 $_{b}$	15.00 $_{c}$	0.45 $_{bc}$	2.67 $_{cd}$	0.00 $_{a}$	10.00 $_{cd}$	0.30 $_{bcd}$	1.67 $_{c}$	0.00 $_{a}$
T13	2.0	0.5	63.33 $_{abc}$	3.81 $_{bc}$	13.98 $_{c}$	16.67 $_{bc}$	26.67 $_{ab}$	0.73 $_{b}$	3.97 $_{b}$	0.00 $_{a}$	18.33 $_{b}$	0.66 $_{b}$	3.33 $_{ab}$	0.00 $_{a}$
T14	2.0	1.0	71.67 $_{a}$	4.53 $_{a}$	18.50 $_{a}$	16.67 $_{bc}$	30.00 $_{a}$	0.80 $_{a}$	4.54 $_{a}$	0.00 $_{a}$	25.00 $_{a}$	0.83 $_{a}$	4.08 $_{a}$	0.00 $_{a}$
T15	2.0	2.0	68.33 $_{ab}$	4.00 $_{b}$	15.10 $_{b}$	13.33 $_{c}$	23.33 $_{b}$	0.60 $_{b}$	3.14 $_{bc}$	0.00 $_{a}$	20.00 $_{ab}$	0.67 $_{b}$	3.42 $_{ab}$	0.00 $_{a}$

* Values expressed as means of 12 replicated experiments, each replicate consisted of 5 cultures.

* Different letters within column (lowercase) indicate a significant difference ($p<0.05$) according to Duncan's multiple range test.

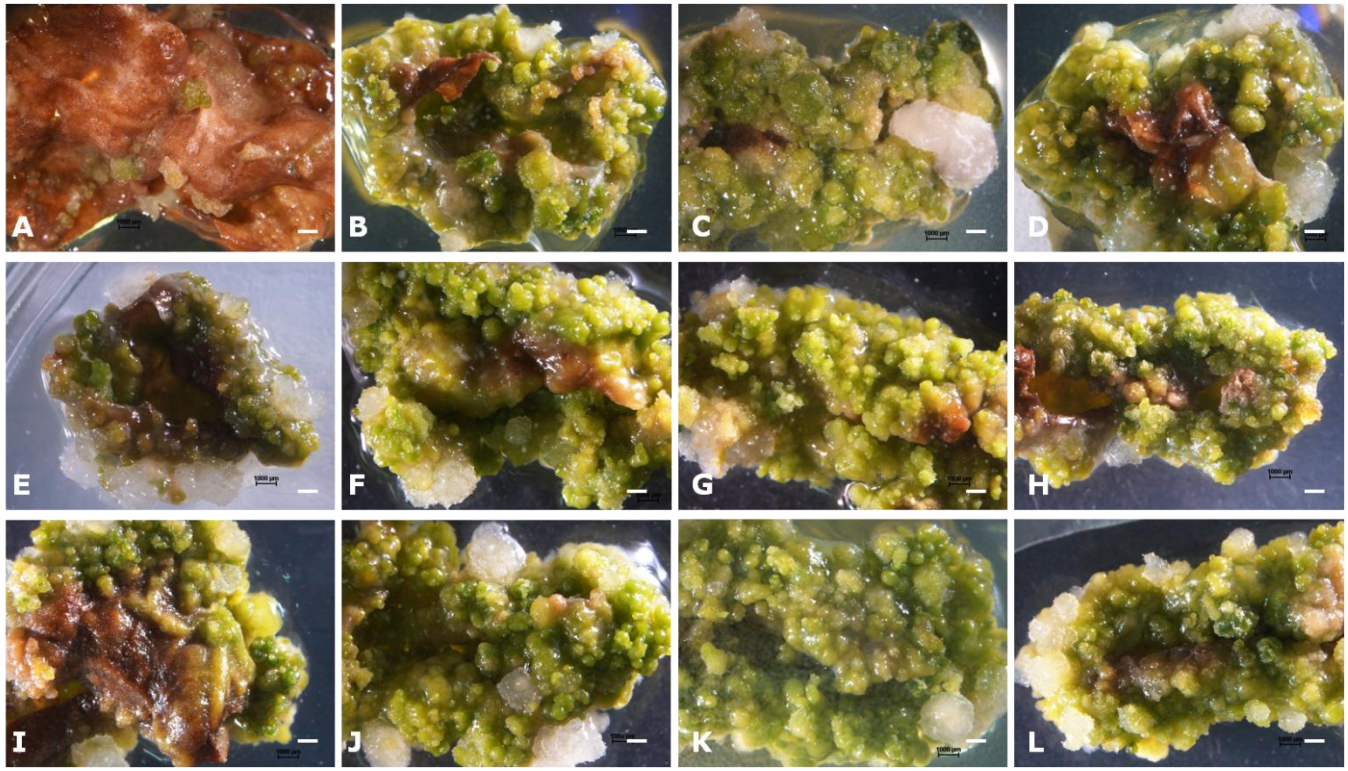


Figure 4.1. Callus and embryo-like structure formation from leaf explants incubated under the light condition on MS medium enriched with NAA and kinetin, 60 days after culture initiation. (A): Browning of leaf explants observed in medium with 0.5 mg/l NAA. (B): 0.5 mg/l NAA and 0.5 mg/l kinetin. (C): 0.5 mg/l NAA and 1.0 mg/l kinetin. (D): 0.5 mg/l NAA and 2.0 mg/l kinetin. (E): 1.0 mg/l NAA. (F): 1.0 mg/l NAA and 0.5 mg/l kinetin. (G): 1.0 mg/l NAA and 1.0 mg/l kinetin. (H): 1.0 mg/l NAA and 2.0 mg/l kinetin. (I): 2.0 mg/l NAA. (J): 2.0 mg/l NAA and 0.5 mg/l kinetin. (K): The highest percentage of callus formation, highest callus weight and highest callus expansion obtained in MS medium enriched with 2.0 mg/l NAA and 1.0 mg/l kinetin. (L): 2.0 mg/l NAA and 2.0 mg/l kinetin. (Bar = 1000 µm).

In this study, leaf explants had a higher callus induction capacity than stem explants, and the difference was significant ($p<0.05$) (Table 4.1). The midrib region of leaf explants exhibited more potential for callus formation, which was in agreement with the results reported by Kumar *et al*. (1992) and Bejoy *et al*. (2008). A relatively low (25.00%) percentage of callus production was obtained from nodal explants when cultured on MS medium supplemented with 2.0 mg/l of NAA and 1.0 mg/l of kinetin (Table 4.1). The variation of callus induction in different explants may be a result of the maturity of leaf and stem explants in response to different growth regulators (Ahmad *et al*., 2010). This is shown by a report indicating that 60% of the juvenile orchid leaf explants responded well with callusing, whereas only 35% of nodal orchid explants formed callus when cultured on MS basal medium containing optimum concentration of 2,4-D and kinetin (Janarthanam and Seshadri, 2008). There are also several reports on callus induction in woody plant species, such as *Gardenia jasminoides* (Al-Juboory *et al*., 1998) and *Rubus* species (Jin *et al*., 1992).

The analysis of callus cultures is usually based on the fresh weight and callus expansion measurements, as it is a quick, easy and non-destructive method for assessing callus growth. There were large differences in terms of the callus fresh weight and callus expansion rate between explants and treatments even after all repetitions done ($p<0.05$). After 60 days of culture initiation, the use of plant growth regulators at 2.0 mg/l NAA and 1.0 mg/l kinetin was recommended for callus induction, as it promoted the highest callus fresh weight (4.53 g) and highest callus expansion rate (18.50 cm^2) in leaf explants (Table 4.1); whereas the results in 2,4-D and kinetin enriched medium ranged only between 0.75 g and 3.95 g of callus fresh weight, and in IAA and BAP enriched medium ranged from 0.38 g to 2.19 g of callus fresh weight (Appendix Table 6 and 7). In other words, the fresh callus weight of all tested explants significantly increased in respect to higher auxin hormonal treatment ($p<0.05$).

Leaf explants formed large portions of calluses, but only a few shoots were observed from these light-induced calluses. When the callus induction percentage is high, the number of shoots per explant reduced, however, other

explants with low callus induction percentage gave rise to more shoots. IAA and 2,4-D appeared to be an unsuitable medium, as high percentage of browning was observed in the leaf explants cultured in this medium (Figure 4.2) (data not shown). One of the many possible reasons may be due to the phytotoxicity of 2,4-D in high concentrations, since many synthetic auxins such as 2,4-D, dicamba and picloram were used commercially as selective herbicides (George and Ravishankar, 1997). Fonnesbech (1972) reported that the use of 2,4-D alone resulted in abnormal growth and disturbed chlorophyll synthesis, whereas NAA is claimed to be less inhibitory to chlorophyll formation than 2,4-D. Besides that, the exudation of phenolic compounds from sweet shoot cells into the medium may also exhibit toxic effect to the callus cells when oxidised (Chugh *et al*., 2009). Palacio *et al*. (2012) found that the phenolic compound production significantly increased when the callus of *Larrea divaricata* underwent organogenesis, during the stage of adventitious shoot development. Hence, it is important to look for a medium that reduces the release of phenolic compounds and can produce maximum number of callus in sweet shoot.

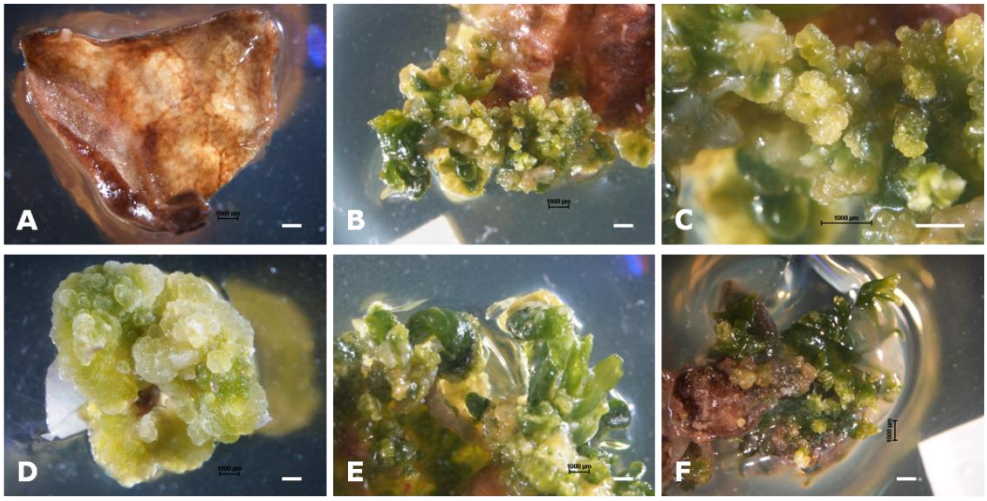


Figure 4.2. Callus formation from leaf explants incubated under the light condition on MS medium supplemented with IAA and BAP. (A): In hormone-free MS medium, leaf explants found to have a higher percentage of browning. (B, C): Direct shoot regeneration from leaf explant on MS medium supplemented with 2.0 mg/l BAP and 0.5 mg/l IAA. (D): Yellow-green friable callus formed on MS medium containing 2.0 mg/l IAA and 0.5 mg/l BAP. (E, F): Observation of leaf like structures after 45 days of culture on 2.0 mg/l BAP and 0.5 mg/l IAA. (Bar = 1000 µm).

4.3.1.1 Morphological observation of light-induced callus cultures

Light-induced callus was subcultured and maintained on the same medium over a period of time for mass propagation of callus. A two-fold ratio of callus proliferation was achieved at every subculture. Two different types of callus were observed on the callus induction medium, namely green friable callus and dark green hard globular callus. Green friable callus was found to maintain its initial growth potential, whereas the hard globular callus showed slow response to the medium. In this study, low percentage of embryo-like structures was produced from the hard globular light-induced callus cultures (Figure 4.3). The embryo-like structures were further developed into organized small bipolar structures which resemble zygotic embryos. However, more research needs to be carried out, in order to confirm the induction and development of somatic embryos from light-induced callus. Serial microscopy observation is needed at the end of each monthly passage for callus and embryogenic cells, in order to determine the number of somatic embryos and to categorize the embryos by stage of development (Purohit, 2003).

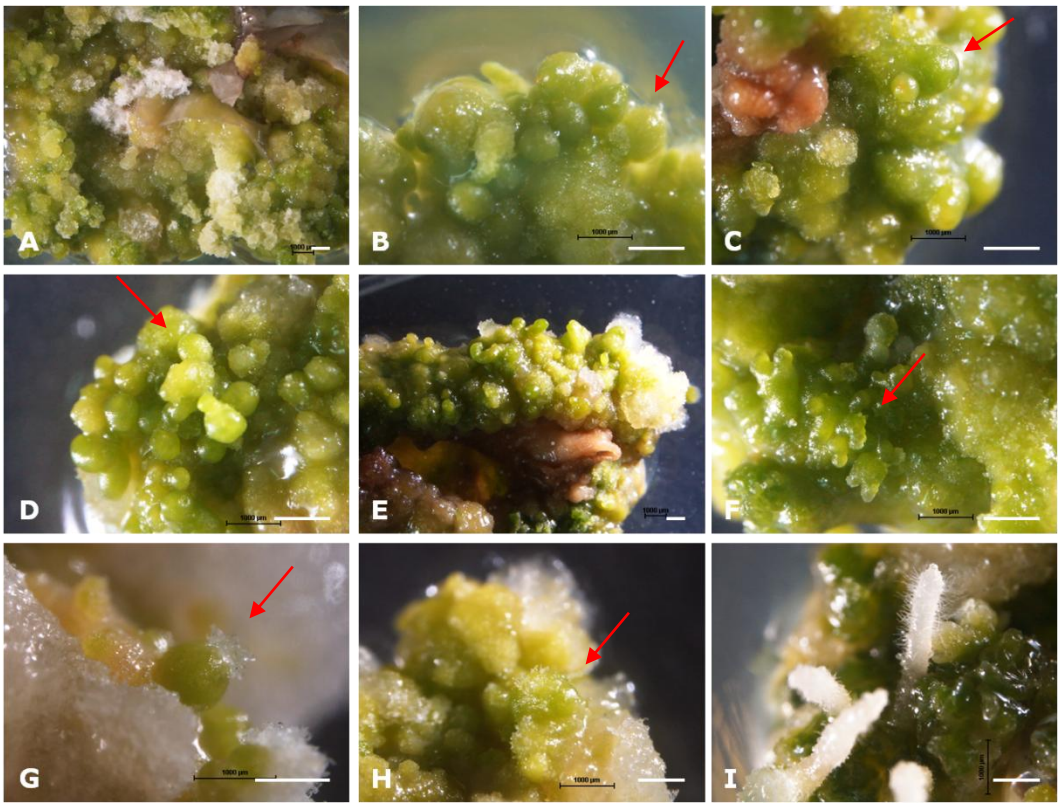


Figure 4.3. The observation of embryo-like structures on MS medium supplemented with NAA and kinetin, 60 days after culture establishement under light condition. (A): The close up structure of friable callus observed in Figure A. (B, C, E): Globular embryo-like structures induced on the leaf explants after 30 days of culture. (D, F): Differentiation was observed from globular embryo to heart-shaped embryo-like structure on medium enriched with NAA and kinetin. (G, H): The indirect regeneration of callus observed on the globular embryo-like structure after 60 days of culture. (I): Globular callus gave rise to the development of roots with root hairs. (Bar = 1000 µm).

4.3.2 Effects of plant growth regulators on the multiplication of dark-induced callus

In this study, the combination of NAA and kinetin were statistically proven to be effective in obtaining dark-induced callus from sweet shoot, as compared to the other combinations of plant growth regulators (IAA-BAP and 2,4-D-kinetin) (Appendix Table 8 and 9). Leaf explants remained the most desirable explants for callus culture due to its high regenerative capacity, followed by internodal and nodal explants ($p<0.05$) (Figure 4.4). After 60 days of culture initiation, the highest number of friable callus was formed on the cut surface of leaf explants when cultured on MS medium supplemented with 2.0 mg/l of NAA and 1.0 mg/l of kinetin (Table 4.2). This medium

promoted the highest frequency of white and friable callus (88.33%) in leaf explants, with the highest callus fresh weight of 4.54 g and the highest callus expansion rate of 13.85 cm^2 (Table 4.2). No callus was formed from leaf and stem explants on MS basal medium without plant growth regulators. The addition of NAA or cytokinin alone was also unable to induce any callus from the tested explants. None of the callus formed shoots directly in this experiment. A similarity was seen between light and dark condition when it came to callus production with auxin. It is found in these two conditions, an increased in auxin concentration enhances callus formation of sweet shoot.

The frequency of regenerating explants markedly increased when all the explants were cultured under dark conditions. With respect to the callus morphology, calluses grown under dark conditions were relatively more friable as compared to that grown in light (Figure 4.4). Hence, this explains why calluses grown in light were more compact and heavier in weight when compared to those grown under dark conditions, however these results were not statistically significant ($p<0.05$). In indica rice cultivar, the explants cultured under light condition promoted higher growth and increase in callus size than those cultured under dark condition (Toki, 1997; Pipatpanukul *et al*., 2004). However, an earlier study carried out by Summart *et al*. (2008) in Thai aromatic rice revealed that callus grown under dark condition had higher cell mass than those under light condition. The differences in callus growth rate seen in the above papers can be attributed to the different types of explant used for their experiment, and may not be related to the medium composition (Thadavong *et al*., 2002).

Light-induced callus grown on MS medium containing 2.0 mg/l NAA and 1.0 mg/l kinetin was induced 30 days after hormonal treatment, whereas explants incubated under the dark conditions successfully proliferated as early as 14 days after culture initiation. Studies of Reddy *et al*. (2011) and Raad *et al*. (2012) on micropropagation of *Anthurium* species also revealed that incubation of leaf explants under dark condition led to better callusing than those incubated under light condition. Callus incubated in the dark formed roots, suggesting that the auxin treatments in high concentration might be exhibiting strong effects on root formation (Figure 4.4).

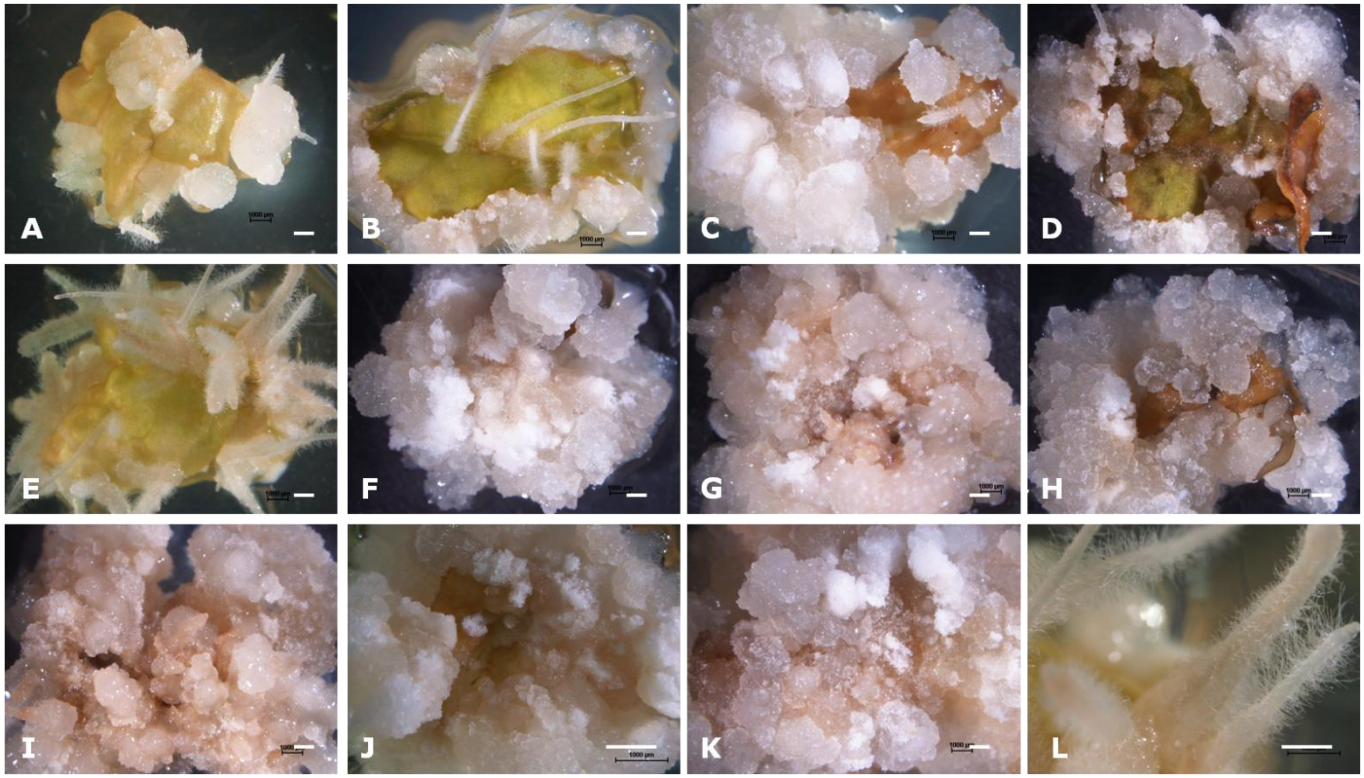


Figure 4.4. Callus formation from leaf explants incubated in the dark condition on MS medium enriched with NAA and kinetin, 60 days after culture initiation. (A): 0.5 mg/l NAA. (B): 0.5 mg/l NAA and 0.5 mg/l kinetin. (C): 0.5 mg/l NAA and 1.0 mg/l kinetin. (D): 0.5 mg/l NAA and 2.0 mg/l kinetin. (E): 1.0 mg/l NAA. (F): 1.0 mg/l NAA and 0.5 mg/l kinetin. (G): 1.0 mg/l NAA and 1.0 mg/l kinetin. (H): 1.0 mg/l NAA and 2.0 mg/l kinetin. (I): 2.0 mg/l NAA. (J): 2.0 mg/l NAA and 0.5 mg/l kinetin. (K): The highest percentage of callus formation, highest callus weight and highest callus expansion observed in MS medium enriched with 2.0 mg/l NAA and 1.0 mg/l kinetin. (L): Friable callus gave rise to the development of roots with root hairs. (Bar = 1000 µm).

Table 4.2. Effect of NAA and kinetin on the incidence (%) of callus formation, callus fresh weight and callus expansion factor of the leaf and stem explants incubated in the dark, 60 days after culture initiation.

Treatment code	Growth regulators (mg/l)		Types of explants											
			Leaf explants				Internodal explants				Nodal explants			
	NAA	KIN	% of callus formation	Estimated callus fresh weight (g)	Callus expansion factor (cm^2)	% of Browning	% of callus formation	Estimated callus fresh weight (g)	Callus expansion factor (cm^2)	% of Browning	% of callus formation	Estimated callus fresh weight (g)	Callus expansion factor (cm^2)	% of Browning
T0	0.0	0.0	0.00 $_{d}$	0.00 $_{i}$	0.00 $_{i}$	100.00 $_{a}$	0.00 $_{d}$	0.00 $_{e}$	0.00 $_{e}$	0.00 $_{a}$	0.00 $_{a}$	0.00 $_{a}$	0.00 $_{a}$	0.00 $_{a}$
T1	0.0	0.5	0.00 $_{d}$	0.00 $_{i}$	0.00 $_{i}$	100.00 $_{a}$	0.00 $_{d}$	0.00 $_{e}$	0.00 $_{e}$	0.00 $_{a}$	0.00 $_{a}$	0.00 $_{a}$	0.00 $_{a}$	0.00 $_{a}$
T2	0.0	1.0	0.00 $_{d}$	0.00 $_{i}$	0.00 $_{i}$	100.00 $_{a}$	0.00 $_{d}$	0.00 $_{e}$	0.00 $_{e}$	0.00 $_{a}$	0.00 $_{a}$	0.00 $_{a}$	0.00 $_{a}$	0.00 $_{a}$
T3	0.0	2.0	0.00 $_{d}$	0.00 $_{i}$	0.00 $_{i}$	100.00 $_{a}$	0.00 $_{d}$	0.00 $_{e}$	0.00 $_{e}$	0.00 $_{a}$	0.00 $_{a}$	0.00 $_{a}$	0.00 $_{a}$	0.00 $_{a}$
T4	0.5	0.0	68.33 $_{c}$	0.54 $_{i}$	3.00 $_{h}$	18.33 $_{bc}$	0.00 $_{d}$	0.00 $_{e}$	0.00 $_{e}$	0.00 $_{a}$	0.00 $_{a}$	0.00 $_{a}$	0.00 $_{a}$	0.00 $_{a}$
T5	0.5	0.5	70.00 $_{bc}$	1.04 $_{h}$	5.72 $_{fg}$	16.67 $_{bc}$	8.33 $_{c}$	0.16 $_{de}$	0.75 $_{de}$	0.00 $_{a}$	0.00 $_{a}$	0.00 $_{a}$	0.00 $_{a}$	0.00 $_{a}$
T6	0.5	1.0	71.67 $_{bc}$	2.02 $_{f}$	7.78 $_{e}$	13.33 $_{bc}$	10.00 $_{bc}$	0.41 $_{cd}$	2.08 $_{cde}$	0.00 $_{a}$	0.00 $_{a}$	0.00 $_{a}$	0.00 $_{a}$	0.00 $_{a}$
T7	0.5	2.0	71.67 $_{bc}$	1.84 $_{fg}$	7.04 $_{ef}$	13.33 $_{bc}$	8.33 $_{c}$	0.27 $_{de}$	1.58 $_{cde}$	0.00 $_{a}$	0.00 $_{a}$	0.00 $_{a}$	0.00 $_{a}$	0.00 $_{a}$
T8	1.0	0.0	70.00 $_{bc}$	1.14 $_{h}$	4.84 $_{g}$	18.33 $_{b}$	10.00 $_{bc}$	0.35 $_{cd}$	1.75 $_{cde}$	0.00 $_{a}$	0.00 $_{a}$	0.00 $_{a}$	0.00 $_{a}$	0.00 $_{a}$
T9	1.0	0.5	80.00 $_{abc}$	3.32 $_{d}$	9.68 $_{d}$	8.33 $_{bc}$	10.00 $_{bc}$	0.38 $_{cd}$	1.92 $_{cde}$	0.00 $_{a}$	0.00 $_{a}$	0.00 $_{a}$	0.00 $_{a}$	0.00 $_{a}$
T10	1.0	1.0	83.33 $_{abc}$	3.65 $_{c}$	11.60 $_{c}$	6.67 $_{bc}$	16.67 $_{ab}$	0.97 $_{bc}$	4.42 $_{bc}$	0.00 $_{a}$	0.00 $_{a}$	0.00 $_{a}$	0.00 $_{a}$	0.00 $_{a}$
T11	1.0	2.0	71.67 $_{bc}$	2.62 $_{e}$	8.14 $_{e}$	6.67 $_{bc}$	11.67 $_{bc}$	0.47 $_{cd}$	2.17 $_{cde}$	0.00 $_{a}$	0.00 $_{a}$	0.00 $_{a}$	0.00 $_{a}$	0.00 $_{a}$
T12	2.0	0.0	71.67 $_{bc}$	1.42 $_{gh}$	5.28 $_{g}$	18.33 $_{b}$	13.33 $_{ab}$	0.73 $_{cd}$	3.08 $_{bcd}$	0.00 $_{a}$	0.00 $_{a}$	0.00 $_{a}$	0.00 $_{a}$	0.00 $_{a}$
T13	2.0	0.5	85.00 $_{ab}$	4.15 $_{b}$	12.91 $_{b}$	6.67 $_{bc}$	20.00 $_{ab}$	1.43 $_{b}$	5.33 $_{b}$	0.00 $_{a}$	0.00 $_{a}$	0.00 $_{a}$	0.00 $_{a}$	0.00 $_{a}$
T14	2.0	1.0	88.33 $_{a}$	4.54 $_{a}$	13.85 $_{a}$	5.00 $_{c}$	23.33 $_{a}$	2.01 $_{a}$	7.46 $_{a}$	0.00 $_{a}$	0.00 $_{a}$	0.00 $_{a}$	0.00 $_{a}$	0.00 $_{a}$
T15	2.0	2.0	73.33 $_{bc}$	2.65 $_{e}$	8.11 $_{e}$	8.33 $_{bc}$	11.67 $_{bc}$	0.42 $_{cd}$	2.08 $_{cde}$	0.00 $_{a}$	0.00 $_{a}$	0.00 $_{a}$	0.00 $_{a}$	0.00 $_{a}$

* Values expressed as means of 12 replicated experiments, each replicate consisted of 5 cultures.

* Different letters within column (lowercase) indicate a significant difference ($p<0.05$) according to Duncan's multiple range test.

4.3.3 Browning and leaf necrosis

A problem commonly encountered when dealing with woody explants is the release of exudates into culture medium which cause browning. The cut surfaces of explants start to discolour and darken after excision, associated with wounding (Figure 4.5). Polyphenolic compounds in trimmed explants might be oxidized by the *polyphenol oxidase* enzymes and cause tissue browning (Chawla, 2002). It is likely that the products of this oxidation process are formed under light condition (Chawla, 2002), and this could be one of the main reasons why browning occurs less in the dark.

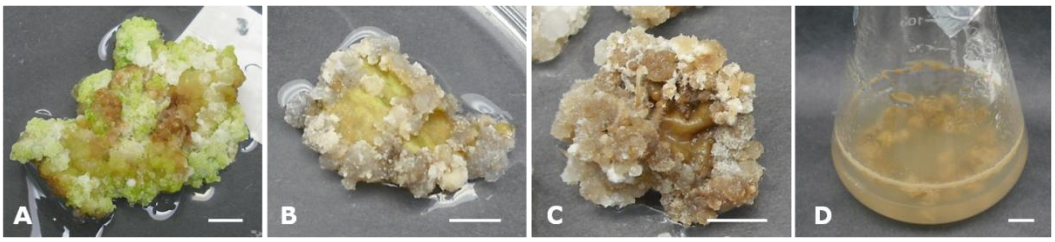


Figure 4.5. Browning effects of *in vitro* leaf explants. (A): The browning of callus observed after several subcultures, due to the exudation of phenolic compounds. (B, C): Tissue blackening and browning observed, 30 days after culture initiation, because of the use of unsuitable medium. (D): The cause of callus browning in cell suspension culture mainly due to the prolonged subculturing periods. (Bar = 1 cm).

In this study, necrosis of callus was observed in all the treatments due to the release of phenolic compounds, albeit in low percentage (<21.67%). The callus mass exhibited a patchy distribution of brown area without any regeneration and eventually became necrotic. It may be due to the differential caulogenic responses of explants towards the plant growth regulators, as documented previously for *Paeonia suffruticosa* (Bouza *et al*., 1994). Hence, a quick transfer of the explants to fresh media is recommended, in order to avoid the possible inhibitory effects of the exudates (Compton and Preece, 1986). The addition of activated charcoal and ascorbic acid may also help to overcome the inhibitory effects of phenolics released into the medium and the additives have been used frequently for many woody species (Purohit, 2003).

Activated charcoal is an amorphous form of carbon (Sathyanarayana and Varghese, 2007), with a high surface area and porosity, which makes it

an ideal medium for the adsorption of many organic and inorganic molecules from culture medium. The addition of activated charcoal was previously reported to promote growth and differentiation in orchids, carrot and tomato tissue culture (Sathyanarayana and Varghese, 2007; Chugh *et al*., 2009). Antioxidants, such as ascorbic acid, are commonly used for rinsing freshly isolated explants to delay the browning process. Ascorbic acid helps in lowering the redox potential of solutions and thus is effective in preventing the browning of plant tissues (Purohit, 2003).

4.3.4 Shoot proliferation and multiplication

Callusing during adventitious shoot regeneration is a common phenomenon observed in sweet shoot, *Morus* species (Lu, 2002) and *Croton urucurana* (Lima *et al*., 2008). New meristems arise from the leaf explants may produce undifferentiated parenchymatous cells without any structural order characteristic of the organ or tissue from which they were derived. Callus generated under light condition was separated using forceps and cultured on MS medium supplemented with 2.0 mg/l BAP and 0.5 mg/l IAA for shoot induction. The earliest sign of shoot bud formation and subsequent elongation was noticeable after seven and 30 days of incubation, respectively (Figure 4.6). After 60 days of shoot initiation, an average 86.67% of light-induced calluses formed adventitious shoots with a mean of 6.23 shoots per callus and an average shoot length of 5.43 cm. The above results are comparable to the adventitious shoot cultures generated from nodal explants using the similar hormonal composition, showing very good shoot proliferation, as mentioned in Section 3.3.2 and 3.4. This proved that this medium is suitable for shoot induction from nodal explants and callus cultures of sweet shoot. Besides that, BAP has also proved not to be a potent cytokinin for promoting callus from woody plant species.

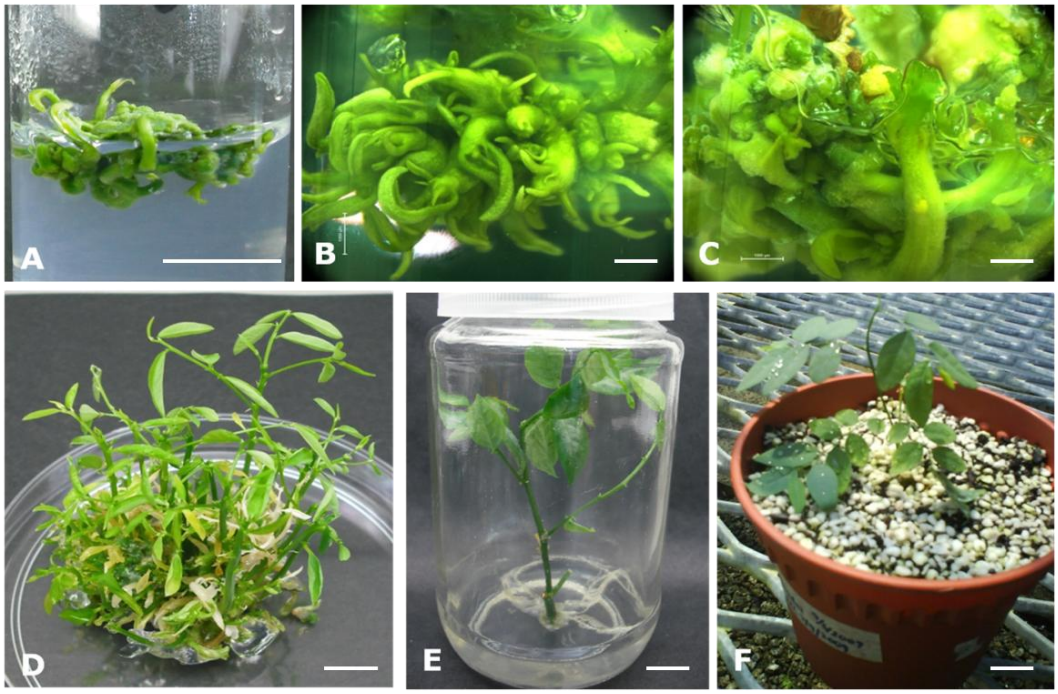


Figure 4.6. Shoot induction from light-induced callus cultures of sweet shoot. (A): Dark green globular and nodular callus formed on MS medium with 2.0 mg/l BAP and 0.5 mg/l IAA after 30 days of shoot initiation (Bar = 1 cm). (B, C): Callus, shoot buds and early stage of adventitious shoots on MS medium with 2.0 mg/l BAP and 0.5 mg/l IAA (Bar = 1000 µm). (D): Development of multiple shoot clusters after 60 days of shoot initiation (Bar = 1 cm). (E): Development of roots on MS medium supplemented with 1.0 mg/l IAA (Bar = 1 cm). (F): Establishment of *in vitro* sweet shoot plant after two months in compost (Bar = 1 cm).

4.3.5 *In vitro* root induction and acclimatization of plantlets

Callus at the basal end of microshoots interfered with the connection between shoots and roots and made rooting difficult, as mentioned by Pattanaik and Chand (1996) in *in vitro* cultures of *Oncimum americanum*. Microshoots were separated from multiple shoot clusters and developed roots after 10 days of culture on half strength MS medium enriched with 1.0 mg/l IAA. High rooting response of 90% was achieved after 10 days of initiation, with an average of 5.95 roots per shoot and an average root length of 5.42 cm. The success rate of root induction via indirect organogenesis is comparable to direct shoot regenerative pathway, as seen in Section 3.3.5 and 3.5.

After one month of acclimatization, survival of 90% was achieved in the shadehouse when transplanted in pots containing a mixture of perlite and compost (Figure 4.6). The inclusion of carbendazim, a systemic benzimidazole fungicide, during the acclimatization of plantlets has successfully reduced the percentage of microbial contamination in the field (Medina *et al*., 2007). It helps to inhibit the polymerisation of free tubulin molecules by binding an arginin residue of the β-tubulin subunit and acts by disrupting cell division through linking to the nuclear spindle, which inhibits fungal growth (IPCS, 1993).

In this experiment, 10% mortality was observed upon transfer of microshoots to *ex vitro* conditions, as the cultured plants may have non-functional stomata, weak root system and poorly developed cuticle (Mathur *et al*., 2008). The controlled conditions induce structural and physiological changes in plants rendering them unfit to survive when transferred directly to the field (Rout *et al*., 2006).

4.4 CONCLUSION

Plant regeneration via indirect organogenesis has been developed for sweet shoot using leaf explants. MS medium supplemented with 2.0 mg/l NAA and 1.0 mg/l kinetin was used in light-induced and dark-induced callus from leaf explants, as it enhanced the callus regeneration capacity (>71.67%), callus fresh weight (>4.53 g) and callus expansion rate (>13.85 cm^2). Callusing response increased as the auxin levels increased. A relatively high percentage (86.67%) of shoot proliferation from light-induced callus was achieved on MS medium supplemented with 2.0 mg/l BAP and 0.5 mg/l IAA. Plantlets rooted on half strength MS medium containing 1.0 mg/l IAA had the highest rooting response (90.00%) with mean number of 5.95 roots per shoot and a mean length of 5.42 cm. All acclimatized plants (90.00%) grew vigorously in compost and showed high homogeneity with no visual evidence of morphological variation. In this study, light and dark-induced calluses were chosen as plant materials for the subsequent antioxidant assays, due to the differing presence of secondary metabolite in these calluses, as mentioned in Section 2.7.6.

CHAPTER 5: INDIRECT SOMATIC EMBRYOGENESIS OF *Sauropus androgynus*

5.1 INTRODUCTION

Somatic embryogenesis is one of the most important technologies for woody plant regeneration, which involves the formation of somatic embryos from a single cell or from clusters of embryogenic cells other than a gamete or the product of gametic fusion (Williams and Maheswaran, 1986). Plant cells generally undergo several mitotic divisions in the presence of an auxin for the histodifferentiation of embryogenic cells, progressing from globular to cotyledonary embryo (Figure 5.1) (Santos *et al*., 2006; Santana-Buzzy *et al*., 2009). Over the past decade, somatic embryogenesis has served as an excellent morphogenetic system, which allowed the scientist to study the histodifferentiation process of somatic embryos (Thorpe, 1995). Besides being a model for early development, somatic embryogenesis is proven to be a powerful system used by non-woody and dicotyledonous species, as a regeneration scheme in mass multiplication of elite genotypes, in large-scale production of synthetic seeds and in genetic transformation studies (Williams and Maheswaran, 1986; Merkle *et al*., 1987). Many scientific papers revealed that the recalcitrant nature of monocotyledonous and woody plant species caused difficulties in generating plantlets via somatic embryogenesis (Dodeman *et al*., 1997; Yeung, 1999; Puigderrajols *et al*., 2000). Up till now, there are only 130 plant species for which detailed protocols for propagation via somatic embryogenesis have been developed, and the best documented system concerning somatic embryogenesis is liquid suspension cultures of carrot (Reinert, 1959; Halperin, 1966).

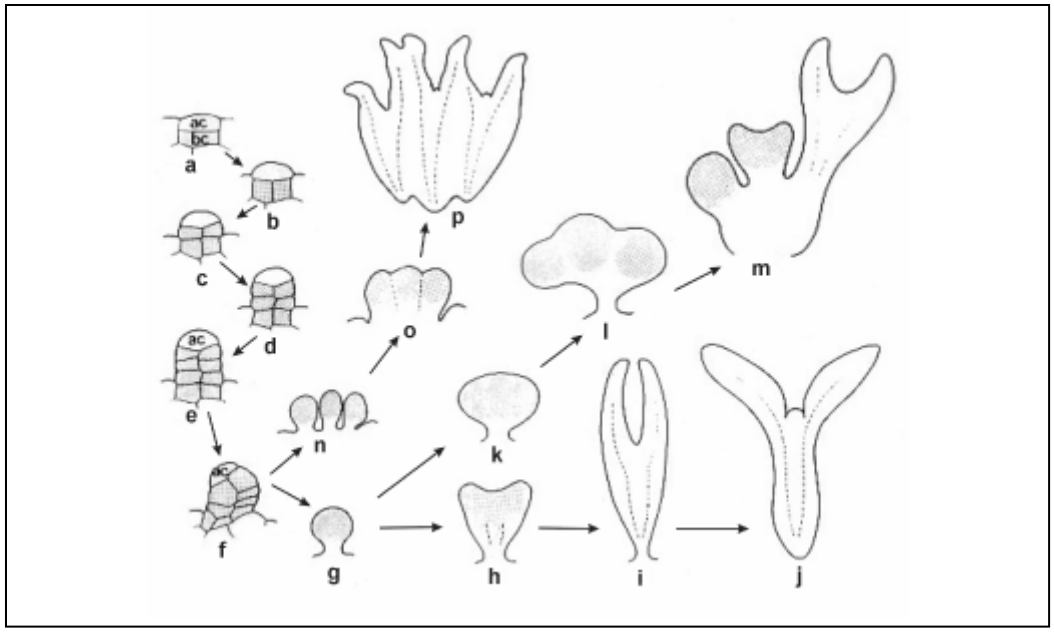


Figure 5.1. Scheme of soybean somatic embryo ontogenetic routes (Santos *et al*. 2006). (a): First division of somatic embryogenic cells. (b): Anticlinal division of the pro-embryo basal cell. (c, d, e, f): Periclinal division in two series of derivatives from basal cell. (g): Globular embryo. (h): Heart-shaped embryo. (i): Torpedo-shaped embryo. (j): Cotyledonary embryo. (k): Enlarged primary globular embryo. (l): Enlarged structure with three growth centers. (m): Differentiation of secondary embryos from an enlarged primary embryo. (n): Early-stage globular embryos originated from adjacent epidermal cells. (o): Fusion of globular embryos. (p): Fasciated cotyledonary embryos.

In sweet shoot, the induction and proliferation of somatic embryos are influenced by a variety of factors, such as the types of explants, plant genotypes, media components and environmental culture conditions. One of the main factors associated with the induction of somatic embryogenesis is the adoption of plant growth regulators in this experiment. Tissues with different levels of competence required precise balance of plant growth regulators, in order to develop a morphogenic response from the plant materials (Silveira *et al*., 2004). Dicamba, NAA and 2,4-D are the most commonly used auxin in inducing the formation of somatic embryos via repetitive cell division, possibly by activating the differential gene expression of plant species (Sudha and Seeni, 2006). Besides that, cytokinin (kinetin, thidiazuron and BAP) is also added into liquid suspension culture for the development of embryogenic cells in dicotyledonous and monocotyledonous

species (Sagare *et al*., 2000). However, the combinatorial effects of auxin and cytokinin were found to suppress the cell differentiation and growth in somatic embryos of certain plant species (Griga, 1998). Therefore, it is crucial to optimize the concentrations of plant growth regulators for an optimum response at each individual stage of sweet shoot somatic embryos.

Histological techniques are frequently used in the study of plant morphogenesis in order to examine the microscopic structural features of cells together with the assembly and arrangement of cells into tissues and organs (Puigderrajols *et al*., 2000). Through histological study, differences in all stages of somatic embryos versus zygotic embryos can be appreciated, and the morphological characteristic of liquid suspension cultures can also be examined. Histological studies describing the anatomy of somatic embryos have been reported earlier on *Olea europea* (Benelli *et al*., 2001), *Quercus robur* (Zegzouti *et al*., 2001) and *Zingiber officinale* (Lincy *et al*., 2009). Despite the benefits of somatic embryogenesis, none of the previous reports described a system of somatic embryo production in sweet shoot within a semi-solid and liquid culture environment. Thus, this study embarks on a pioneer objective to examine the ontogeny of sweet shoot somatic embryos derived from leaf-derived callus, in order to highlight the possible causes of developmental failure and to determine the feasibility of plantlet regeneration via somatic embryogenesis.

5.2 MATERIALS AND METHODS

5.2.1 Plant materials

Leaf explants of sweet shoot were surface sterilized using 70% (v/v) ethanol for 1 minute, followed by 20% (v/v) Clorox for 20 minutes (Section 3.2.3). The embryogenic callus was induced from aseptic leaf explants cultured on MS medium supplemented with 3.0% (w/v) sucrose, 0.25% (w/v) Phytagel™, 2.0 mg/l NAA and 1.0 mg/l kinetin, as previously mentioned in Section 4.3.2 and 4.4. Embryogenic calluses were incubated in the dark at 26±2^{o}C for three to four months for mass multiplication of sweet shoot. The embryogenic calluses were subcultured every four weeks on same fresh medium to maintain the embryogenic state of cell lines.

5.2.2 Effects of plant growth regulators and cultivation systems on somatic embryogenesis

5.2.2.1 Semi-solid medium cultivation system

After three months of callus induction, inoculation density of 1.0 g (Mujib and Samaj, 2006) of friable embryogenic callus was transferred onto 30 ml MS medium enriched with 3.0% (w/v) sucrose, 0.25% (w/v) Phytagel™ and different concentrations of NAA and kinetin (0.0, 0.5, 1.0 and 2.0 mg/l). After four weeks on the induction medium, the globular embryos were transferred onto histodifferentiation medium containing different levels of NAA and kinetin (0.0, 0.5, 1.0 and 2.0 mg/l) for the development of heart-shaped and torpedo-shaped somatic embryos. Individual torpedo-shaped embryos obtained from MS medium containing 1.0 mg/l NAA and 0.5 mg/l kinetin were subsequently transferred onto hormone-free MS medium for the maturation of somatic embryos, 12 weeks after the induction of somatic embryo from leaf-derived callus. Each petri plate was sealed with parafilm and incubated at 26±2^{o}C under dark conditions.

Individual cotyledonary somatic embryos grown on hormone-free MS medium were then subcultured directly onto shoot induction medium for shoot proliferation of sweet shoot. All germinated microshoots were incubated for eight weeks as described in Section 3.2.4.1. Data on (a) the percentage of primary embryogenesis, number of (b) globular, (c) heart-shaped, (d) torpedo-shaped somatic embryos per g of callus, (e) number of somatic embryos germinated and (g) number of somatic embryos with secondary embryogenesis, were recorded after 16 weeks of embryo induction.

5.2.2.2 Liquid medium cultivation system

The establishment of embryogenic cell suspension culture in sweet shoot was initiated by inoculating 1.0 g of three-month-old embryogenic callus (Mujib and Samaj, 2006) onto 30 ml liquid MS medium supplemented with 3.0% (w/v) sucrose and different strengths of NAA and kinetin (0.0, 0.5, 1.0 and 2.0 mg/l). All the embryogenic cell suspension cultures were agitated continuously at 100 rpm on an orbital shaker (Tech-Lab, Malaysia) and incubated continuously in darkness at 26±2^{o}C. Embryogenic cell suspensions were maintained by transferring 10 ml of old suspension to 20 ml of fresh

liquid MS medium at three week intervals to avoid any accumulation of phenolic compounds (Ong *et al*., 2008). The growth of embryogenic cells was determined by measuring the packed cell volume (PCV) in cell suspension culture, every seven days until constant growth was achieved. The measurement of PCV was recorded by allowing the cell suspensions to precipitate in a sterile centrifuge tube.

After three weeks of embryogenic cell induction, embryogenic cell clumps were filtered through a 200 µm stainless steel sieve (Choi *et al*., 1999) and the filtered embryogenic cell clumps (0.5 g) were transferred aseptically into 100 ml Erlenmeyer flask containing 30 ml of liquid MS medium enriched with different levels of NAA and kinetin (0.0, 0.5, 1.0 and 2.0 mg/l), for the induction of globular embryos. After three weeks on histodifferentiation medium, embryogenic cell suspensions consisting of globular embryos were subcultured in liquid MS medium containing various strengths of NAA and kinetin (0.0, 0.5, 1.0 and 2.0 mg/l), for the development of heart-shaped and torpedo-shaped somatic embryos in sweet shoot.

Individual torpedo-shaped embryos obtained from MS medium supplemented with 1.0 mg/l NAA and 0.5 mg/l kinetin were then transferred onto fresh hormone-free MS medium, in order to facilitate the maturation of somatic embryos. Mature somatic embryos grown in hormone-free MS medium were removed from Erlenmeyer flasks using forceps and transferred directly onto shoot induction medium for shoot induction. The elongated shoots were incubated for eight weeks as previously mentioned in Section 3.2.4.1. Data on (a) the percentage of primary embryogenesis, number of (b) globular, (c) heart-shaped, (d) torpedo-shaped somatic embryos per g of callus, (e) number of somatic embryos germinated and (g) number of somatic embryos with secondary embryogenesis, were recorded after 12 weeks of embryo induction

5.2.3 Histological analysis of somatic embryos

Somatic embryos of sweet shoot at different developmental stages (globular, heart-shaped, torpedo-shaped and cotyledonary somatic embryos)

were removed from the liquid MS medium and fixed for 24-48 hours at room temperature in a Glutaraldehyde-Paraformaldehyde-Caffeine (GPC) (Sigma-Aldrich, USA) fixative solution [50 ml 0.2 M phosphate buffer, pH 7.2; 20 ml 10% (v/v) Paraformaldehyde; 4 ml 25% (v/v) Glutaraldehyde; 1 g caffeine and 26 ml distilled water to a total volume of 100ml]. Samples of somatic embryos were subsequently dehydrated in ascending ethanol series (v/v): 30% for 30 min; 50% for 45 min; 70% for 45 min; 80% for 60 min; 90% for 60 min; 95% for 60 min and twice in absolute ethanol for 60 min. Extensive exposure to absolute ethanol was avoided during the dehydration process, in order to prevent tissue hardening which may cause difficulty in sectioning the samples using microtome. A vacuum machine was used for 5-10 minutes, to ensure the complete infiltration of solution in somatic embryo samples.

The samples of somatic embryos were then prepared for infiltration with basic resin (Leica Historesin Embedding Kit, Germany) for 24-48 hours at 4^{o}C under low pressure (vacuum). The infiltration process was completed when the sample appeared to be slightly translucent and precipitated at the bottom of the vessel. After infiltration, each individual sample was subsequently embedded in a mold and the resin was allowed to polymerize fully for a day before installing the holders (Figure 5.2). Serial sections of 4 µm were sliced using Leica RM2165 rotary microtome (Leica Microsystems Nussloch GmbH, Germany) and delicately placed on the clean microscope slides (Figure 5.2). The microscope slides were allowed to dry overnight on MH6616 slide drying bench (Electrothermal, UK). An optional of 0.5 % (w/v) toluidine blue staining reagent (Sigma, USA) (0.5 g toluidine blue in 100 ml 0.2 M sodium acetate buffer, pH 4.6) was used to confirm the resin slices are laid properly on the microscope slides, before further staining is done.

Figure 5.2. Equipment needed for histological examination of somatic embryo. (A): Leica RM2165 rotary microtome was used for sample sectioning, resin sections were placed in the distilled water container and delicately placed on the microscope slides. Microscope slides were allowed to dry overnight on slide drying bench. (B): Installation of holder with resin on a mold. (C): Automated research microscope was used to examine the structure of somatic embryo cultures.

Good sections were stained with 1% periodic acid for 5 minutes. After rinsing four times with distilled water (pH 4.5), stained sections were continuously submerged in Schiff's reagent (1 g basic fuchsin, 2 g disodium metabisulfite in 1 N HCl, 0.5 g neutralized activated charcoal) for 20 minutes in the dark, to detect the presence of polysaccharide (starch reserves) in somatic embryo cultures. The stained slides were rinsed again for four times with distilled water at pH 4.5. Counter staining was done using Naphthol Blue Black reagent (Sigma-Aldrich, USA) (1 g Naphthol Blue Black in 100 ml 7% (v/v) acetic acid) at 60^{o}C for 5 minutes, to enable the nucleus, soluble and reserve proteins to be visualized under light microscopy. The addition of 1N HCl in distilled water is critical to prevent the resins from turning pink and indigo, if the pH is too high. Stained slides were rinsed under running water and left overnight to dry on MH6616 slide drying bench (Electrothermal, UK).

For preservation purposes, the microscope slides were mounted with Cytoseal™ 60 mounting medium and allowed to dry before examining under an automated Leica DM6000 B research microscope (Leica Microsystems Nussloch GmbH, Germany) (Figure 5.2). All images were photographed using a microscope camera (ProgRes® C10, Jenoptik Laser, Optik, Systeme GmbH,

Germany) (Figure 5.2). All of the major steps involved in histological examination of somatic embryo cultures are summarized in Figure 5.3.

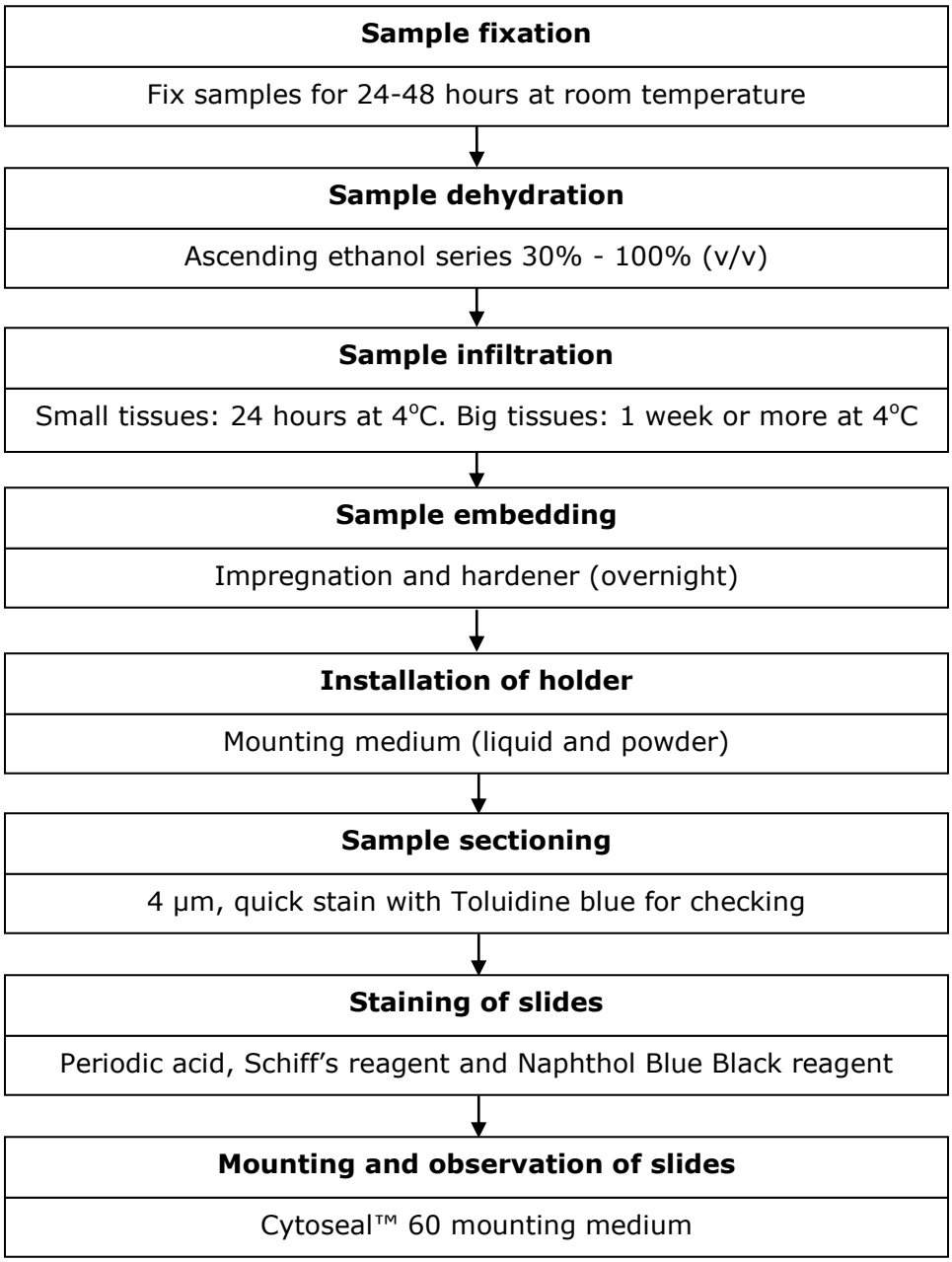


Figure 5.3. The flowchart above listed the major steps involved in histological examination of sweet shoot somatic embryo cultures.

5.2.4 Embryo germination and plant regeneration

Individual cotyledonary embryos grown in hormone-free MS medium were transferred onto shoot induction medium containing 2.0 mg/l BAP and

0.5 mg/l IAA, for shoot induction and proliferation as stated earlier in Sections 3.2.4.1, 3.4, 4.2.3 and 4.4. The elongated shoots were subcultured onto the same fresh medium every four weeks for mass multiplication of sweet shoot. The cultures were incubated as described in Section 3.2.4.1.

After 2 months of shoot induction, elongated shoots generated from somatic embryos were aseptically transferred onto root elongation medium supplemented with 1.0 mg/l IAA. Rooted microshoots with 5-6 cm in height, were transplanted to pots with a mixture of perlite and compost (1:1) and then acclimatized to shadehouse conditions, as stated in Section 3.2.7 and 4.2.6. The survival percentage of plantlets was recorded, one month after the acclimatization in shadehouse.

5.2.5 Statistical analysis

The experiments were performed two times with a total of 15 explants per treatment each time. All data were analyzed statistically by one-way ANOVA followed by Duncan's multiple range tests (DMRT) at a significance level of $p<0.05$ using Statistical Package for Social Sciences (SPSS) software version 16.0.

5.3 RESULTS AND DISCUSSION

5.3.1 Effects of plant growth regulators and semi-solid medium cultivation system on somatic embryogenesis

In the present study, the morphogenetic potential of sweet shoot is strongly dependant on the type of explants. Leaf explants were proven to induce higher growth rate of friable and opaque-white embryogenic callus than internodal and nodal explants, as mentioned earlier in Section 4.4. The variation in callus responses displayed by different types of explants may be attributed to the physiological state of the explants (Jimenez, 2005). The friable and opaque-white callus obtained from leaf explant was found to be more responsive in somatic embryo induction, due to its ability in segregating into uniform cell aggregates on semi-solid and liquid MS medium (Buiteveld *et al*., 1994; Burns and Wetzstein, 1997). Hence, leaf-derived callus appeared to be a better source of explant for the initiation of somatic embryogenesis in sweet shoot. This observation is in line with the studies done by Michler and

Bauer (1991), Pickens *et al*. (2005) and Alcantara *et al*. (2014) in tissue cultures of *Populus* species, *Euphorbia pulcherrima* and sugarcane.

Based on the result shown in Table 5.1, different hormonal treatments were found to be statistically significant for the incidence of somatic embryo formation ($p<0.05$). Embryogenic callus initiated in the dark on MS medium supplemented with 2.0 mg/l NAA and 1.0 mg/l kinetin, was transferred onto semi-solid MS medium enriched with 1.0 mg/l NAA and 0.5 mg/l kinetin, and with this, a higher proportion (83.33%) of embryogenic callus produced higher yields of globular somatic embryos (6.40 embryos/g callus), after four weeks of embryo induction (Table 5.1). A relatively high number of globular somatic embryos (6.40 embryos/g callus) differentiated into heart-shaped (6.20 embryos/g callus) and torpedo-shaped somatic embryos (5.80 embryos/g callus) using the same hormonal composition, after 12 weeks of embryo induction (Table 5.1). The superiority of NAA in initiating the embryogenic pathway may be attributed to its ability to induce stress genes, which has been shown to contribute to the cellular reprogramming of carrot somatic cells in somatic embryogenesis as it triggered changes in the morphology, physiology and metabolism of somatic cells (Kitamiya *et al*., 2000). Therefore, in most species further development of somatic embryos usually requires reduction of auxin level (Jimenez, 2005), as observed in the present work.

Table 5.1. Effect of medium composition on the induction, development and germination of somatic embryos in semi-solid medium of sweet shoot.

Treatment code	Growth regulators (mg/l)		Stages of somatic embryogenesis				
			Somatic embryo induction (4 weeks after embryo induction)			Development	
	NAA	KIN	% of responded callus	% of embryo formation	Number of globular embryo per g of callus	Number of heart-shaped embryo per g of callus (8 weeks after embryo induction)	Number of torpedo-shaped embryo per g of callus (12 weeks after embryo induction)
T0	0.0	0.0	16.67 $_{c}$	16.67 $_{c}$	2.00 $_{d}$	1.00 $_{d}$	1.00 $_{d}$
T1	0.0	0.5	0.00 $_{d}$	0.00 $_{d}$	0.00 $_{e}$	0.00 $_{e}$	0.00 $_{e}$
T2	0.0	1.0	0.00 $_{d}$	0.00 $_{d}$	0.00 $_{e}$	0.00 $_{e}$	0.00 $_{e}$
T3	0.0	2.0	0.00 $_{d}$	0.00 $_{d}$	0.00 $_{e}$	0.00 $_{e}$	0.00 $_{e}$
T4	0.5	0.0	66.67 $_{ab}$	50.00 $_{b}$	4.33 $_{bc}$	3.33 $_{c}$	2.50 $_{c}$
T5	0.5	0.5	50.00 $_{b}$	33.33 $_{bc}$	4.50 $_{bc}$	3.00 $_{c}$	2.00 $_{c}$
T6	0.5	1.0	33.33 $_{bc}$	33.33 $_{bc}$	3.33 $_{cd}$	2.50 $_{cd}$	1.00 $_{d}$
T7	0.5	2.0	33.33 $_{bc}$	16.67 $_{c}$	2.00 $_{d}$	2.00 $_{cd}$	1.00 $_{d}$
T8	1.0	0.0	66.67 $_{ab}$	66.67 ^{ab}	5.00 $_{b}$	4.25 $_{b}$	3.50 $_{bc}$
T9	1.0	0.5	83.33 $_{a}$	83.33 $_{a}$	6.40 $_{a}$	6.20 $_{a}$	5.80 $_{a}$
T10	1.0	1.0	66.67 $_{ab}$	50.00 $_{b}$	5.33 $_{b}$	4.67 $_{b}$	4.00 $_{b}$
T11	1.0	2.0	50.00 $_{b}$	33.33 $_{bc}$	3.50 $_{cd}$	3.00 $_{c}$	2.50 $_{c}$
T12	2.0	0.0	50.00 $_{b}$	50.00 $_{b}$	4.00 $_{c}$	3.33 $_{c}$	2.33 $_{c}$
T13	2.0	0.5	66.67 $_{ab}$	66.67 ^{ab}	3.50 $_{cd}$	3.25 $_{c}$	2.00 $_{c}$
T14	2.0	1.0	66.67 $_{ab}$	50.00 $_{b}$	3.00 $_{cd}$	2.33 $_{cd}$	1.33 $_{cd}$
T15	2.0	2.0	0.00 $_{d}$	0.00 $_{d}$	0.00 $_{e}$	0.00 $_{e}$	0.00 $_{e}$

* Values expressed as means of 15 replicated experiments, each replicate consisted of 1 culture.

* Different letters within column (lowercase) indicate a significant difference ($p<0.05$) according to Duncan's multiple range test.

It was noted that a relatively low percentage (16.67%) of somatic embryos was formed on hormone-free MS medium in this study. The reason for such observation may be attributed to the residual effect of exogenous hormone carried by the callus from its previous induction medium containing 2.0 mg/l NAA and 1.0 mg/l kinetin (Rajasekaran *et al*., 1987). Furthermore, semi-solid MS medium enriched with kinetin alone also failed to induce somatic embryogenesis from the embryogenic callus (Table 5.1), as found earlier in tissue cultures of *Feijoa sellowiana* (Cruz *et al*., 1990), *Arabidopsis thaliana* (Luo and Koop, 1997) and *Quercus robur* (Zegzouti *et al*., 2001). The synergistic effects of auxin and cytokinin in enhancing somatic embryogenesis of sweet shoot was also reported in somatic embryo cultures of coffee (Neuenschwander and Baumann, 1992) and *Rauvolfia micrantha* (Sudha and Seeni, 2006). Thus, the above results suggest that the incorporation of NAA plays a significant role in inducing somatic embryos from sweet shoot.

Out of 60 torpedo-shaped somatic embryos transferred to hormone-free MS medium, maturation rate of 80% (48 cotyledonary embryos) was achieved after 16 weeks of embryo induction from leaf-derived callus. With longer period of culture (>16 weeks), secondary globular embryos (50%) were formed on the surface of primary somatic embryos, without going through an intervening callus phase (Figure 5.4; Figure 5.5). Many reports have highlighted that the presence of kinetin hastens the secondary somatic embryo induction, and this finding is supported by Lincy *et al.* (2009) in somatic embryos of *Zingiber officinale*. All the germinated somatic embryos showed good structural organization, such as normal shoot meristem, well structured vascular system and higher accumulation of starch in the cotylendonary-shaped somatic embryos (Figure 5.4). The formation of leaf-like structures was also observed on hormone-free MS medium, after 16 weeks of culture initiation.

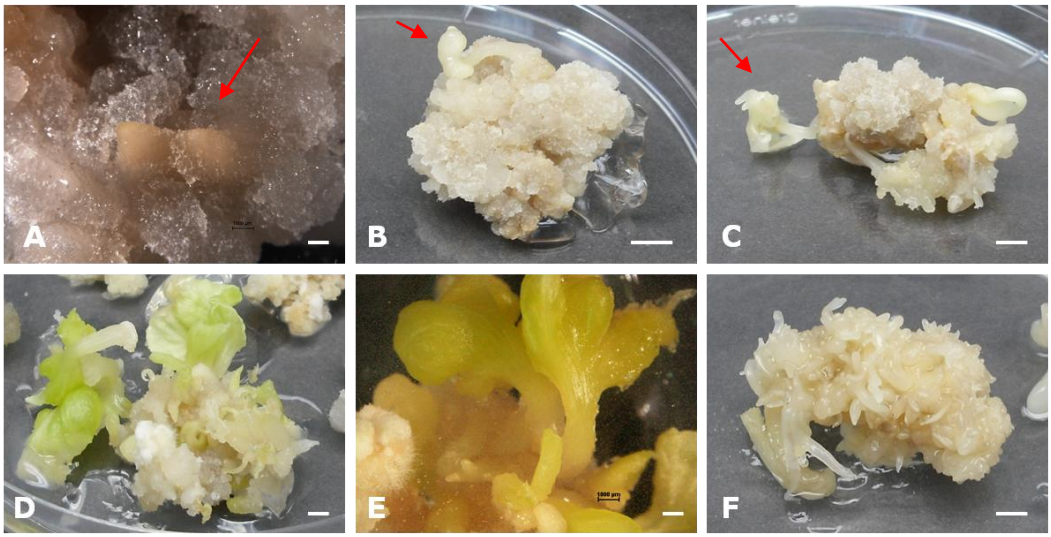


Figure 5.4. The developmental stages of somatic embryos generated in semi-solid medium enriched with NAA and kinetin in the dark. (A): Formation of globular embryos from friable callus after four weeks of embryo induction on MS medium supplemented with 1.0 mg/l of NAA and 0.5 mg/l of kinetin (Bar = 1000 µm). (B) The development of heart-shaped and torpedo-shaped embryos obtained on semi-solid MS medium supplemented with 1.0 mg/l of NAA and 0.5 mg/l of kinetin (Bar = 1 cm). (C): The germination of dicotyledonary embryos developed from torpedo-shaped embryos on hormone-free MS medium, after 16 weeks of embryo induction (Bar = 1 cm). (D, E): The formation of green leaf-like structures observed in MS hormone-free medium after 16 weeks of embryo induction (Bar = 1 cm). (F): Secondary somatic embryogenesis occurred on the MS medium containing NAA and kinetin, after more than 16 weeks of embryo induction (Bar = 1 cm).

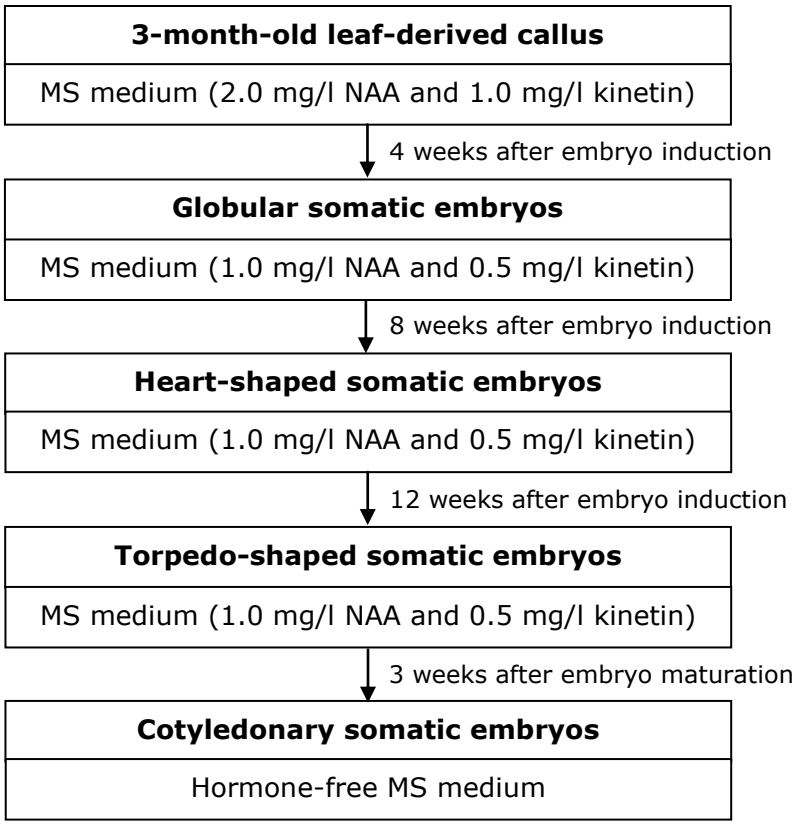


Figure 5.5. Summary of somatic embryogenesis in sweet shoot cultured in semi-solid MS medium.

5.3.2 Effects of plant growth regulators and liquid medium cultivation system on somatic embryogenesis

Three-month-old embryogenic callus (1.0 g) was randomly selected and suspended in liquid MS medium supplemented with different concentrations of NAA and kinetin (0.0, 0.5, 1.0 and 2.0 mg/l) for the production of embryogenic cell suspension cultures (Figure 5.6). In this study, the dispersed callus cells that undergo embryogenic status proliferated into morphologically distinct cells, which were spherical cell masses (Figure 5.6). These cell masses were capable of organizing into multi-cellular aggregates, resembling the pro-embryogenic mass in woody plant species. These induced multi-cellular structures enlarged gradually to become globular embryos, and developed into heart-shaped, torpedo-shaped and cotyledonary embryos, after 12 weeks of embryo induction (Figure 5.5). Several attempts have been made to induce somatic embryo differentiation from woody plant species using the same cultivation system, as evidenced by liquid cultures of *Cajanus cajan* (Anbazhagan and Ganapathi, 1999), *Vigna* species (Premanand

et al., 2000), *Macrotyloma uniflorum* (Varisai *et al*., 2004) and *Musa acuminata* (Jalil *et al*., 2008).

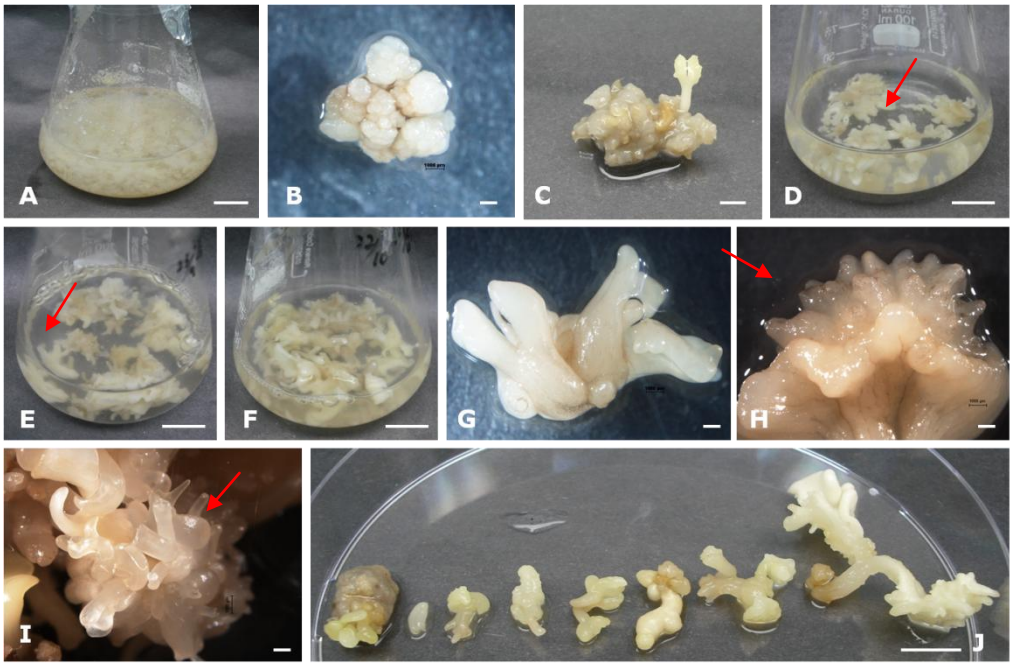


Figure 5.6. The developmental stages of somatic embryos generated in liquid medium enriched with NAA and kinetin in the dark. (A): Embryogenic cell suspension cultures established on MS medium containing 2.0 mg/l of NAA and 1.0 mg/l of kinetin (Bar = 1 cm). (B): Embryogenic cell aggregates observed on cell suspension induction medium (2.0 mg/l NAA and 1.0 mg/l kinetin) after three weeks of callus inoculation (Bar = 1000 µm). (C): Heart-shaped embryos were observed after six weeks of embryo induction on MS medium enriched with 1.0 mg/l NAA and 0.5 mg/l kinetin (Bar = 1 cm). (D, E, F): Huge number of torpedo-shaped embryos observed in MS medium supplemented with 1.0 mg/l NAA and 0.5 mg/l kinetin (Bar = 1 cm). (G): Germination of somatic embryos observed on hormone-free MS medium (Bar = 1000 µm). (H): Secondary somatic embryos formed on the surface of primary embryos on a medium containing 0.5 mg/ of NAA (Bar = 1000 µm). (I): Different developmental stages of secondary somatic embryos on liquid medium lacking growth regulators (Bar = 1000 µm). (J): The overall developmental stages of sweet shoot somatic embryos generated in liquid medium containing NAA and kinetin (Bar = 1 cm).

The present study revealed that the formation of embryogenic cell suspension cultures depended on the exogenous application of NAA and kinetin. The growth of embryogenic cells increased from a packed cell volume of 0.6 ml to 5.20 ml, as the concentration of NAA increased from 0.5 mg/l to

2.0 mg/l ($p<0.05$) (Table 5.2; Figure 5.6). Amongst the treatments tested, the highest embryogenic capacity (83.33 %) of cell suspension culture was achieved in 2.0 mg/l NAA and 1.0 mg/l kinetin at a rotating speed of 100 rpm (Table 5.2). Moreover, the similar hormone composition was also found to be effective in inducing cell growth with the highest packed cell volume of 5.20 ml (Table 5.2). From Figure 5.7, the growth profile of cell suspension cultures exhibited a sigmoidal growth curve throughout the five weeks of initiation on MS medium supplemented with 2.0 mg/l NAA and 1.0 mg/l kinetin. During the first week of culture initiation (lag phase), minimal growth (1.1 ml) was observed due to the adaptation of embryogenic cells to the newly inoculated environment (Figure 5.7) (Yen *et al*., 1999; Silveira *et al.*, 2004). As embryogenic callus entered the log phase, the cells achieved maximum density (5.2 ml) and started to grow exponentially for the subsequent two weeks of culture (Figure 5.7). By the fourth week of culture, linear and stationary phase were observed, most likely due to the depletion of nutrients in liquid MS medium leading to cell death (Figure 5.7) (Ong et *al*., 2008). This indicates that the optimum subculture interval for cell suspension cultures of sweet shoot is three weeks. The embryogenic competency was maintained for more than 3 years by repeated subculture.

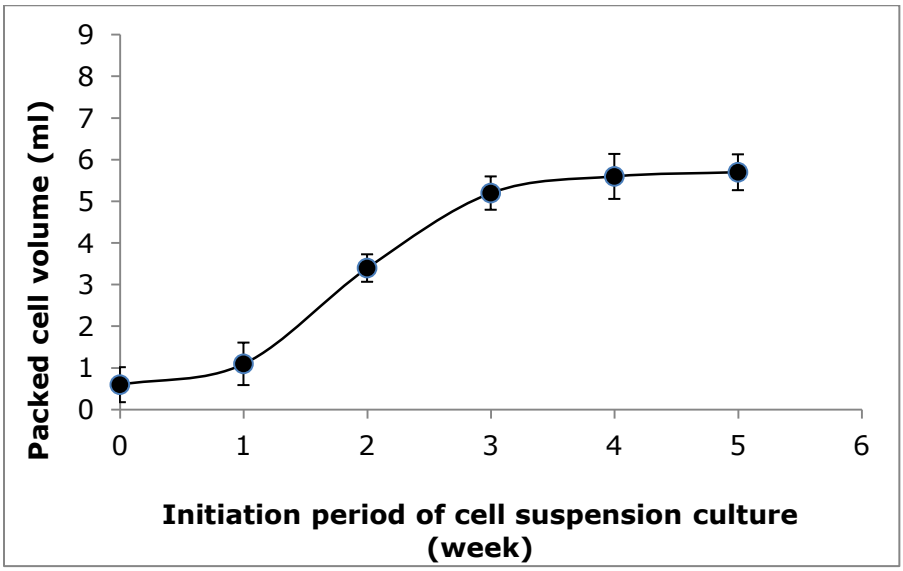


Figure 5.7. Growth curve of sweet shoot cell suspension culture after cultured for 5 weeks in MS medium supplemented with 2.0 mg/l NAA and 1.0 mg/l kinetin based on the measurement of packed cell volume. (Bar = standard deviation).

Table 5.2. Effect of hormonal treatments on the induction, development and germination of somatic embryos in liquid medium of sweet shoot.

Treatment code	Growth regulators (mg/l)		Stages of somatic embryogenesis					
			Pack cell volume (ml pellet/ml culture) (3 weeks after induction	Somatic embryo induction (6 weeks after embryo induction)			Development	
	NAA	KIN		% of responded callus	% of embryo formation	Number of globular embryo	Number of heart-shaped embryo (9 weeks after embryo induction)	Number of torpedo-shaped embryo (12 weeks after embryo induction)
T0	0.0	0.0	0.00 $_{e}$	0.00 $_{d}$	0.00 $_{e}$	0.00 $_{f}$	0.00 $_{g}$	0.00 $_{g}$
T1	0.0	0.5	0.00 $_{e}$	0.00 $_{d}$	0.00 $_{e}$	0.00 $_{f}$	0.00 $_{g}$	0.00 $_{g}$
T2	0.0	1.0	0.00 $_{e}$	0.00 $_{d}$	0.00 $_{e}$	0.00 $_{f}$	0.00 $_{g}$	0.00 $_{g}$
T3	0.0	2.0	0.00 $_{e}$	0.00 $_{d}$	0.00 $_{e}$	0.00 $_{f}$	0.00 $_{g}$	0.00 $_{g}$
T4	0.5	0.0	0.00 $_{e}$	66.67 $_{b}$	50.00 $_{bc}$	10.00 $_{c}$	9.34 $_{cd}$	7.34 $_{d}$
T5	0.5	0.5	1.10 $_{d}$	66.67 $_{b}$	33.33 $_{c}$	10.66 $_{c}$	9.00 $_{cd}$	7.00 $_{d}$
T6	0.5	1.0	0.60 $_{d}$	50.00 $_{bc}$	16.67 $_{d}$	8.00 $_{cd}$	4.00 $_{e}$	4.00 $_{e}$
T7	0.5	2.0	0.00 $_{e}$	33.33 $_{c}$	16.67 $_{d}$	4.00 $_{e}$	2.00 $_{f}$	2.00 $_{f}$
T8	1.0	0.0	0.70 $_{e}$	66.67 $_{b}$	66.67 $_{b}$	14.00 $_{b}$	13.50 $_{b}$	11.50 $_{b}$
T9	1.0	0.5	3.50 $_{b}$	83.33 $_{a}$	83.33 $_{a}$	15.60 $_{a}$	14.80 $_{a}$	13.20 $_{a}$
T10	1.0	1.0	1.40 $_{d}$	66.67 $_{b}$	33.33 $_{c}$	11.00 $_{c}$	10.00 $_{c}$	9.00 $_{c}$
T11	1.0	2.0	0.00 $_{e}$	50.00 $_{bc}$	16.67 $_{d}$	6.00 $_{de}$	4.00 $_{e}$	2.00 $_{f}$
T12	2.0	0.0	1.25 $_{d}$	66.67 $_{b}$	50.00 $_{bc}$	10.00 $_{c}$	8.66 $_{cd}$	6.00 $_{d}$
T13	2.0	0.5	2.50 $_{c}$	66.67 $_{b}$	66.67 $_{b}$	9.00 $_{cd}$	7.50 $_{cd}$	6.50 $_{d}$
T14	2.0	1.0	5.20 $_{a}$	83.33 $_{a}$	66.67 $_{b}$	8.00 $_{cd}$	6.00 $_{d}$	4.00 $_{e}$
T15	2.0	2.0	0.00 $_{e}$	0.00 $_{d}$	0.00 $_{e}$	0.00 $_{f}$	0.00 $_{g}$	0.00 $_{g}$

* Values expressed as means of 15 replicated experiments, each replicate consisted of 1 culture.

* Different letters within column (lowercase) indicate a significant difference ($p<0.05$) according to Duncan's multiple range test.

This current study also showed that hormone-free MS medium and those supplemented with kinetin alone failed to induce embryogenic cell suspension culture from leaf-derived callus, after 3 weeks of callus inoculation (Table 5.2). This finding is in agreement with the observation made for *Cassia angustifolia*, which emphasize the great importance of incorporating auxin into MS medium for the growth of embryogenic cell suspension culture (Agrawal and Sardar, 2007). A study done by Dodeman *et al*. (1997) in *Trifolium repens* also explained the effectiveness of cytokinin and auxin in promoting embryogenic cell formation from the epidermis of immature zygotic embryos. For that reason, both cytokinin and auxin were proven to be essential for somatic cell division, as documented in somatic cells of Norway spruce, carrot and lentil (Wilson *et al*., 1974; Stals and Inze, 2001). The addition of 2.0 mg/l NAA in combination with 1.0 mg/l kinetin was then suggested for somatic embryogenesis of sweet shoot for the maintenance and proliferation of embryogenic cell suspension cultures.

Recent evidence showed that exogenous auxins, like NAA and 2,4-D may have the ability to down-regulate the gene expression patterns of leaf-derived callus via DNA methylation and replaced it with an embryogenic program (Vergara *et al*., 1990). The down-regulation of gene expression that occurs upon exposure to exogenous auxins was found to resemble those which occur during sporogenesis (meiotic divisions), as proven in cell suspension cultures of carrot, soybean, and Siberian ginseng (LoSchiavo *et al*., 1989; Bonnelle *et al*., 1990; Chakrabarty *et al*., 2003). The exact effect of cytokinin and auxin during induction process, however, proves difficult to ascertain, as there are plenty of variations that can affect the induction of embryogenic state, such as explant types, plant genotypes, medium components and culture environment.

The positive interactive effects between NAA and kinetin were shown in the present study, as the embryogenic capacity of these cell lines remained intact and entered into another cycle of new embryogenic cell production (repetitive embryogenesis), even after several passages of subcultures with the same hormone composition (2.0 mg/l NAA and 1.0 mg/l kinetin) (Table 5.2). A similar phenomenon was also observed in embryogenic suspension cultures of carrot, one of the model plants for

somatic embryogenesis, whereby the repetitive embryogenesis of pro-embryogenic cell clumps was initiated in a medium containing high auxin concentration (McWilliam *et al*., 1974). Once auxin concentration was reduced below a certain threshold, the repetitive cycles of somatic embryogenesis ceased, and the histodifferentiation of carrot embryos began (Halperin and Wetherell, 1964). This shows that differing concentrations of auxin and cytokinin appear to be responsible in controlling different developmental stages of somatic embryos.

The induction of somatic embryogenesis from embryogenic cell suspension culture was positively affected by the association between auxin and cytokinin used in this experiment, as shown in Table 5.2 ($p<0.05$). Table 5.2 revealed that the highest frequency (83.33%) of somatic embryo formation was obtained from subculture of senile suspension cells on MS medium fortified with 1.0 mg/l NAA and 0.5 mg/l kinetin, after three weeks of embryo induction. This histodifferentiation medium was significantly different ($p<0.05$) compared to the other treatments, as it promoted the highest yield of globular somatic embryos with a mean number of 15.60 embryos per g of callus (Table 5.2; Figure 5.6). In the same medium, most of the globular embryos successfully proliferated into heart-shaped and torpedo-shaped embryos after nine weeks of embryo induction, with an average number of 14.80 and 13.20 embryos per g of callus, respectively (Table 5.2; Figure 5.6; Figure 5.8). The majority of the embryos re-callused prior to transfer to the conversion medium. A similarity was seen between semi-solid and liquid cultures, whereby a reduction of auxin and cytokinin concentrations from the induction medium permitted the proper development of somatic embryo in sweet shoot.

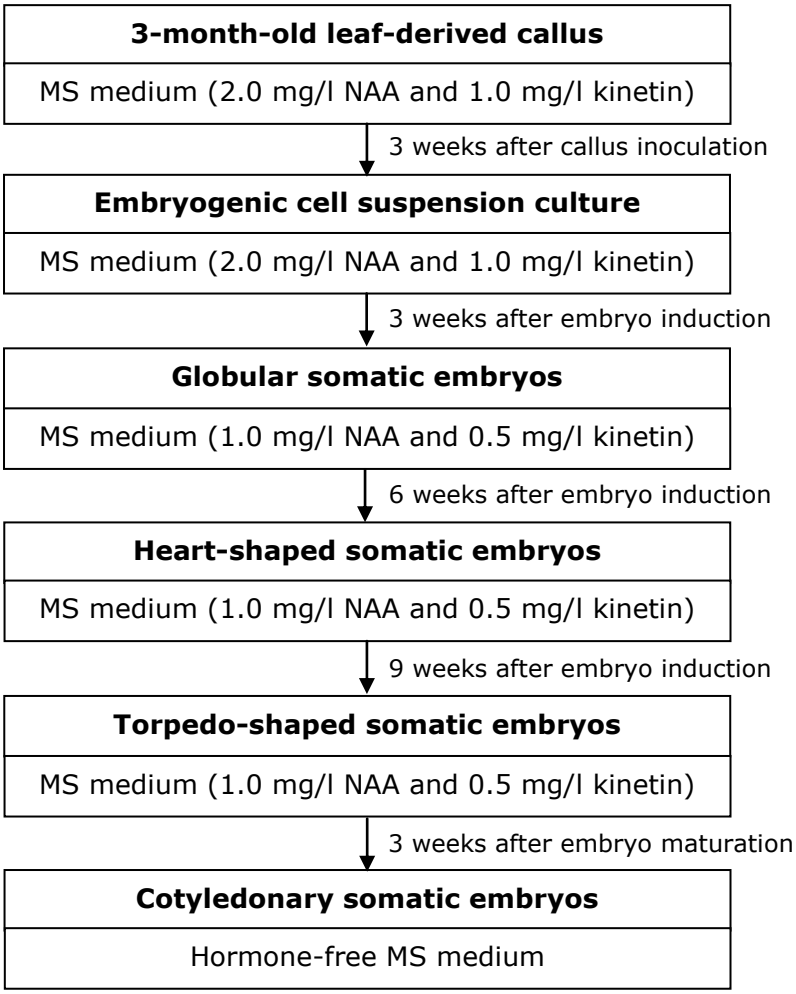


Figure 5.8. Summary of somatic embryogenesis in sweet shoot cultured in liquid MS medium.

For sweet shoot, the use of NAA alone as a histodifferentiation medium was unable to induce high amounts of good quality heart-shaped and torpedo-shaped somatic embryos (Table 5.2). It was observed that continued exposure of somatic embryos to high auxin level produced abnormal embryos with stunted polarity and poorly developed trumpet-shaped cotyledons with irregular leaf-like structure (data not shown). Sixty torpedo-shaped embryos, when transferred to hormone-free MS medium, greatly improved the maturation rate (90%) of embryos (54 cotyledonary embryos) after three weeks of transfer from the histodifferentiation medium (Table 5.2; Figure 5.6). The absence of plant growth regulators in conversion medium suggests that the embryos of sweet shoot may have the ability to synthesize hormones required for successful maturation during its embryo development, as previously reported by Thorpe (1995) in woody plant species. After two months of shoot induction, the mature somatic embryos formed shoots and roots

from the exact poles on semi-solid MS medium supplemented with 2.0 mg/l BAP and 0.5 mg/l IAA. The outcome of this study proved that plant regeneration of sweet shoot can be established via indirect somatic embryogenesis using both liquid and semi-solid MS medium supplemented with optimal concentration of NAA and kinetin.

During the maturation of somatic embryos, continued presence of NAA and kinetin in the conversion medium resulted in morphologically abnormal embryos such as fused cotyledons, fused embryos, and multiple cotyledons and radicals (data not shown), albeit in low percentage (<10.67%). These abnormal somatic embryos rarely generated into plantlets, as evidenced in embryogenic cultures of soybean (Buchheim *et al.*, 1989) and alfalfa (Xu and Bewley, 1992). One of the many possible reasons may be due to the detrimental effects of auxin on apical and lateral meristem development, which led to the reduction of embryo germination capacity in the later stage of somatic embryogenesis (Piccioni *et al*., 1997). A study done by von Arnold *et al*. (2002) on carrot somatic embryogenesis showed that the frequency of DNA methylation increased as the auxin concentration increased in the conversion medium, which gave rise to the development of abnormal embryos. Furthermore, the addition of cytokinin in conversion medium has been reported to suppress the development of main embryo axis, as described by Aung *et al.* (1982) and Danin *et al*. (1993) in somatic embryogenic cultures of soybean and celery. Hence, treatments such as inclusion of activated charcoal and abscisic acid in the conversion medium could lead to the recovery of somatic embryos with a normal morphology and help to increase the germination ability of somatic embryos in woody plant species, simply by absorbing the auxins and cytokinins released from developing tissues (Buchheim *et al*., 1989; Ebert and Taylor, 1990). Future studies using physiological, biochemical and molecular markers may enable scientists to localize the abnormal structure of somatic embryo easily, when compared to direct visualisation.

Overall, a marked increase in somatic embryo production was observed in liquid MS medium containing NAA and kinetin, as compared to those obtained on semi-solid medium. This could be due to the poor diffusion rate of nutrients through the solid matrix (Romberger and Tabor, 1971), low water content (Debergh *et al*., 1981) and the modification of

the elemental composition of the medium (Singha *et al*., 1985). Hence, the use of suspension cultures is an important component in the scale-up and synchronization of embryogenic cultures. This was proven in a study done by Lulsdorf *et al*. (1992) showing that *Picea glauca-engelmannii* and *Picea mariana* yielded 4000 and 6000 pro-embryos when grown on liquid MS suspension culture, respectively.

5.3.3 Histological examination of somatic embryo cultures

Somatic embryogenesis is an important regenerative pathway for woody plant species, by which the somatic cells undergo a developmental sequence similar to those seen in zygotic embryos (Dudits *et al*., 1991). In the last decade, there are many comprehensive reviews dealing with different aspects of somatic embryogenesis, especially on the manipulation of medium compositions and plant growth regulators for optimal production of somatic embryos in carrot, soybean, pea and many non-woody plant species (Williams and Maheswaran, 1986; Carman, 1990; Thorpe, 1995). However, only a few detailed articles have been reported on the structural aspects of somatic embryogenesis, with regards to characterization of ontogenetic stages in somatic embryos (Fransz and Schel, 1991; Toonen *et al*., 1994; Yeung *et al*., 1996). Thus, it is imperative to monitor the morphological and anatomical characteristic changes during embryo development, since no research has been conducted on the histological study of sweet shoot somatic embryos.

In this study, somatic embryogenesis of sweet shoot involves control of four consecutive steps, namely (1) induction of embryogenic cell lines from cell suspension culture, (2) maintenance and multiplication of embryogenic cell lines, (3) maturation of somatic embryos, and (4) conversion of somatic embryos into viable plantlets. Histological sections prepared from embryogenic cultures at different developmental stages revealed the complete ontogeny of somatic embryos in sweet shoot, starting with globular embryos through to cotyledonary embryos, and these were similar to the zygotic counterparts. The concepts of cell competence, induction and determination used by animal biologists (Ammirato, 1987) were adopted in the histological observation of sweet shoot, to have a better insight in the early events leading to somatic embryo formation. Somatic cells in suspension cultures of sweet shoot were dedifferentiated to enable them to become competent and respond

to specific signals prior to the induction process, which corresponds to the study done by Halperin (1966) in carrot embryogenic cultures. The inductive signals mentioned above were in the forms of plant growth regulators, such as auxin and cytokinin used in this experiment. The results emphasize the importance of auxin in channeling cells into the final determined state with the capacity to form the somatic embryos of sweet shoot. This finding was supported by the work done by Nomura and Komamine (1985), in which auxin accelerated the acquisition of embryogenic potential of carrot embryos.

This study also attempted to compare the fine structure of quiescent and growing somatic cells in pro-embryogenic cell mass and non-embryogenic cell mass of sweet shoot in cell suspension cultures. After 21 days of culture incubation on MS medium fortified with 2.0 mg/l NAA and 1.0 mg/l kinetin, visualization under light microscope revealed a peripheral region of pro-embryogenic cell mass consisting of small, dense cytoplasmic cells with prominent nuclei, whereas the non-embryogenic cells were large in size and highly vacuolated in appearance (Figure 5.9). An accumulation of starch granules was abundant within the cytoplasm of pro-embryogenic mass in sweet shoot (Figure 5.9). The quantity of starch granules present in the cytoplasm was found to be closely related to the mitotic activity of pro-embryogenic cells, as observed in *Lycopodiella inundata*, where the number of starch granules varied from 20 starch granules during the S phase of cell cycle, reduced to 10 units at the end of the telophase and then increased to 40 starch granules during the interphase of cell cycle (Atmane *et al*., 2000). Additionally, the histological observation of sweet shoot also revealed the presence of numerous organelles within the dense cytoplasm of pro-embryogenic mass that were capable of rapid cell division (Figure 5.9), as observed in *Ranunculus sceleratus*, pea and carrot embryogenic culture (Halperin and Jensen, 1967; Thomas *et al*., 1972; Tautorus *et al*., 1991). It is proven that mitogenic activity is an essential property of embryogenic cells in sweet shoot. Polarized development occurred early in somatic embryogenesis of sweet shoot suggesting pattern formation has begun and this process is essential to subsequent histodifferentiation within the embryo proper.

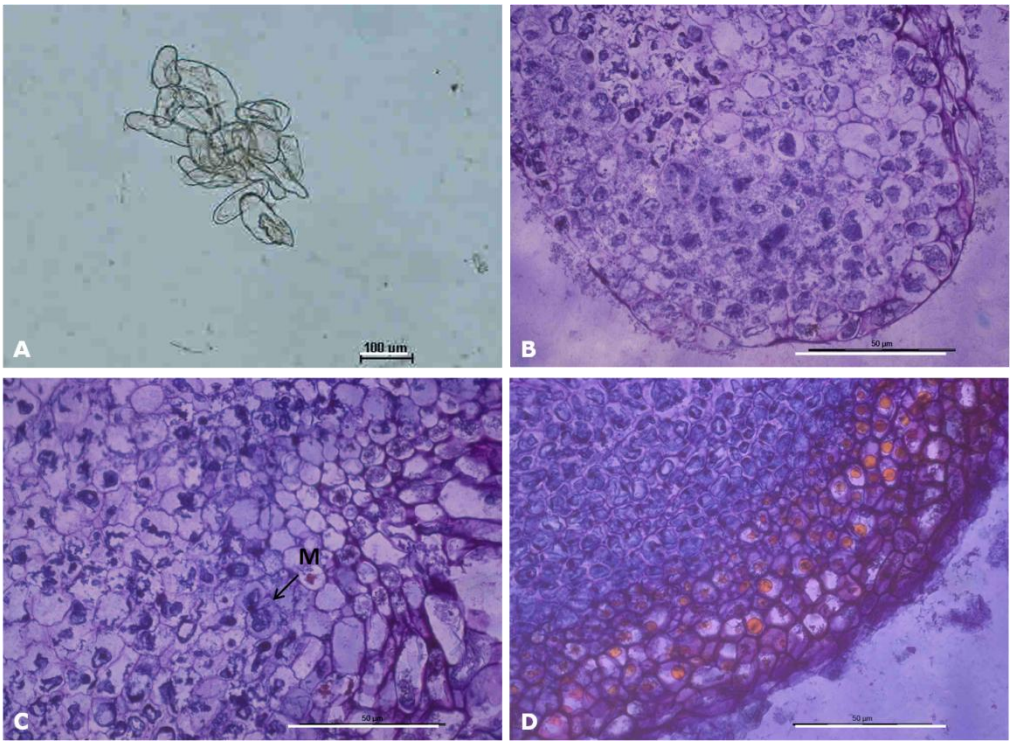


Figure 5.9. Comparison of non-embryogenic and embryogenic cell clusters in cell suspension cultures of sweet shoot. (A): Non-embryogenic cells were large in size and highly vacuolated in appearance (Bar = 100 µm). (B): Embryogenic cell mass consisted of small, dense cytoplasmic cells with prominent nuclei (Bar = 50 µm). (C): Meristematic mass showing individualized groups of cells delimited by thickened cell walls. Mitotic activity of embryogenic cells resulted in the formation of globular embryo (M = mitotic activity) (Bar = 50 µm). (D): Starch granules abundant within the cytoplasm (Bar = 50 µm).

Based on the results shown in Figure 5.10, primary somatic embryos of sweet shoot proliferated from a cluster of embryogenic cells via tissue growth and fragmentation. These embryogenic cells showed continuous mitotic division in suspension cultures and formed globular masses (pro-embryoids) that exhibited a distinct epidermis after three weeks of culture initiation (Figure 5.10). These results were consistent with the studies done by Halperin (1970), McWilliam *et al*. (1974), and Jones and Rost (1989). As illustrated in these studies, carrot and rice somatic embryos derived from cellular aggregates were not directly from a single, free floating cell but from many embryogenic cells. In this study, there were only a few somatic embryos of sweet shoot produced from a single pro-embryogenic cell mass. This may be due to the increased competition in metabolic processes, which prevented the further development of some embryogenic cells in favour of others (Jones and Rost, 1989). As embryogenic cells are tightly aggregated and have similar

metabolic processes, metabolic co-operation between these cells were more efficient in carrying out single tasks, and these metabolic processes could have led to fewer number of somatic embryos being formed. Hence, the strategy involved in the regulation of continual embryo development may be quite different from that involved in the induction and determination events.

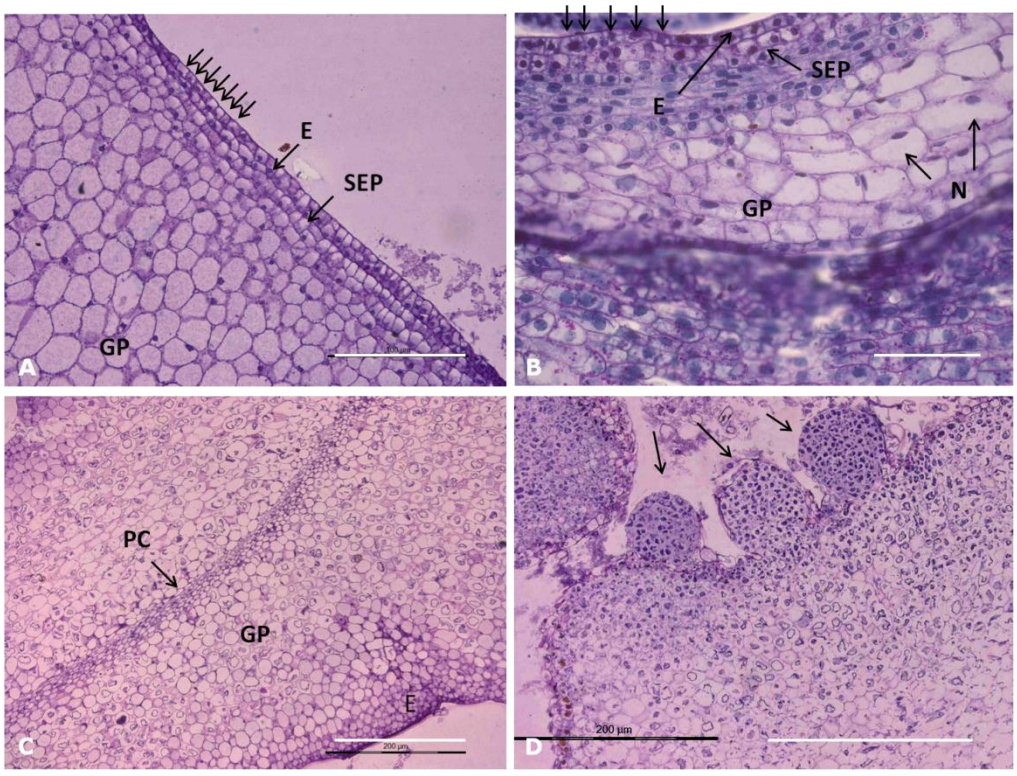


Figure 5.10. A light micrograph showed longitudinal sections of pro-embryogenic cell mass in liquid suspension culture of sweet shoot. (A, B): Transverse section showing periclinal division of embryogenic cell (arrows) in explant epidermis (E = epidermis; SEP = subepidermis; GP = ground parenchyma; N = nucleus) (Bar = 100 µm). (C): Longitudinal section of embryogenic cells mass showing the initiation of procambium. (PC = procambium; GP = ground parenchyma) (Bar = 200 µm). (D): Differentiation of pro-embryogenic globules from meristematic mass, after six weeks of culture initiation (arrows) (Bar = 200 µm).

Upon the transfer of the embryogenic cells onto liquid MS medium with reduced plant growth regulators (1.0 mg/l NAA and 0.5 mg/l kinetin), histodifferentiation of somatic embryos in sweet shoot occurred after six weeks of culture induction. The originally smooth embryogenic cell mass took on a more undulated appearance, as superficial embryogenic cells began to divide and gave rise to globular somatic embryos of sweet shoot (Figure 5.10). Interestingly, this current study showed that the primary

somatic embryo originated from a periclinal division of one epidermal cell, and this marked the early expression of a new developmental sequence from the epidermal cell of a pro-embryogenic cell mass (Figure 5.10). The histodifferentiation of somatic embryos in sweet shoot continued with formation of spherical globules delimited by the epidermal layer, which further developed into typical somatic embryos. The terminal cell of spherical globules gave rise to the embryo proper while the basal cell formed the suspensor. These structural characteristics of primary somatic embryos corresponded to the somatic embryogenesis as described by Sharp *et al*. (1980), Maheswaran and Williams (1986) and Wann (1988).

Changes in mitotic activity helped to establish polarity of somatic embryos within a pro-embryogenic cell mass, as shown in this study. Further differentiation of these cell structures in globular embryos led to the formation of heart-shaped, torpedo-shaped and cotyledonary embryos on the surface of an embryogenic callus. Besides that, the organelles and starch grains present in globular embryos of sweet shoot became even more abundant as compared to the embryogenic cells described above (Figure 5.11). The premitotic increase in cellular activity may be reflected by the increase in number of organelles and changes in the nuclear morphology and vacuolar structures (Schulz, 1988). These changes reflect premitotic enhancement of respiration, protein and carbohydrate synthesis, and a turnover of membranes, similar to the cytoplasmic reactivation process in the root cortical cells of pea during vascular differentiation (Schulz, 1988).

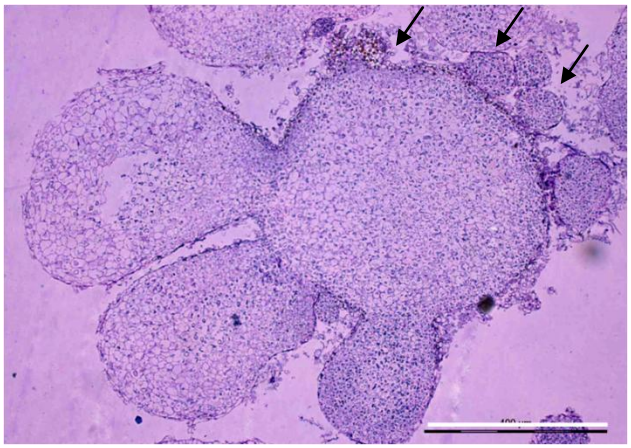


Figure 5.11. The formation of early globular somatic embryos from embryogenic cell mass of sweet shoot, after three weeks of culture induction on MS medium supplemented with 1.0 mg/l NAA and 0.5 mg/l kinetin. The number of starch granules (orange) increased in globular somatic embryos, which was responsible for the cellular activity of embryogenic cells (Bar = 400 µm).

The transition of sweet shoot pro-embryoids to pre-globular and globular stages was also characterized by the formation of concentric rings of cells with a small vacuole and a prominent cytoplasm (Figure 5.12). The globular embryos of sweet shoot were attached to the embryogenic callus with a prominent multicellular stalk called the suspensor, which showed long vacuolated cells with a slightly stained cytoplasm (Figure 5.12). In the study of suspensor function on *Phaseolus* (Yeung, 1980) and maize (Schel *et al*., 1984), the suspensor was shown to serve as a conduit of nutrients for the young developing embryos. In this study, the suspensor was absent in some samples, replaced by a few layers of more densely-stained cells that supported the connection between the globular embryo and embryogenic callus. These cells could represent either a disorganized suspensor or a differential developmental pattern of this structure, as previously reported by Santos *et al*. (2006). The differences in suspensor morphology may be due to the timing of polarity establishment. Zygotic embryos of woody plant species have strong polarity and thus clear suspensor formation; whereas, during the early formation of somatic embryos, the polarity is ill-defined and prevented the formation and development of a structurally defined suspensor.

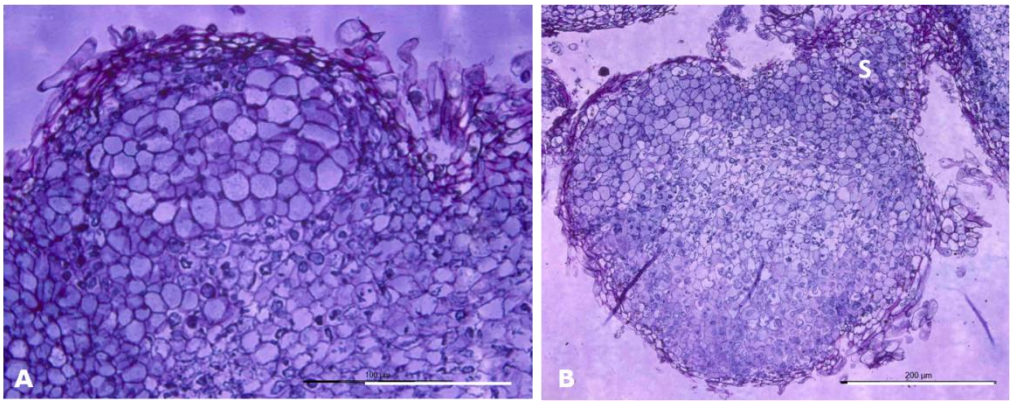


Figure 5.12. (A): The presence of a ring of cells with small vacuole and prominent cytoplasm during the formation of preglobular embryo (Bar = 100 µm). (B): The suspensor formed through successive divisions of base cells (S = suspensor) (Bar = 200 µm).

As somatic embryo of sweet shoot developed, the globular embryo elongated to form a heart-shaped embryo, after nine weeks of embryo induction (Figure 5.13). Prior to the formation of heart-shaped embryo, axial elongation of the inner isodiametric cells of globular embryo led to the formation of a longitudinal extension near the lower end of an embryo. The ground meristem of heart-shaped embryo began to vacuolate and this transition event continued throughout embryo maturation. Similar observations were reported by Schiavone and Cooke (1985) in carrot embryogenic cultures.

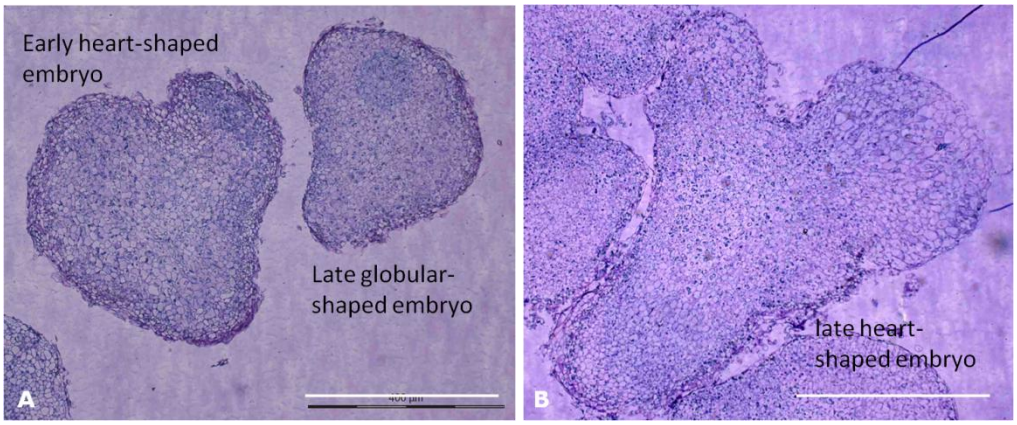


Figure 5.13. (A): The globular somatic embryo began to elongate into heart-shaped embryo of sweet shoot with well defined protoderm, after nine weeks of culture (Bar = 400 µm). (B): Axial elongation of the inner isodiametric cells of globular embryo led to the formation of a longitudinal extension near the lower end of heart-shaped embryo (Bar = 400 µm).

One of the most important structural features in heart-shaped somatic embryos is the differentiation of protoderm, the outermost layer

of a developing embryo (Figure 5.13). The protoderm of sweet shoot was distinguished in the early stage of heart-shaped embryo, but it was better defined in the later stage of heart-shaped embryo, after an incubation period of nine weeks (Figure 5.13). In sweet shoot, the protoderm cells of the heart-shaped embryos were not as tightly packed as the zygotic embryos in woody plant species. The studies done by McWilliam *et al*. (1974) and Santos *et al*. (1983) indicated the tendency of protoderm to form secondary embryos in carrot and alfalfa, due to the absence of a fully differentiated protoderm. It was also observed that abnormal protoderm formation led to the arrest of sweet shoot somatic embryos. The problems associated with arrested somatic embryos could be overcome by identifying the glycoproteins which were responsible for the proper development of protoderm (Sterk *et al*., 1991; Von Engelen *et al*., 1991; De Jong *et al*., 1992). These histological results clearly indicate the importance of protoderm in the further development of sweet shoot somatic embryo.

The formation of early torpedo-shaped embryo is the first sign of incipient formation of procambium, shoot and root apical meristem in sweet shoot (Figure 5.14; Figure 5.15). The conversion of heart-shaped embryos to torpedo-shaped embryos coincided with the differentiation of procambium-like cells in the central core towards the root pole (Figure 5.14). According to Schiavone and Cooke (1985), the change in growth axis of procambium served as an important step in the morphogenesis of woody plant species, as it involved changes of cytoskeletal pattern of cells, such as cell size and shape. The results obtained in this study were in agreement with the study done on soybean, whereby the differentiated internal structures, such as procambium, shoot and root apical meristems can only be found in torpedo-shaped embryos (Santos *et al*., 2006).

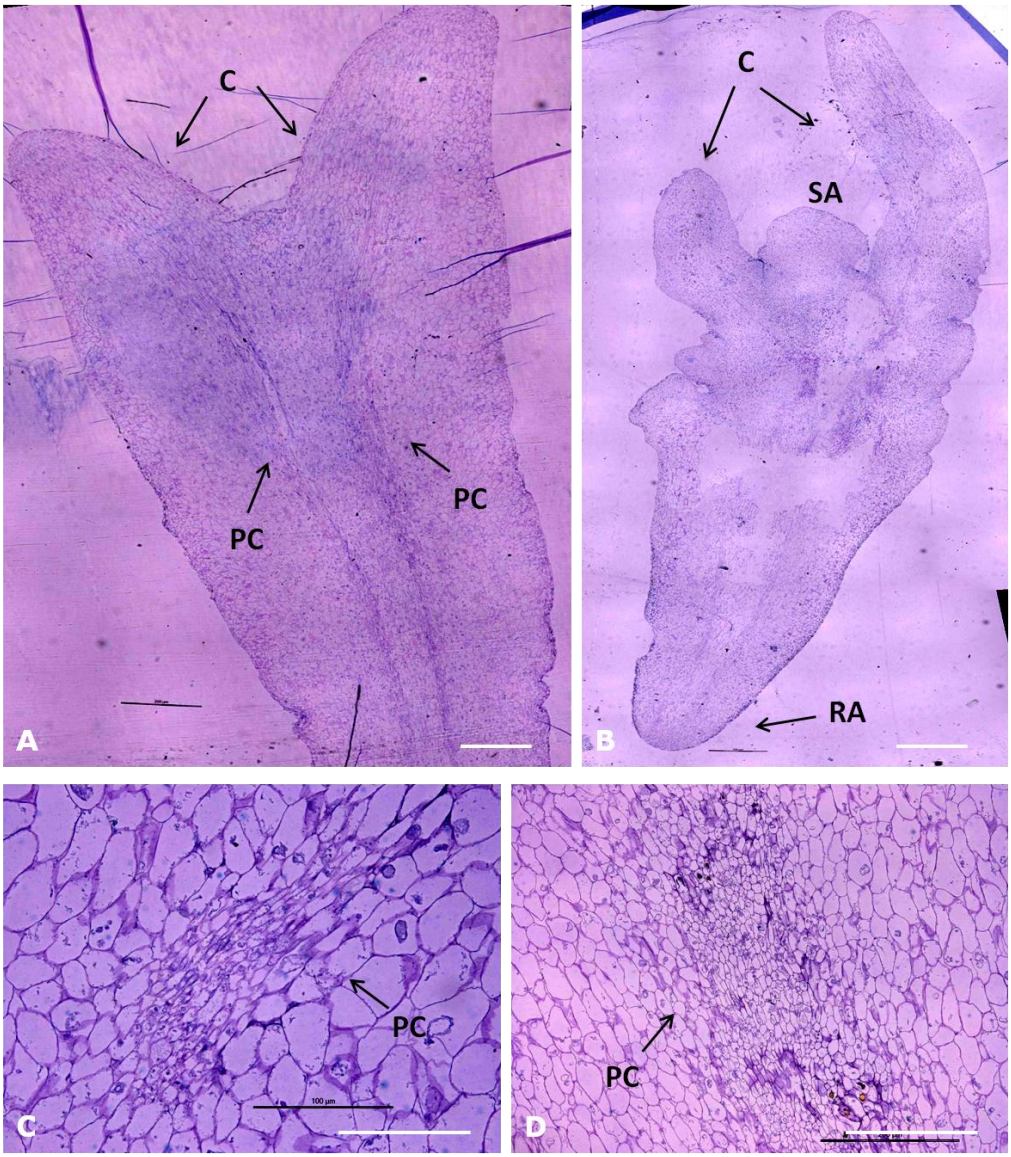


Figure 5.14. (A): Longitudinal section of early torpedo-shaped embryo showing the formation of bipolar structure from heart-shaped embryo with thickened procambium (PC = procambium; C = cotyledon) (Bar = 400 µm). (B): The initial formation of cotyledon primordia, shoot and root apical meristems in late torpedo-shaped embryos, after 12 weeks of embryo induction on MS medium fortified with 1.0 mg/l NAA and 0.5 mg/l kinetin (SA = shoot apical meristem; RA = root apical meristem) (Bar = 400 µm). (C): Initial development of procambium in torpedo-shaped somatic embryos of sweet shoot (PC = procambium) (Bar = 100 µm). (D): A slightly more developed procambium was observed at the later stage of torpedo-shaped embryo (PC = procambium) (Bar = 200 µm).

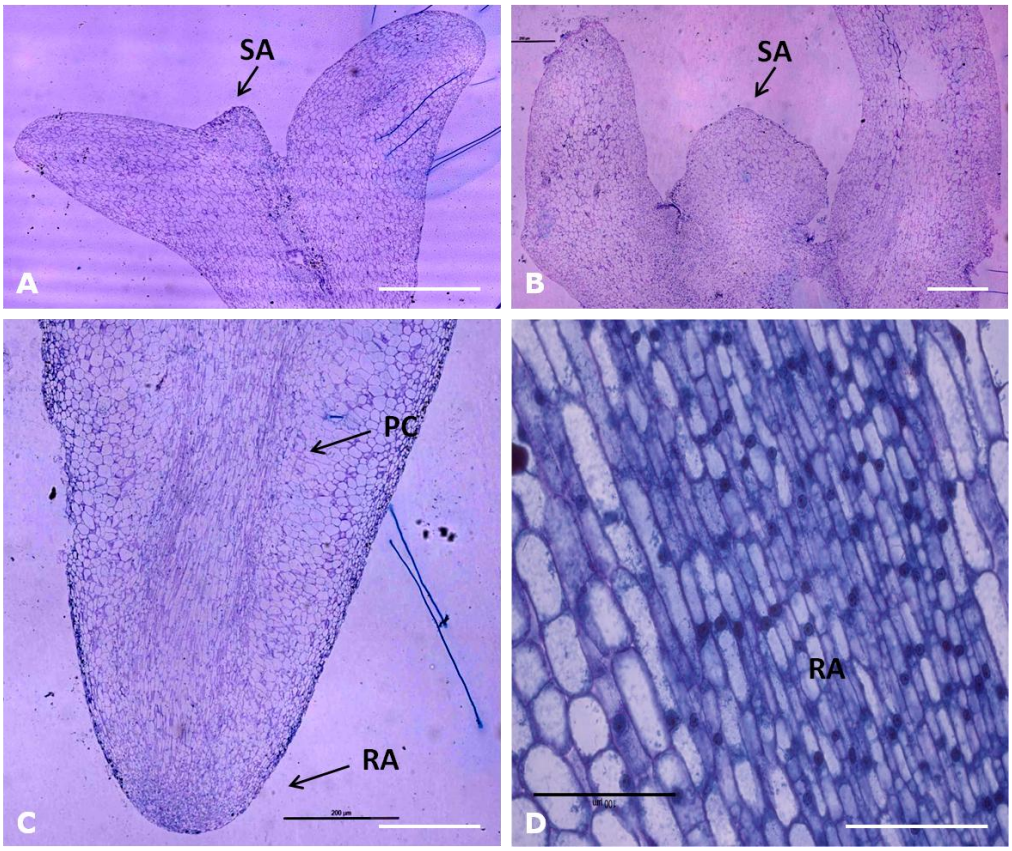


Figure 5.15. (A): The development of shoot apical meristem was first detected in early torpedo-shaped somatic embryo of sweet shoot (SA = shoot apical meristem) (Bar = 200 µm). (B): Detail of shoot apical meristem of late torpedo-shaped embryo (SA = shoot apical meristem) (Bar = 200 µm). (C): Longitudinal sections showing the initiation of root apical meristem coupled with well defined procambium at the later stage of torpedo-shaped embryo (RA = root apical meristem; PC = procambium) (Bar = 200 µm). (D): Longitudinal sections showing the cells of root apical meristem with prominent nucleus (RA = root apical meristem) (Bar = 200 µm).

The next important morphogenetic event in somatic embryogenesis of sweet shoot is the formation of cotyledons and well defined embryonic axes (root and shoot apical meristems) (Figure 5.16). The initiation of cotyledon primordia and apical meristems denoted the beginning of cotyledonary stage of embryo development. From the structural standpoint, the differentiation of these respective structures was judged from their position, cell profiles and cytoplasmic density. The cotyledon primordia of sweet shoot was composed of dense isodiametric cells with conspicuous nuclei and it arose as a small protrusion from the peripheral region of the terminal end of somatic embryos. The protoderm cells of cotyledonary embryo were tightly packed and remained cytoplasmic, whereas the cells of ground parenchyma tissue were highly vacuolated

with small intercellular spaces. The center core of cotyledonary embryo was occupied by the procambium, which traversed the ground parenchyma and bifurcated into the cotyledons at the shoot pole (Figure 5.16). Vascular tissues were derived from the procambium at later stage of cotyledonary embryos (Figure 5.16). The shoot apical meristem consisted of a small group of cytoplasmic cells occupying the apical notch between the cotyledons. The root apical meristem occupied the region between the cap and the procambium poles. The regeneration patterns of *Arabidopsis* and carrot somatic embryos suggested that the development of root apical meristem may be tightly coupled to the differentiation of shoot apical meristem, whereby the shoot pole of embryo was found to regulate the growth of the root pole (Schiavone and Racusen, 1990; Barton and Poethig, 1993). The somatic embryos generated in this study showed normal developed cotyledons and the ontogenetic route of somatic embryos resembled closely the development of carrot zygotic embryos (Schiavone, 1988). The work of Schiavone and Racusen (1990) suggested the importance of cotyledons in somatic embryo regeneration, as different regions of carrot somatic embryos showed strong regenerative powers due to the presence of spatially-specific proteins in the upper and lower half of the embryo (Racusen and Schiavone, 1988). Since the cotyledon constituted most of the major organs in cotyledonary embryo, it is most likely the candidate for regeneration and maintenance of observed polarity in somatic embryos of sweet shoot.

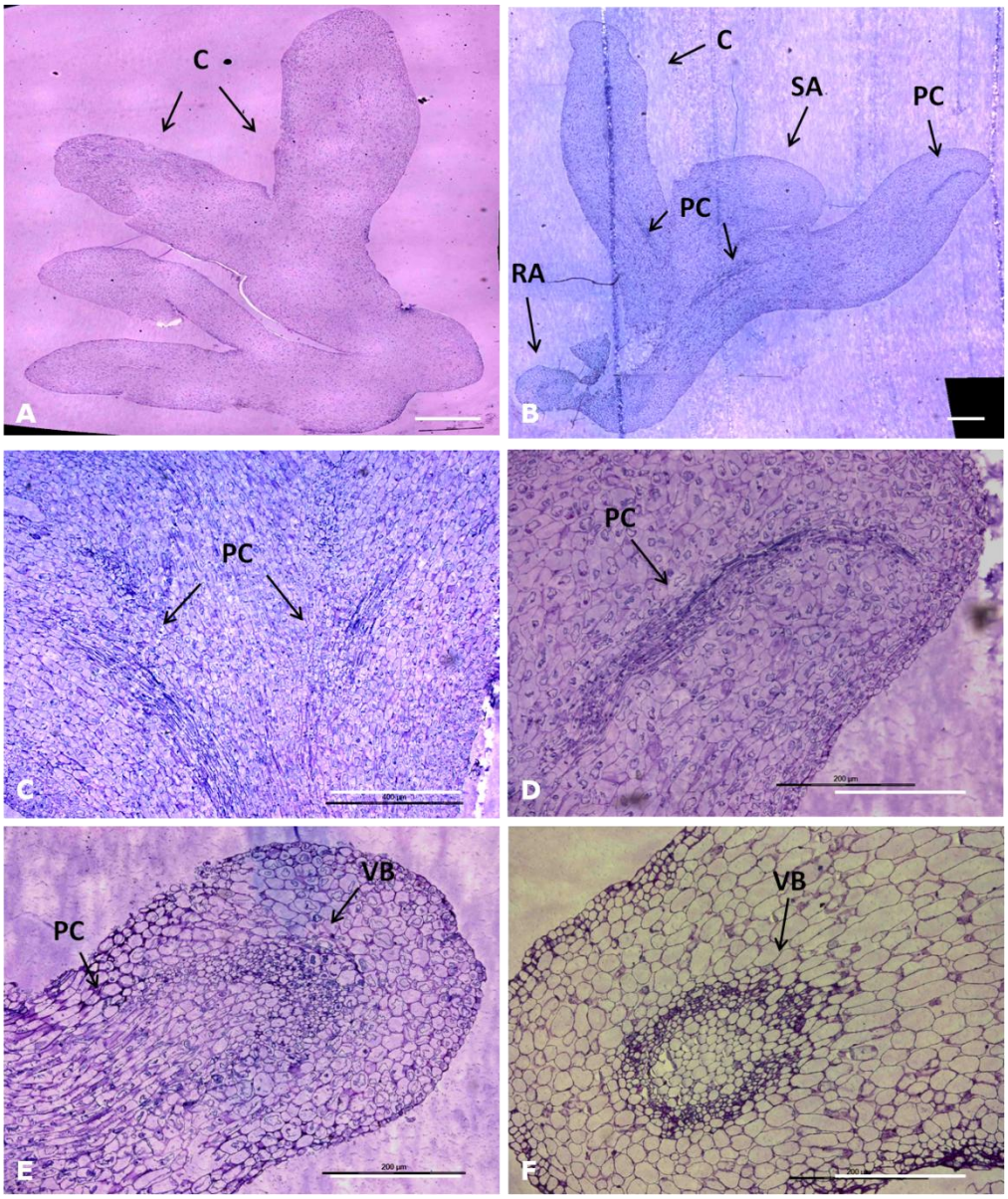


Figure 5.16. (A): Torpedo-shaped somatic embryo of sweet shoot was successfully differentiated into cotyledonary embryo, when transferred to hormone-free MS medium (C = cotyledon) (Bar = 400 µm). (B): The main embryogenic characteristics such as nuclei (N), cytoplasmic structures, meristematic tissues, cotyledons (C), procambium (PC), shoot and root apical meristem (SA and RA) were observed in this stage of somatic embryo (Bar = 400 µm). (C, D): The procambium located at the center core of cotyledonary embryo were bifurcated into the cotyledons at the shoot pole (Bar = 200 µm). (E, F): Vascular tissues (VB) were derived from the procambium at the later stage of cotyledonary embryos (Bar = 200 µm).

5.3.4 Plant regeneration from somatic embryos of sweet shoot

The formation of shoot and root apical meristems is one of the most important events in somatic embryogenesis. In sweet shoot, the germinability of somatic embryo depended on the proper development of a

normal and functional meristem. Cotyledonary somatic embryos of sweet shoot were successfully germinated into shoot clumps after transfer to MS medium containing 2.0 mg/l BAP and 0.5 mg/l IAA. A positive result was obtained in this study for adventitious shoot proliferation, as high frequency of shoot formation (75%) was achieved after two months of initiation from the cotyledonary embryos, with a mean of 6.45 shoots per embryo and an average shoot length of 5.69 cm. Out of 60 plantlets transplanted *ex vitro*, survival of 76.67% was achieved after one months of acclimatization in the shadehouse. Although the histological origin of these shoots was not monitored in this study, it is believed that sweet shoot may undergo the same process of meristem initiation and development that originates somatic embryos. These results showed that the experiment is reproducible, as it managed to generate the same positive findings as stated in Section 3.4 and 4.4.

In this study, about 25% of sweet shoot embryos and plantlets displayed poor and aberrant germination, growth and vigor, which may be due to the absence of a functional apical meristem (Thorpe, 1995). This abnormality may result from the presence of pronounced vacuolation in the apical notch of abnormal embryos, thus leading to shoot necrosis, as mentioned in the studies of embryogenic cultures of grape (Gray, 1992) and *Daucus carota* (Nickle and Yeung, 1993). Furthermore, the germination of embryos may also be inhibited by the residual auxin from the previous maturation treatment (Rajasekaran *et al*., 1987). Therefore, experimental manipulations such as the addition of abscisic acid and silver nitrate, the removal of ethylene and osmotic stress, and desiccation treatment can be used to improve the organization of apical meristem and its subsequent conversion step (Kong and Yeung, 1992; Nickle and Yeung, 1993).

5.4 CONCLUSION

A reliable and reproducible protocol for indirect somatic embryogenesis of sweet shoot was successfully established from leaf-derived callus cultures using both semi-solid medium and liquid medium together with the optimal concentrations of NAA and kinetin. Somatic embryos obtained from semi-solid medium resulted in the expansion of embryogenic callus without any production of fine embryogenic suspension, whereas the dedifferentiated somatic cells in liquid medium

became competent and proliferated into embryogenic cell suspension after responding to optimal NAA and kinetin concentrations. The cultivation of somatic embryos via liquid medium is preferable over semi-solid medium, as it induced the highest packed cell volume of embryogenic cell suspension and produced the highest number of somatic embryos at each individual stage of sweet shoot. This study showed that the supplementation of NAA and kinetin helped to regulate the growth of embryogenic cells, greatly enhanced the embryo histodifferentiation process, and led to the complete embryo maturation and development of sweet shoot. The highest embryogenic capacity (83.33%) of embryogenic cell suspension culture was achieved in 2.0 mg/l NAA and 1.0 mg/l kinetin, with an average packed cell volume of 5.2 ml. MS medium containing 1.0 mg/l NAA and 0.5 mg/l kinetin was used as an induction medium to induce the highest yield of globular somatic embryos (7.80 embryos), after three weeks of embryo induction. The histological results also showed that most of the globular embryos successfully differentiated into heart-shaped (6.20 embryos) and torpedo-shaped embryos (5.80 embryos) using the same hormonal composition, after nine weeks of embryo induction. Therefore, NAA synthetic auxin in combination with kinetin is compatible with the expression of indirect somatic embryogenesis pathway of sweet shoot. After 12 weeks of embryo initiation from embryogenic cell suspension cultures, a maximum maturation rate (90%) of cotyledonary embryo was obtained on hormone-free MS medium. A conversion rate of 75% was attained for sweet shoot, and 76.67% of the regenerated plants survived in the shadehouse. As shown in the histological study done in Section 5.3.3, the somatic embryos of sweet shoot progressed through the globular, heart, torpedo and cotyledonary stages, similar to the zygotic embryos and somatic embryos of carrot and soybean (Figure 5.1). The main embryogenic characteristics such as starch grains, nuclei, procambium, cytoplasmic structures and meristematic tissues are seen in all stages of sweet shoot. This report conclusively describes the complete protocol for somatic embryogenesis of sweet shoot from leaf-derived callus all the way to an established plantlet, and is also the only report of its kind. This is also a novel study on sweet shoot histology that is able to provide new in-depth understanding on the ontogenesis of indirect somatic embryogenesis.

CHAPTER 6: DETERMINATION OF TOTAL PHENOLIC CONTENT, TOTAL FLAVONOID CONTENT, ANTIOXIDANT ACTIVITIES AND ENZYMATIC ACTIVITIES OF ELICITED AND NON-ELICITED CULTURES OF *Sauropus androgynus*

6.1 INTRODUCTION

In the past decade, antioxidant compounds have been shown to exhibit great potential for prevention of many diseases in humans, such as cancer-related diseases, neurodegenerative conditions and coronary heart diseases (Gordon, 1996; Leutner, 2001; Matkowski, 2008). Antioxidants such as flavonoids and phenolic acids can be found virtually in all plant parts, especially the photosynthesizing plant cells of woody plant species (Kumar and Pandey, 2013). Several qualitative tests were conducted on sweet shoot and were documented to possess antioxidant activities due to the presence of phenolic and flavonoid compounds in methanolic leaf extracts (Miean and Mohamed, 2001; Wong *et al*., 2006; Benjapak *et al*., 2008; Maisuthisakul *et al*., 2008; Nahak and Sahu, 2010; Andarwulan *et al*., 2010; Lee *et al*., 2011; Shubha *et al*., 2011; Andarwulan *et al*., 2012). However, the amounts of these antioxidant compounds in field-grown plants of sweet shoot were negligible for promotion as a source of antioxidants for human consumption. Therefore, plant tissue culture technology served as an alternative method to enhance the production of antioxidant compounds, in order to maximize the full potential of sweet shoot.

Even though plant tissue cultures hold great promises for the antioxidant production in sweet shoot, there is still a lack of basic knowledge on the biosynthetic pathway and the mechanisms for the production of antioxidant metabolites (Namdeo, 2007). Many studies have shown that the low productivity of antioxidant metabolites were mainly due to the lack of specific precursors in the metabolic pathway (Rao and Ravishankar, 2002; Kim *et al.*, 2006). Therefore, the addition of elicitors, precursors and intermediate metabolites were believed to be useful in increasing the yield of antioxidant compounds (Lila *et al.*, 2005). In most cases, methyl jasmonate (MJ) and salicylic acid (SA) are potent elicitors, which help to trigger the signal transduction pathway responsible for biochemical and physiological processes in plants (Creelman and Mullet, 1997; Koca and Karaman, 2014). The use of MJ and SA elicitation method

was reported for the production of anthocyanins in soybean (Franceschi and Grimes, 1991), phenolics in sweet basil (Singh *et al.*, 1998), alkaloids in *Nicotiana* species (Keinanen *et al.,* 2001) and terpenoids in *Hyoscyamus muticus* (Martin *et al.*, 2002).

Besides that, precursor treatment with phenylalanine (Phe) is also another possible approach used in influencing the biosynthetic pathway in plant cell system (Kovacik *et al.*, 2007; Shinde *et al.,* 2009a). According to Fraser and Chapple (2011), Phe is an end product of shikimate pathway and also served as an upstream metabolic precursor in phenylpropanoid pathway as shown in Figure 6.1. From Phe, the biosynthesis of phenolics and flavonoids proceeds through a series of enzymatic reactions, such as phenylalanine ammonia-lyase (PAL), cinnamic acid 4-hydroxylase (C4H) and 4-coumarate:CoA ligase (4CL) to yield 4-coumaroyl-CoA (Ritter and Schulz, 2004). The condensation of one molecule of 4-coumaroyl-CoA and three molecules of malonyl-CoA facilitated the production of naringenin chalcone (Kao *et al*., 2002). This reaction is performed by *chalcone synthase* (*CHS*) and leads to the production of chalcone scaffolds from which all flavonoids are derived from (Ferreyra *et al*., 2012) (Figure 6.2). In year 2004, Sivakumar *et al.* displayed the highest PAL enzymatic activity (increment of 2.25 fold) and colchicine contents (higher by 67.5 fold) after the exogenous application of Phe (30 mM) and tyrosine (30 mM) in callus cultures of *Gloriosa superba*. Furthermore, Phe could be the preferred precursor compared to other compounds (tyrosine, proline and glutamine) in the phenylpropanoid pathway as it is inexpensive and more effective in the inducement of phenolic and flavonoid accumulation in woody plant species (Li and Zhu, 1990). Hence, the addition of Phe has the economical advantage to enhance the production of both flavonoid and phenolic compounds in sweet shoot.

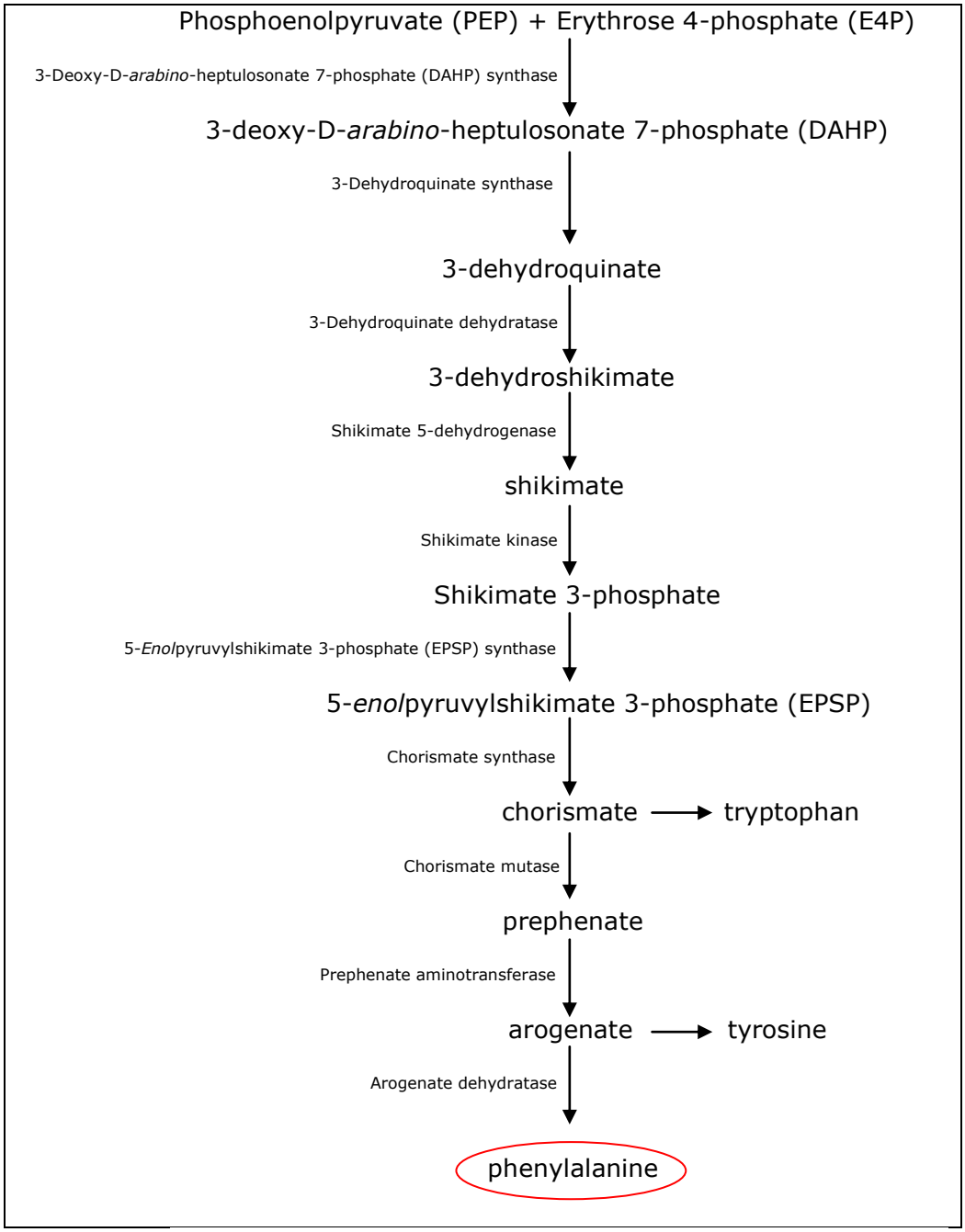


Figure 6.1. Metabolic intermediates of shikimate pathway in *Arabidopsis* plants. Phenylalanine is the end products of shikimate pathway (Fraser and Chapple, 2011).

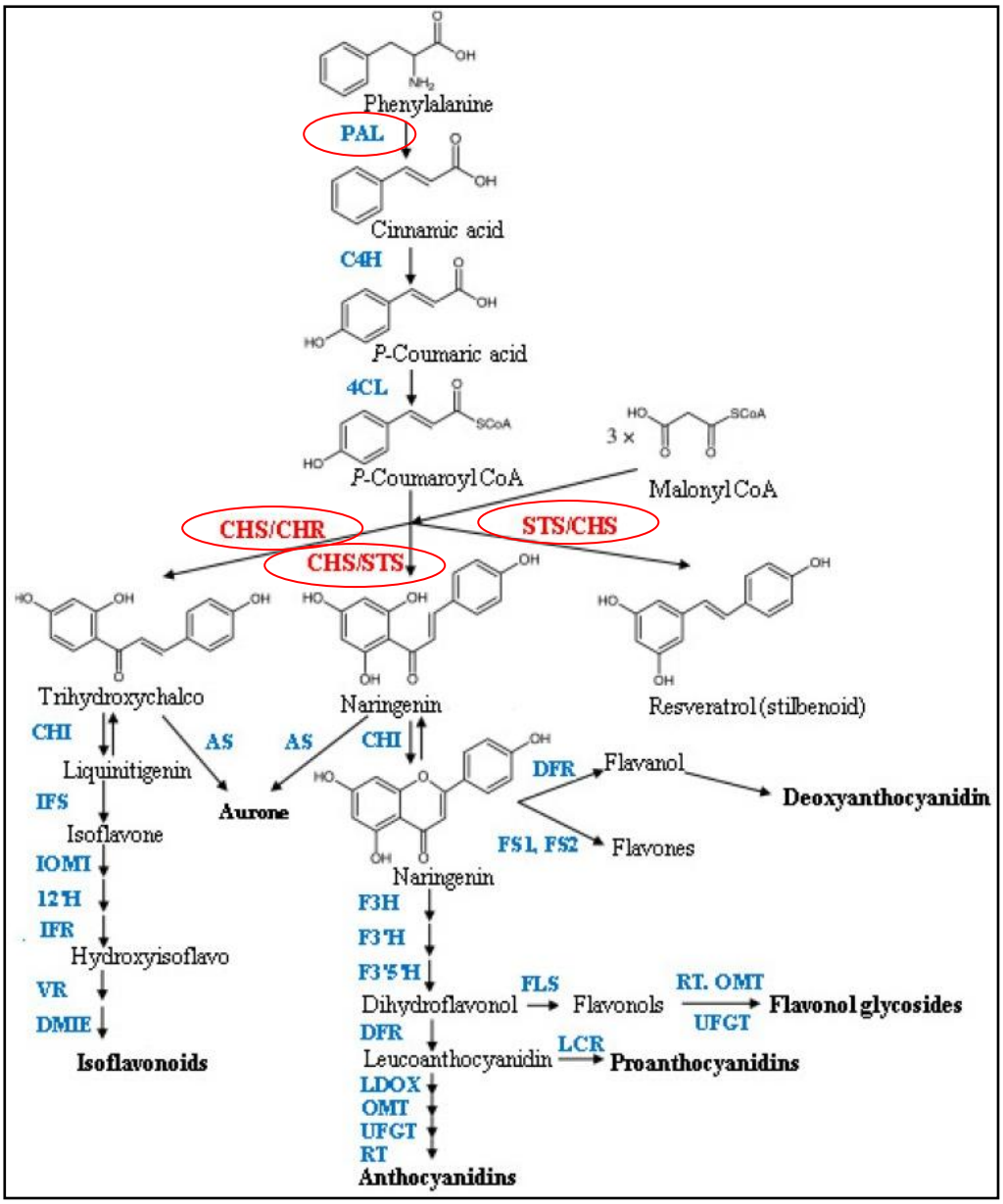


Figure 6.2. Phenylpropanoid pathway and flavonoid biosynthetic pathway of *Zingiber officinale* (Ghasemzadeh *et al*., 2012). Phenylalanine is converted to trans-cinnamic acid via PAL enzyme. CHS enzyme catalyzed the *p*-coumaroyl CoA to naringenin and other related flavonoids.

Up till now, there is no literature review or information on the experimentation of elicitation and precursor feeding in *in vitro* cultures of sweet shoot. This is the first report studying the effects of various elicitor and precursor application (MJ, SA and Phe) on the production of flavonoid and phenolic compounds, and its relationship to antioxidant (DPPH and FRAP assay) and enzymatic activities (PAL and CHS analysis), in a time-dependent manner. Correlative analysis was also performed in this study, to determine the relationships between secondary metabolites, antioxidant levels and enzymatic activities in tissue cultures of sweet shoot.

6.2 MATERIALS AND METHODS

6.2.1 Chemicals

For the elicitor and precursor treatments of sweet shoot cultured tissues, the analytical reagents of MJ and SA were purchased from Sigma-Aldrich, USA (purity ≥ 95%), while Phe (analytical grade) was obtained from Merck, USA (purity ≥ 99%). All of these chemicals were employed without further purification and the stock solutions were prepared as mentioned in Section 6.2.3. As for the plant extraction step, HPLC-grade methanol and hydrochloric acid (HCl) were provided from Fisher Scientific, USA. Tert-butylhydroquinone (TBHQ) (analytical grade) was acquired from Sigma-Aldrich, USA and was used without further purification. To determine total phenolic and total flavonoid content of sweet shoot, the analytical grade reagents of Folin-Ciocalteau reagent, sodium carbonate, sodium nitrite, sodium hydroxide, aluminium chloride, gallic acid and rutin were obtained from BD, USA; Merck, USA; Sigma-Aldrich, USA; Classical Chemicals Sdn Bhd, Malaysia and R&M Marketing, UK. The chemical reagents used in the antioxidant assays of sweet shoot were 2,2-diphenyl-1-picrylhydrazyl (DPPH), ascorbic acid, sodium acetate, 2,4,6-tri (2-pyridyl)-1,3,5-triazine (TPTZ), iron (III) chloride hexahydrate ($FeCl_3.6H_2O$) and iron sulphate from BD, USA; Merck, USA; Sigma-Aldrich, USA; Classical Chemicals Sdn Bhd, Malaysia and R&M Marketing, UK. For the assay of PAL activity, the analytical grade reagents, such as sodium hydroxide, boric acid and cinnamic acid were purchased from Sigma-Aldrich, USA. The analytical grade reagents for CHS assay, such as sodium hydroxide, boric acid, 2-mercaptoethanol, Dowex 1x4 resin, potassium cyanide, chalcone and polyethylene glycol were purchased from Sigma-Aldrich, USA. Tris-HCl buffer was obtained from Promega, USA.

6.2.2 Plant materials

6.2.2.1 Shadehouse-grown plants of sweet shoot

Containerized plants of *Sauropus androgynus* (sweet shoot) were collected from UPM, Serdang Malaysia and it was identified by Dr. Mahmud bin Tengku Muda Mohamed, as mentioned earlier in Section 3.2.1. The stem cuttings were done conventionally in the shadehouse for mass propagation of sweet shoot. These cuttings served as plant materials for the elicitor and precursor treatment of sweet shoot. Stem cuttings, such as nodal cuttings were prepared from the upper part of juvenile sweet shoot. Selected nodal cuttings were trimmed into 8.0 ± 2.0 cm long with

at least 1 well matured bud and had a stem diameter of 0.2 ± 0.1 cm. The top most leaf was retained and cut transversely into half. These trimmed cuttings were then placed in rows into a depth of 2.5 cm perlite-compost (1:1) mixture in 50 x 35 cm^2 size plastic trays (Figure 6.3). Misting was done for one minute hourly during daytime, to ensure the soil was constantly moist with high relative humidity. One to four layers of nursery net were used to reduce the light intensity by 50% to 96% of ambient, respectively (Pacholczak *et al*., 2005). When new shoots reached about 5 cm, the nursery nets were gradually removed and the shoots were acclimatized to ambient conditions for two weeks before transferring to new pots for further shoot development. The well developed plantlets were cultivated for six months (Santos-Gomes *et al*., 2003) in the shadehouse (Section 3.2.1) prior to treatment with salicylic acid (abiotic elicitor), methyl jasmonate (abiotic elicitor) or phenylalanine (precursor), respectively.

Figure 6.3. Stem cuttings of *Sauropus androgynus* (sweet shoot). (A): Sweet shoot was propagated via stem cuttings, the cuttings were trimmed to 8 cm in length with a diameter of 0.2 cm. (B, C): Plantlets regenerated after 1 months of cultivation. (D): Six-month-old sweet shoot served as plant sources for the elicitor and precursor treatment of sweet shoot.

6.2.2.2 *In vitro* cultures of sweet shoot

Cultured tissues of sweet shoot were established according to the procedure described in Section 3.4 for *in vitro* shoot cultures, Section 4.4 for dark-induced callus and light-induced callus cultures, and Section 5.4 for somatic embryogenic cultures. All well developed cultured tissues were maintained for six months in the culture room (Biotechnology Research Center, the University of Nottingham Malaysia Campus) before elicitation experiment was conducted using phenylalanine, salicylic acid or methyl jasmonate separately (Santos-Gomes *et al*., 2003).

For shoot induction, nodal explants were inoculated aseptically onto MS medium (Murashige and Skoog, 1962) supplemented with 2.0 mg/l BAP, 0.5 mg/l IAA, 3.0% (w/v) sucrose and 0.25% (w/v) Phytagel™ (Figure 6.4). The media were adjusted to pH 5.8±0.1 and autoclaved at 121^{o}C for 15 minutes. All cultures were incubated at 26±2^{o}C under 16-hour light and 8-hour dark cycle. The adventitious shoot formed was subcultured onto the same fresh MS medium at intervals of four weeks for multiplication.

The dark-induced callus and light-induced callus cultures of sweet shoot were initiated from aseptic leaf explants when cultured on MS medium containing 2.0 mg/l NAA, 1.0 mg/l kinetin, 3.0% (w/v) sucrose and 0.25% (w/v) Phytagel™ (Figure 6.3). In this study, the light-induced callus cultures were kept in the culture room at 26±2^{o}C under 16-hour light photoperiod, whereas the dark-induced callus cultures were maintained in the dark at 26±2^{o}C. The callus formed was subcultured onto the same fresh MS medium every four weeks for mass multiplication of callus.

Three months after callus induction in the dark, inoculation density of 1.0 g friable embryogenic callus were transferred onto 30 ml liquid MS medium enriched with 3.0% (w/v) sucrose, 2.0 mg/l NAA and 1.0 mg/l kinetin. The embryogenic cell suspensions were transferred to liquid MS medium fortified with 1.0 mg/l NAA and 0.5 mg/l kinetin to induce globular somatic embryos of sweet shoot, after three weeks of initiation. Torpedo-shaped somatic embryos were then transferred onto fresh hormone-free MS medium, in order to facilitate the maturation of somatic embryos (Figure 6.4). Suspension cultures were agitated continuously at

100 rpm on an orbital shaker and incubated at 26±2°C in darkness. Somatic embryos of sweet shoot were maintained by transferring 10 ml of old suspension to 20 ml of fresh medium at 3 week intervals to avoid any accumulation of phenolic compounds.

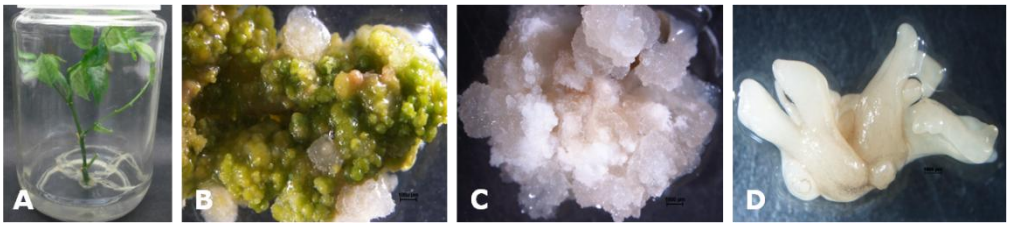


Figure 6.4. *In vitro* cultures for elicitor and precursor treatment of sweet shoot. (A): *In vitro* shoot culture. (B): Light-induced callus culture. (C): Dark-induced callus culture. (D): Somatic embryo culture.

6.2.3 Preparation of elicitors and precursor

In order to obtain high yields of secondary metabolites suitable for commercial exploitation, many efforts have been focused on isolating the biosynthetic activities of cultured tissues, achieved by employing precursor feeding and optimizing the elicitation conditions. MJ, SA and Phe were used in this study for sustainable metabolite production and antioxidant capacity improvement of sweet shoot. It is crucial to identify the most suitable type of elicitor, its optimal concentration and the period of contact between elicitor and *in vitro* cultures of sweet shoot.

6.2.3.1 Methyl jasmonate

A stock solution (100 µM) of MJ was prepared by transferring 1.12 µl of MJ to 49.99 ml of 95% (v/v) ethanol. The stock solution of MJ was then sterilized in the laminar flow cabinet using 0.2 µm syringe filter (Minisart, Sartorius) and stored at 4°C for further use.

6.2.3.2 Salicylic acid

In order to prepare a 100 µM stock solution of SA, about 6.9 mg of SA were dissolved in 40 ml of 95% (v/v) ethanol with gentle heating and constant stirring. The stock solution was made up to 50 ml with 95% (v/v) ethanol. The sterilization of SA stock solution was similar to MJ preparation, as mentioned in Section 6.2.3.1.

6.2.3.3 Phenylalanine

A 1 mg/l of Phe stock solution was prepared by adding 100 mg of Phe to 100 ml of purified water. Gentle heating and constant stirring were required to completely dissolve the crystals. Similar syringe filter sterilization technique was employed for Phe stock solution, as stated in Section 6.2.3.1.

6.2.4 Elicitor and precursor treatment of sweet shoot

6.2.4.1 Shadehouse-grown plant of sweet shoot

Stock solutions prepared in Section 6.2.3 were diluted with 1000 ml of purified water to a final concentration of 0, 50, 100 and 200 µM for MJ and SA; whilst for Phe a final concentration of 0, 5, 10 and 20 mg/l were used in this study (Table 6.1). The elicitation experiment was carried out by spraying each individual six-month-old shadehouse-grown plant (Santos-Gomes *et al*., 2003) with differing concentrations of MJ, SA or Phe (Figure 6.5). Shadehouse-grown plants sprayed with distilled water were served as control in this experiment. All of the elicitor and precursor treatments were applied individually to shadehouse-grown plants in the early morning hours by using manual sprayer and the volume of spray solution has to completely cover the plant foliage till it dripped (Dolatabadian *et al*., 2009). The sprayed shadehouse-grown plants were immediately covered with a vinyl pack for 1 hour (Kim *et al*., 2006). After 1 hour of incubation, the plants treated with elicitors and precursor were isolated from the control plants treated with only purified water. The treated shadehouse-grown plants were left in open air for 2 hours to completely remove the remaining elicitors and precursor. A time course study of the influence of elicitors and precursor on metabolite production was conducted by harvesting the leaves at 7 days intervals for 3 weeks.

Table 6.1. Elicitor concentrations and its elicitation period used for yield enhancement of secondary metabolites in sweet shoot.

Treatment	Elicitors (methyl jasmonate and salicylic acid)	
	Concentration (μM)	Elicitation period (week)
1	0	0
2	0	1
3	0	2
4	0	3
5	50	0
6	50	1
7	50	2
8	50	3
9	100	0
10	100	1
11	100	2
12	100	3
13	200	0
14	200	1
15	200	2
16	200	3

* Similar experimental layout was adopted for phenylalanine treatment of sweet shoot. The concentrations for phenylalanine were 0, 5, 10 and 20 mg/l. Five biological replicates were assigned for each treatment. Each biological replicate equaled to one explant. Three technical replicates were performed for each biological replicate.

Figure 6.5. Procedures for elicitor and precursor treatment of shadehouse-grown sweet shoot. (A): Exogenous application of elicitor and precursor onto shadehouse-grown plants using manual sprayer. (B): Plants treated with elicitors and precursor were incubated for 1 hours.

6.2.4.2 *In vitro* cultures of sweet shoot

In this study, light-induced callus, dark-induced callus, somatic embryo and *in vitro* shoot cultures of sweet shoot were treated individually

with different types of elicitors and precursor, such as SA, MJ and Phe. For elicitor and precursor treatment, approximately 15 g of six-month-old cultured tissues (Santos-Gomes *et al*., 2003) were inoculated onto 30 ml of hormone-free MS medium supplemented with 30 g/l (w/v) sucrose, 0.25% (w/v) Phytagel™ and different concentration of elicitors and precursor, similar to the shadehouse-grown plants (Section 6.2.4.1). Culture medium without elicitors and precursor was used as the control in this experiment. Metabolite accumulation in elicited and non elicited cultures of sweet shoot was then evaluated over the given elicitation period (0, 1, 2 and 3 weeks).

6.2.5 Plant extraction

Secondary metabolites of sweet shoot, such as phenolic acids and flavonoids were extracted and quantified by a modified method by Hertog *et al.* (1992), Miean and Mohamed (2001), and Andarwulan *et al*. (2010). Shadehouse-grown plants and *in vitro* cultures of sweet shoot were cleansed and blotted dry on filter paper before macerating with pestle and mortar. A 15 g fresh weight of the sample was extracted with 20 ml of 62.5% (v/v) aqueous methanol (HPLC grade) containing 2.0 g/l of TBHQ as an antioxidant. To every 20 ml of aqueous methanol used, 5 ml of 1.2 M HCl was added to the extract, in order to hydrolyze the glycosides and to release the bound flavonoids. Each plant sample was then refluxed at 90^{o}C for 2 hours and allowed to cool on ice. This similar procedure was repeated thrice with fresh solvents. Crude extracts were filtered through 0.45 µm Whatman filter paper (Whatman, England) and stored at -20^{o}C prior to analysis.

6.2.6 Determination of total phenolic content

Total phenolic content of sweet shoot was determined using a modified method of Folin-Ciocalteau assay from Singleton and Rossi (1965), which measured the redox properties of polyphenols in plant extracts (Ainsworth and Gillespie, 2007). Phenolics in plant extracts underwent a complex redox reaction with phosphomolybdate and phosphotungstate present in Folin reagent, in order to form a blue complex that was quantified by visible-light spectrophotometer (Schofield *et al*., 2001).

Briefly, 50 µl of crude extracts were mixed with 3.0 ml of purified water and 0.25 ml of undiluted Folin-Ciocalteau's phenol reagent. After 8 minutes, 0.75 ml of 7% (w/v) sodium carbonate and 0.95 ml of purified water were added to the reaction mixture and allowed to stand in the dark for 2 hours at 30-40°C. The absorbance of reaction mixture was measured at 765 nm using a UV-Vis spectrophotometer (UVLine 9400, Schott® instruments, SI analytical GmbH, Germany). Total phenolic content of sweet shoot was calculated from a standard calibration curve using gallic acid (GAE) ($y = 0.0221x + 0.092$, $R^2 = 0.9993$) and expressed as µg GAE equivalent per 10 g fresh weight samples (FW).

6.2.7 Determination of total flavonoid content

Total flavonoid content of sweet shoot was evaluated by aluminium chloride colourimetric assay according to modified protocol of Subhasree *et al*. (2009), whereby the aluminium chloride formed acid stable complexes with the C-4 keto group and either with the C-3 or C-5 hydroxyl group of flavonoids (Pallab *et al*., 2013). An aliquot of 250 µl of crude extracts was added into a test tube containing 1.5 ml of purified water and 0.25 ml of 5% (w/v) sodium nitrite. The reaction mixture was mixed well using vortex and allowed to react at room temperature for 5 minutes. Following this, 0.3 ml of 10% (w/v) aluminium chloride was added and left to stand for 6 minutes. Lastly, 1.0 ml of 1 M sodium hydroxide was added and the reaction mixture was diluted with purified water to make the final volume up to 5 ml. The absorbance of reaction mixture was read at 510 nm. Rutin was used to calculate the standard calibration curve ($y = 0.0226x + 0.145$, $R^2 = 0.9937$) and the results were expressed as µg rutin (RE) equivalent per 10 g fresh weight samples (FW).

6.2.8 Determination of antioxidant activities

6.2.8.1 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay

DPPH assay was determined based on the antioxidant's ability of plant extracts to quench the stable free radical DPPH by donating either electron or hydrogen atoms (Sahin *et al.*, 2004). The hydrogen atom or electron donation ability of plant extract was measured from the degree of bleaching of the purple-coloured methanolic solution of stable DPPH radical (Sanchez-Moreno, 2002). This assay has been widely used for the screening of antioxidant activities in fruit and vegetable extracts, since it has the ability to accommodate many samples in a relatively short period

of time and it is a highly sensitive method in detecting bioactive antioxidants at low concentrations (Villano *et al*., 2007).

The scavenging activity of sweet shoot on DPPH radicals was assayed according to Nahak and Sahu (2010) with some modifications. Briefly, 0.1 mM DPPH solution in 95% (v/v) methanol was prepared freshly and measured at 517 nm. An aliquot (5.0 ml) of crude extract was added to 0.5 ml of 0.1 mM methanolic DPPH solution and mixed well. The change in absorbance at 517 nm was then measured after 30 minutes of incubation in the dark. DPPH dissolved in 95% (v/v) methanol served as a blank to obtain the auto zero base line. The percentage inhibition of DPPH free radical scavenging activity was calculated using the following equation, whereby "Ac" is the average absorbance of control reaction (DPPH in methanol, except the crude extract) and "As" is the average absorbance of DPPH solution containing crude extract at 30 minutes.

$$\text{Free radical scavenging activity } (\%) = \left(1 - \frac{\text{As}}{\text{Ac}}\right) \text{x } 100\%$$

6.2.8.2 Ferric reducing antioxidant potential (FRAP) assay

FRAP assay employs the ability to measure crude extracts by reducing the ferric tripyridyltriazine (Fe(III)-TPTZ) complex to form ferrous tripyridyltriazine (Fe(II)-TPTZ) by using a reductant at low pH (Wong *et al*., 2006). In FRAP assay, the yellow-coloured test solution changed to various shades of green and blue, depending on the reducing power of antioxidants in plant extracts (Zou *et al*., 2011).

The ability of crude extracts to reduce ferric ions was measured using a modified protocol conducted by Wong *et al*. (2006). This method was developed to measure the ferric reduction ability of plant samples at a low pH. FRAP reagent was freshly prepared by mixing 300 mM sodium acetate (pH 3.6), 10 mM 2,4,6-tripyridyl-1,3,5-triazine (TPTZ) dissolved in 40 mM of HCl, and 10 mM iron (III) chloride hexahydrate ($FeCl_3.6H_2O$) in a ratio of 10:1:1. A total of 200 µl of extracts was gently mixed with 3 ml of FRAP reagent and incubated at 37^{o}C in water bath for 30 minutes. The increase in absorbance at 593 nm was measured after 30 minutes against FRAP reagent blank. Standards of known $FeSO_4$ were used to generate a calibration curve ($y = 0.1211x + 0.105$, $R^2 = 0.9769$) and the results were expressed as µg $FeSO_4$ equivalent per 10 g fresh weight samples (FW).

6.2.9 Determination of enzymatic activities

6.2.9.1 Assay of phenylalanine ammonia-lyase (PAL) activity

The PAL activity of sweet shoot samples was measured according to the protocol described by Kovacik and Klejdus (2012) and Havir (1987) with some minor modification. Crude enzyme extract was prepared by homogenizing 0.5 g fresh weight of sweet shoot samples in an ice-cold pestle and mortar with 2 ml of 0.1 M sodium borate buffer (pH 8.8). The stock solution (1 M) of sodium borate buffer was prepared by dissolving 10 g of sodium hydroxide and 61.83 g of boric acid in 500 ml of purified water. The homogenates were centrifuged at 10,000 rpm for 20 minutes at 4°C. The supernatant were collected as crude enzyme source for PAL analysis and stored on ice prior to usage.

For assay of PAL activity, the reaction mixtures consisting of 350 µl of homogenates and 500 µl of 0.1 M sodium borate buffer (pH 8.8) were pre-incubated at 40°C for five minutes before adding 300 µl of 50 mM L-phenylalanine. The reaction mixture was then incubated at 37^{o}C for one hour. The reaction was terminated by adding 50 µl of 5 N HCl and the absorbance of reaction mixture was read at 275 nm using a UV-Vis spectrophotometer (UVLine 9400, Schott® instruments, SI analytical GmbH, Germany). A sample without L-phenylalanine was used as a blank. The PAL activity of plant extracts was determined based on the rate of cinnamic acid production ($y = 0.7436x + 0.062$, $R^2 = 0.9619$) and the results were expressed as mmol of cinnamic acid (CA) equivalent per g fresh weight samples (FW).

6.2.9.2 Assay of chalcone synthase (CHS) activity

The CHS activity of sweet shoot samples was assayed spectrophotometrically using a modified procedure described by Obinata *et al*. (2003) and Ghasemzadeh *et al*. (2012). Crude enzyme extracts were prepared at 4^{o}C by homogenizing 0.5 g fresh weight of sweet shoot samples in a pre-chilled pestle and mortar with 1 ml of 0.1 M sodium borate buffer (pH 8.8) containing 1 mM 2-mercaptoethanol. The homogenates were treated with 0.1 g of Dowex 1x4 resin for 10 minutes before centrifuging at 15,000 rpm for 10 minutes at 4^{o}C, to remove any residual cell, cell debris and resin. After 10 minutes, a 0.2 g of Dowex 1x4 resin was added to supernatant and allowed to incubate for another 15 minutes at 4^{o}C. Following this, the resin was removed by centrifugation at

15,000 rpm for 15 minutes at 4°C. The resultant supernatant were collected as crude enzyme source for CHS analysis and stored on ice prior to usage.

To assay the CHS activity, a total of 100 µl of enzyme extracts was gently mixed with 1.98 ml of 50 mM Tris-HCl buffer (pH 7.6) containing 10 mM potassium cyanide. The enzyme reaction was allowed to incubate for 1 minute at 30°C after adding 10 mg of chalcone in 10 µl of 1.5% polyethylene glycol (PEG 20000). The absorbance of reaction mixture was read at 370 nm using a UV-Vis spectrophotometer (UVLine 9400, Schott® instruments, SI analytical GmbH, Germany). The protein concentration was determined from a standard calibration curve by Bradford (Bradford, 1976) method ($y = 0.0578x + 0.0181$, $R^2 = 0.9749$) and the CHS activity of sweet shoot was expressed as nanokatal (nkat) per mg protein.

6.2.10 Statistical analysis

These experiments were conducted in a completely randomized block design with five biological replicates for each treatment. For each biological replicate, three technical replicates were performed. Each experiment was repeated twice. All data were subjected to analysis of variance (ANOVA) using Statistical Package for Social Sciences (SPSS) software version 16. The significant differences among the treatments at $p<0.05$ were determined by Duncan's multiple range tests (DMRT) using SPSS software. Pearson's correlation coefficient test was used to assess the correlations between total phenolic content, total flavonoid content, antioxidant activities (DPPH and FRAP assay) and enzymatic activities (PAL and CHS assay) of sweet shoot. Assumptions of the normally distributed residuals were assessed using model-checking plots in SPSS software.

6.3 RESULTS AND DISCUSSION

6.3.1 Determination of total phenolic and total flavonoid content

The biosynthesis of secondary metabolites shows tissue specificity. Each part of the plant generates its own unique set of secondary metabolites (Matkowski, 2008). The preliminary study showed that sweet shoot leaf extracts exhibited higher total phenolic and total flavonoid contents than stem extracts (Appendix Table 10). This observation can be explained by the differences in antioxidant distribution between leaves and stems of sweet shoot. These results were also similar to the findings by

Siddhuraju *et al.* (2002), whereby the leaf extracts of *Cassia fistula* were superior to stem extracts for flavonoid production. Besides that, del Bano *et al*. (2004) also stated that certain antioxidative compounds were selectively biosynthesized in the leaves and not in the stems. For that reason, sweet shoot leaves treated with either a precursor or elicitor were subjected to methanol extraction to maximize the total phenolic and flavonoid contents of sweet shoot. Methanol was chosen as an extraction solvent in this study, since preliminary studies showed that ethanol, diethyl ether, chloroform and ethyl acetate extracts of sweet shoot leaves produced lower levels of total phenolic and flavonoid contents (Appendix Table 10).

6.3.1.1 Shadehouse-grown sweet shoot

As shown in Table 6.2, the application of MJ, SA and Phe onto shadehouse-grown plants significantly influenced the accumulation of total phenolic and total flavonoid contents of sweet shoot ($p<0.05$). At week 0, total phenolic content of 61.20 µg/10g FW and total flavonoid content of 193.62 µg/10g FW were detected in untreated shadehouse-grown plants (baseline). The addition of Phe showed a higher increase in the production of phenolic and flavonoid compounds compared to (a) MJ-elicited plants, (b) SA-elicited plants and (c) non-elicited shadehouse-grown plants. Results presented in Table 6.2 showed that the highest total phenolic (132.29 µg/10g FW) and total flavonoid (398.31 µg/10g FW) contents in shadehouse-grown plants were the ones treated with 20 mg/l of Phe, after 3 weeks of treatment. This corresponded to 2.16 and 2.06 folds increase in total phenolic and total flavonoid contents than baseline, and was also 2.96 and 2.24 folds higher in phenolic and flavonoid concentrations respectively, when compared to untreated plants harvested at week 3. These results were comparable with previous reports for buckwheat (Margna *et al*., 1985), *Brugmansia candida* (Spollansky *et al*., 2000) and *Salvia miltiorrhiza* (Dong *et al*., 2010), where Phe treatment was more effective than the other elicitors (MJ and SA) in enhancing the production of phenolic and flavonoid compounds in shadehouse-grown plants.

Table 6.2. Comparison of total phenolic and total flavonoid contents in shadehouse-grown sweet shoot elicited with methyl jasmonate, salicylic acid and phenylalanine, after a treatment period of 21 days.

Types of elicitors/precursors	Concentration	Total Phenolic Content (µg GAE/10 g FW)			Total Flavonoid Content (µg RE/10 g FW)		
		week 1	week 2	week 3	week 1	week 2	week 3
Methyl jasmonate	0µM	31.27 $_{d}$	34.32 $_{d}$	44.70 $_{bc}$	124.96 $_{e}$	137.05 $_{cde}$	177.31 $_{bc}$
	50µM	31.87 $_{d}$	32.85 $_{d}$	56.10 $_{ab}$	131.12 $_{e}$	138.60 $_{cde}$	185.13 $_{ab}$
	100µM	42.59 $_{cd}$	33.01 $_{d}$	59.37 $_{ab}$	158.57 $_{cd}$	140.39 $_{cde}$	189.74 $_{ab}$
	200µM	43.09 $_{bcd}$	42.53 $_{cd}$	64.92 $_{a}$	172.24 $_{bcd}$	166.69 $_{bcd}$	198.62 $_{a}$
Salicylic acid	0µM	31.27 $_{cd}$	34.32 $_{bcd}$	44.70 $_{ab}$	124.96 $_{c}$	137.05 $_{bc}$	177.31 $_{a}$
	50µM	27.34 $_{d}$	31.86 $_{cd}$	33.60 $_{bcd}$	117.11 $_{c}$	130.39 $_{bc}$	136.66 $_{bc}$
	100µM	41.29 $_{bc}$	33.27 $_{cd}$	49.55 $_{a}$	155.32 $_{ab}$	135.63 $_{bc}$	180.89 $_{a}$
	200µM	40.19 $_{bc}$	46.26 $_{ab}$	46.98 $_{ab}$	149.73 $_{ab}$	171.36 $_{ab}$	179.69 $_{a}$
Phenylalanine	0 mg/l	31.27 $_{e}$	34.32 $_{e}$	44.70 $_{cde}$	124.96 $_{f}$	137.05 $_{e}$	177.31 $_{cde}$
	5 mg/l	52.39 $_{cd}$	57.63 $_{c}$	80.87 $_{bc}$	186.36 $_{cd}$	180.29 $_{cde}$	263.61 $_{bc}$
	10 mg/l	46.27 $_{cde}$	69.75 $_{bc}$	98.77 $_{b}$	179.22 $_{cde}$	207.26 $_{cd}$	318.70 $_{b}$
	20 mg/l	58.95 $_{c}$	88.33 $_{bc}$	132.29 $_{a}$	193.10 $_{cd}$	287.63 $_{b}$	398.31 $_{a}$

* Values for total phenolic content expressed as µg gallic acid equivalent (GAE) per 10 g fresh weight samples (FW).
* Values for total flavonoid content expressed as µg rutin equivalent (RE) per 10 g fresh weight samples (FW).
* Values expressed as means of five biological replicated experiments and each biological replicate consisted of three technical replicates.
* Different letters within row and column (lowercase) indicate a significant difference ($p<0.05$) according to Duncan's multiple range test.

From this study, Phe was superior to MJ and SA in it's ability to induce production of phenolic and flavonoids via a series of enzymatic reactions in phenylpropanoid pathway of sweet shoot, as previously mentioned in Section 6.1 (Reymond and Farmer, 1998) ($p<0.05$). This metabolic flux phenomenon may enhance the production of downstream flavonoid and phenolic compounds, thus resulting in higher concentrations as observed in this study.

Amongst the two elicitors tested, the levels of total phenolic and total flavonoid contents for plants treated with MJ were significantly higher than those for SA ($p<0.05$). This observation could be due to the activation of a higher number of defense-related genes in shadehouse-grown plants of sweet shoot treated with MJ (Kombrik and Somssich, 1997). A similar finding was noted in *Arabidopsis* plants by Reymond and Farmer (1998), in which 18 defence-related genes were reported to be MJ-inducible and 8 of them were reported to be SA-inducible. Based on the report done by the same authors, the pathogenesis-related proteins (PR) such as thionin (THI2.1), defensin (FDF1.2), class I chitinase (PR-3) (CHIA1) and class III chitinase (PR-8) (CHIB1) were successfully identified in *Arabidopsis* plants treated with MJ, but all of these genes were absent in SA-treated plants. In addition, MJ also has the ability to activate the enzymes, which were responsible for the production of phenolic and flavonoid compounds, such as DAHP synthase (DHS1) in shikimate pathway, CHS in flavonoid biosynthesis pathway, phenylalanine ammonia-lyase (PAL1) in phenylpropanoid pathway and tyrosine aminotransferase (TAT) in tyrosine biosynthesis pathway (McConn *et al*., 1997; Vijayan *et al*., 1997). None of these enzymes were induced by SA in *Arabidopsis* plants. Thus, these results point towards the importance of MJ as a defence gene regulator compared to SA, as also seen in the studies done by Kim *et al*. (2006), Wang *et al*. (2008), Benedec *et al.* (2012) and Koca *et al*. (2014).

Based on the results shown in Table 6.2, shadehouse-grown plants treated with 200 µM of MJ produced significantly higher phenolic (64.92 µg/10g FW) and flavonoid (198.62 µg/10g FW) compounds at week 3 of elicitation, when compared to those treated with SA (Table 6.2) ($p<0.05$). Amongst the SA treatments, the highest production of phenolic and flavonoid compounds was detected in plants elicited with 100 µM of SA

after 3 week of elicitation (49.55 µg/10g FW and 180.89 µg/10g FW, respectively). However, SA concentration of 50 µM led to lower phenolic and flavonoid contents in shadehouse-grown sweet shoot (27.34 µg/10g FW and 117.11 µg/10g FW, respectively). This may be attributed to the feedback inhibition in the metabolic pathway, as shown in Figure 6.1 (Zia *et al*., 2007).

There were studies that exhibited negative results on the accumulation of plant biomass with higher concentrations and longer duration of both precursor and elicitor treatment in tissue culture of woody plant species. For example, Skrzypczak-Pietraszek *et al*. (2014) revealed that MJ concentrations higher than 800 µM caused severe disadvantageous effects on the growth of *Exacum affine*. This observation has also been reported in many studies showing higher concentration of MJ can impair the growth of woody plant species (Heijari *et al*., 2005; Moreira *et al*., 2009). Despite this, in this study, the supplementation of Phe, MJ and SA at the all levels of concentration and contact duration did not significantly cause variation on the growth of sweet shoot (Appendix Table 11) ($p>0.05$). There were no significant differences observed in growth rate of sweet shoot under different treatments of elicitors and precursor (Appendix Table 11). This observation was supported by Saw *et al.* (2010) and Syklowska-Baranek *et al*. (2012), in which the growth rate of *Vitis vinifera* and *Hydrocotyle bonariensis* was not affected by the addition of Phe.

6.3.1.2 *In vitro* shoot culture of sweet shoot

The analysis of variance showed significant effects of MJ, SA and Phe treatments on the biosynthesis of phenolic and flavonoid compounds in shoot cultures of sweet shoot ($p<0.05$) (Table 6.3). The total amount of phenolic compounds in sweet shoot ranged from 32.77 µg/10g FW for shoot cultures treated with SA to 160.57 µg/10g FW for shoot cultures treated with Phe (Table 6.3). The total flavonoid content of shoot cultures varied from 135.08 µg/10g FW to 512.15 µg/10g FW (Table 6.3). The baseline value of 50.31 µg/10g FW and 185.61 µg/10g FW was recorded for phenolic and flavonoid content in untreated shoot cultures harvested at week 0, respectively.

Table 6.3. Comparison of total phenolic and total flavonoid contents in shoot cultures of sweet shoot elicited with methyl jasmonate, salicylic acid and phenylalanine, after a treatment period of 3 weeks.

Types of elicitors/precursors	Concentration	Total Phenolic Content (µg GAE/10 g FW)			Total Flavonoid Content (µg RE/10 g FW)		
		week 1	week 2	week 3	week 1	week 2	week 3
Methyl jasmonate	0µM	46.91 $_{cd}$	49.19 $_{cd}$	60.46 $_{bc}$	176.85 $_{bc}$	182.40 $_{bc}$	192.99 $_{bc}$
	50µM	37.79 $_{cde}$	45.07 $_{cde}$	91.56 $_{a}$	144.74 $_{d}$	178.42 $_{bc}$	299.11 $_{a}$
	100µM	47.86 $_{cd}$	35.93 $_{e}$	68.61 $_{b}$	179.71 $_{bc}$	140.59 $_{d}$	202.08 $_{b}$
	200µM	43.88 $_{cde}$	46.45 $_{cd}$	55.60 $_{bc}$	173.71 $_{bcd}$	187.29 $_{bc}$	180.29 $_{bc}$
Salicylic acid	0µM	46.91 $_{bc}$	49.19 $_{bc}$	60.46 $_{ab}$	176.85 $_{bc}$	182.40 $_{bc}$	192.99 $_{ab}$
	50µM	32.77 $_{e}$	42.01 $_{cd}$	53.72 $_{bc}$	135.08 $_{d}$	156.88 $_{bcd}$	179.21 $_{bc}$
	100µM	39.99 $_{cd}$	45.79 $_{bc}$	69.05 $_{a}$	152.05 $_{bcd}$	174.88 $_{bc}$	204.43 $_{a}$
	200µM	33.77 $_{e}$	38.35 $_{cde}$	60.22 $_{ab}$	135.63 $_{d}$	145.38 $_{bcd}$	191.51 $_{ab}$
Phenylalanine	0 mg/l	46.91 $_{e}$	49.19 $_{e}$	60.46 $_{cd}$	176.85 $_{e}$	182.40 $_{e}$	192.99 $_{de}$
	5 mg/l	49.04 $_{e}$	63.62 $_{cd}$	89.75 $_{bc}$	178.87 $_{e}$	195.63 $_{de}$	300.23 $_{cd}$
	10 mg/l	60.58 $_{cd}$	79.66 $_{bcd}$	160.57 $_{a}$	193.69 $_{de}$	254.93 $_{cd}$	512.15 $_{a}$
	20 mg/l	82.27 $_{bcd}$	100.62 $_{bc}$	116.09 $_{b}$	270.65 $_{cd}$	320.08 $_{c}$	384.11 $_{b}$

* Values for total phenolic content expressed as µg gallic acid equivalent (GAE) per 10 g fresh weight samples (FW).
* Values for total flavonoid content expressed as µg rutin equivalent (RE) per 10 g fresh weight samples (FW).
* Values expressed as means of five biological replicated experiments and each biological replicate consisted of three technical replicates.
* Different letters within row and column (lowercase) indicate a significant difference ($p<0.05$) according to Duncan's multiple range test.

The results of colourimetric analysis (Table 6.3) revealed that the highest phenolic (160.57 µg/10g FW) and flavonoid (512.15 µg/10g FW) content was achieved by incorporating 10 mg/l of Phe to MS medium, after 3 weeks of precursor treatment ($p<0.05$). This medium significantly improved the production of phenolic and flavonoid content in shoot cultures by 3.19 and 2.65 fold respectively, when compared to baseline. Additionally, it was also 1.92 and 1.99 fold higher in both phenolic and flavonoid contents when compared to untreated *in vitro* shoots at third week of inoculation. These values were also significantly better than MJ and SA elicitor treatments in all levels of concentration and duration of contact ($p<0.05$). Similar observations were also observed by Roy and Mukhopadhyay (2012) in shoot cultures of *Mentha arvensis*, whereby the addition of Phe (10 mg/l) significantly increased the production of rosmarinic acid by 2.03 fold. The positive effects of Phe shown in this study were also similar to the findings from shoot cultures of strawberry on anthocyanin production (Edahiro *et al*., 2005) and sweet basil (Kim *et al*., 2006) on polyphenol production.

The application of Phe exogenously resulted in a slight increase in tissue growth (Appendix Table 11) ($p>0.05$). The reason for this observation may be due to the excess supplies of carbon level that can be used for phenol accumulation (Ilvessalo and tuomi, 1988; Tisserat and Berhow, 2003). As mentioned in Section 6.3.1.1, the enhancement of flavonoid and phenolic content in shoot cultures of sweet shoot may be due to the stimulation of key enzymes PAL and CHS, which is the gateway for phenylpropanoid pathway (Ghosh *et al.*, 2006; Mukhopadhyay and Mukhopadhyay, 2008). Previous study demonstrated that actively growing tissues of *Coleus blumei* converted more than 20% of exogenously supplied Phe (477 µCi/µmol) to caffeoyl ester and this high rate of biosynthesis coincided with an increase in the PAL activity, which in turn produced rosmarinic acid (Razzaque and Ellis, 1977). Other enzymatic activities such as peroxidase (POD) and polyphenol oxidase (PPO) activity were also elicited in callus cultures of *Artemisia absinthium* treated with 12.5 mg/l of Phe (Zia *et al.*, 2007).

In this study, there was a dramatic increase in metabolites seen in *in vitro* shoot cultures when compared to shadehouse-grown plants of sweet shoot. This may represent the variations of inducibility of different

plant cell culture systems in the production of phenolic and flavonoid content (Ong *et al*., 2002). The more differentiated a culture became, the least the response to elicitation was, as evidenced in *in vitro* shoot cultures of *Ruta graveolens* (Bohlmann and Eilert, 1994), *Artemisia judaica* (Liu *et al*., 2004) and *Taxus brevifolia* (Khosroushahi *et al.*, 2011). This observation was also seen in the present study, whereby shadehouse-grown plants lacked inducible metabolite accumulation, when compared to shoot cultures of sweet shoot.

Based on the results shown in Table 6.3, similarities were observed between shadehouse-grown plants and shoot cultures, in which the production of phenolics and flavonoids responded well in a linear manner to a certain Phe concentration and with longer contact duration between cultures and elicitors ($p<0.05$). As the concentration of Phe increased from 0 mg/l to 10 mg/l, the total phenolic and flavonoid contents increased in shoot cultures of sweet shoot (Table 6.3). However, the production of phenolic and flavonoid compounds decreased at the concentration of 20 mg/l of Phe (Table 6.3). These results were in line with the studies done by Shinde *et al*. (2009b) in hairy root cultures of *Psoralea corylifolia*, where the production of daidzein and genistein were greatly inhibited when concentration of Phe increased from 2 mM to 10 mM. This phenomenon was also seen in MJ and SA elicitation of sweet shoot cultured plants, and this could be attributed to the feedback inhibition in metabolic pathway (Zia *et al*., 2007), as mentioned earlier in Section 6.3.1.1. Therefore, it is essential to optimize the concentration of elicitors and precursor, in order to maximize the production of phenolic and flavonoid contents in shoot cultures of sweet shoot.

Plant growth regulators are known to be able to regulate the metabolism in plant cell cultures, as it affects both the growth and secondary metabolite production in woody plant species (Lian *et al*., 2002). However, this statement does not hold true in this study. The preliminary data showed that the simultaneous administration of elicitor and plant growth regulators markedly reduced the flavonoid and phenolic contents compared to those shoot cultures treated with only elicitors and precursor. It showed that shoot cultures treated with elicitors and plant growth regulators had 1.5 times lower concentration of the above mentioned metabolites (Appendix Table 12). A similar observation was

seen in another study on callus cultures of *Larrea divaricata,* where 2.0 mg/l of BAP also reduced the production of lignan and flavonoid compounds compared to control cultures (Palacio *et al*., 2012). There was also another study on lentil seeds, which showed reduced total antioxidant capacity, total phenol content and catechin content by 1.03, 1.34, 2.42 folds after treated with 20 mg/l of IAA, when compared to control seeds (Giannakoula *et al*., 2012). Due to the negative effects of plant growth regulators on the production of metabolites in sweet shoot, the results for tissue cultures treated with plant growth regulators in conjunction with elicitors will not be reported in this study.

6.3.1.3 Light-induced callus culture of sweet shoot

In this study, the lowest amount of phenolic (35.28 µg/10g FW) and flavonoid (137.04 µg/10g FW) compounds were recorded in the untreated light-induced callus cultures harvested at week 0 (baseline). Statistical analysis, as shown in Table 6.4, revealed that higher amounts of phenolic and flavonoid compounds were produced in response to higher elicitor concentrations at longer elicitation period ($p<0.05$). The most suitable concentration for the peak production of these metabolites was at 20 mg/l of Phe, where the total phenolic content averaged at 246.62 µg/10g FW and the flavonoid content averaged at 636.26 µg/10g FW, after 3 weeks of incubation ($p<0.05$) (Table 6.4), which were both 6.99 and 4.64 fold greater than baseline. These results were also higher in comparison to those callus cultures harvested at week 3 by 3.72 (phenolic) and 3.03 (flavonoid) fold. The stimulatory effects of Phe observed in this study were supported by studies done by Shinde *et al*. (2009a), Masoumian *et al*. (2011) and Indu *et al.* (2013), which showed that the addition of Phe significantly enhanced flavonoid and phenolic production in light-induced callus cultures of *Psoralea coryfolia* (higher by 1.3 fold using 0.5 mM Phe), *Hydrocotyle bonariensis* (increment of 23% using 3 mg/l Phe) and *Anthocephalus indicus* (increased by 4.98 fold using 100 mg/l Phe), when compared to control cultures. Thus, this data demonstrated that light-induced callus cultures of sweet shoot were able to retain the potential to synthesize flavonoids and phenolics in higher amounts when exposed to Phe. As mentioned in Section 6.3.1.1, Phe is actively involved in the enzymatic conversion reactions in phenylpropanoid pathway for the production of antioxidants (Reymond and Farmer, 1998).

Table 6.4. Comparison of total phenolic and total flavonoid contents in light-induced callus cultures of sweet shoot treated with methyl jasmonate, salicylic acid and phenylalanine, after 3 weeks of elicitor treatment.

Types of elicitors/precursors	Concentration	Total Phenolic Content (µg GAE/10 g FW)			Total Flavonoid Content (µg RE/10 g FW)		
		week 1	week 2	week 3	week 1	week 2	week 3
Methyl jasmonate	0µM	39.25 $_{e}$	52.07 $_{de}$	66.31 $_{cd}$	146.97 $_{f}$	178.29 $_{def}$	209.67 $_{cd}$
	50µM	42.61 $_{de}$	51.50 $_{de}$	77.78 $_{c}$	163.40 $_{ef}$	171.36 $_{ef}$	245.55 $_{c}$
	100µM	52.27 $_{de}$	65.52 $_{cd}$	98.00 $_{b}$	178.15 $_{def}$	200.50 $_{cd}$	310.10 $_{b}$
	200µM	59.49 $_{cde}$	72.67 $_{c}$	135.27 $_{a}$	184.61 $_{def}$	218.03 $_{cd}$	446.96 $_{a}$
Salicylic acid	0µM	39.25 $_{cd}$	52.07 $_{ab}$	66.31 $_{a}$	146.97 $_{cd}$	178.29 $_{bc}$	209.67 $_{a}$
	50µM	39.74 $_{cd}$	45.62 $_{bc}$	53.55 $_{ab}$	150.19 $_{cd}$	174.57 $_{bc}$	179.03 $_{bc}$
	100µM	44.65 $_{bc}$	47.13 $_{bc}$	60.25 $_{ab}$	173.72 $_{bc}$	177.85 $_{bc}$	192.66 $_{b}$
	200µM	35.68 $_{d}$	39.33 $_{cd}$	48.96 $_{bc}$	139.96 $_{d}$	147.31 $_{cd}$	177.66 $_{bc}$
Phenylalanine	0 mg/l	39.25 $_{f}$	52.07 $_{ef}$	66.31 $_{de}$	146.97 $_{f}$	178.29 $_{def}$	209.67 $_{de}$
	5 mg/l	57.61 $_{def}$	66.25 $_{de}$	103.19 $_{cd}$	184.15 $_{def}$	209.12 $_{de}$	323.91 $_{cd}$
	10 mg/l	68.95 $_{de}$	87.03 $_{cde}$	148.83 $_{b}$	200.99 $_{de}$	293.20 $_{cd}$	463.70 $_{b}$
	20 mg/l	100.45 $_{cd}$	131.61 $_{bc}$	246.62 $_{a}$	321.78 $_{cd}$	392.91 $_{c}$	636.26 $_{a}$

* Values for total phenolic content expressed as µg gallic acid equivalent (GAE) per 10 g fresh weight samples (FW).
* Values for total flavonoid content expressed as µg rutin equivalent (RE) per 10 g fresh weight samples (FW).
* Values expressed as means of five biological replicated experiments and each biological replicate consisted of three technical replicates.
* Different letters within row and column (lowercase) indicate a significant difference ($p<0.05$) according to Duncan's multiple range test.

In the two elicitors tested, SA is weaker when it came to enhancing the total phenolic and total flavonoid content in light-induced callus culture of sweet shoot ($p<0.05$). It is noted to be even poorer than the untreated light-induced callus cultures at third week of incubation (Table 6.4). The possible reason would be, while most metabolites induced by elicitation were involved in plant defense systems, SA may have a tendency to either amplify or interrupt the plant defense signaling pathway (Chen *et al*., 2006a). Several reports have also shown similar unsuccessful results using SA as an elicitor, such as tropane production in *Atropa belladonna* (Lee *et al*., 2001) and mitragynine production in *Mitragyna speciosa* (Zuldin *et al*., 2013). Hence, unsuitable elicitor (SA) may cause unsuccessful elicitation, which indicates that a successful elicitation is a challenging process that requires intense standardization.

MJ has been shown to be the better elicitor than SA in this study (135.27 µg/10g FW for total phenolic content and 446.96 µg/10g FW for total flavonoid content), which corresponded to 2.04 and 2.13 fold higher than SA-elicited callus cultures ($p<0.05$). This finding was supported by many reports, such as those by Ali *et al*. (2007) (200 µM MJ), Antognomi *et al*. (2007) (100 µM MJ), Kim *et al.* (2007) (200 µM MJ) and Jalalpour *et al.* (2014) (100 µM MJ), which observed an increase in phenolic and flavonoid compounds under MJ treatments. It was hypothesized that this elevation may be attributed to the activation of plant's defence response system via MJ, as previously seen in Section 6.3.1.1.

The formation of secondary metabolites is an integration of the cell differentiation process. The cell differentiation process may follow specific biochemical and morphological principles. This phenomenon was similarly seen in this study, whereby the light-induced callus culture with Phe had higher flavonoid and phenolic contents than those induced in shadehouse-grown plants and *in vitro* shoot cultures of sweet shoot. The superiority of light-induced callus cultures for the production of secondary metabolites in sweet shoot may be attributed to the development-dependent differentiation process. Several studies showed that the differentiation of tracheary elements and laticifer cells in callus culture led to the production of metabolites in woody plant species (Oluk, 2006; Matkowski, 2008). These findings are consistent with the results of Palacio *et al.* (2012), who showed that the amount of flavonoid compounds present in callus cultures

were significantly greater (9.9 fold) than those in shoot cultures of *Larrea divaricata*. Similar findings were also reported for phenolic and flavonoid production in *Scopolia parviflora* (Tabata *et al*., 1972), *Momordica charantia* (Agarwal and Kamal, 2007) and *Morinda citrifolia* (Deshmukh *et al*., 2011). Thus, light-induced callus cultures served as an alternative plant source than conventional shadehouse-grown plants for the production of phenolic and flavonoid compounds in sweet shoot.

6.3.1.4 Dark-induced callus culture of sweet shoot

In the present study, the exogenous application of Phe was proven statistically to be effective in enhancing the total phenolic and total flavonoid contents of dark-induced callus cultures ($p<0.05$) (Table 6.5). For the untreated dark-induced callus culture at week 0, the total phenolic and total flavonoid contents of sweet shoot were 45.40 µg/10g FW and 174.44 µg/10g FW, respectively (baseline). In the cultures fed with Phe, levels of phenolics and flavonoids rapidly rose and were best at concentration of 10 mg/l of Phe (70.07 µg/10g FW and 212.40 µg/10g FW, respectively), after 3 weeks of contact duration (Table 6.5). At these phenolic and flavonoid concentrations, it was 1.54 and 1.22 folds higher than baseline respectively. Furthermore, when compared to untreated callus cultures at week 3, total phenolic and flavonoid contents induced on MS medium fed with 10 mg/l Phe increased by 2.01 and 1.56 folds respectively. The positive results obtained in this study may follow the 'phenylalanine pool' hypothesis as discussed earlier in Section 6.3.1.1 (Kakegawa *et al*., 1995), whereby the incorporated Phe is used not only as a precursor but also as a concentration-dependent signal to stimulate phenolic and flavonoid production by inducing enzyme activities. These results concurred with those experiments by callus cultures of *Coleus blumei* and *Salvia officinalis* (Ellis and Towers, 1970; Ibrahim, 1987).

Table 6.5. Comparison of total phenolic and total flavonoid contents in dark-induced callus cultures of sweet shoot elicited with methyl jasmonate, salicylic acid and phenylalanine, after 21 weeks of elicitor treatment.

Types of elicitors/precursors	Concentration	Total Phenolic Content (µg GAE/10 g FW)			Total Flavonoid Content (µg RE/10 g FW)		
		week 1	week 2	week 3	week 1	week 2	week 3
Methyl jasmonate	0µM	20.21 $_{e}$	20.61 $_{e}$	34.79 $_{bc}$	93.67 $_{e}$	94.52 $_{e}$	135.77 $_{bc}$
	50µM	23.09 $_{cd}$	20.73 $_{e}$	38.27 $_{bc}$	100.24 $_{cd}$	96.85 $_{de}$	145.92 $_{bc}$
	100µM	25.19 $_{cd}$	18.85 $_{e}$	46.69 $_{a}$	106.51 $_{cd}$	88.57 $_{e}$	176.63 $_{a}$
	200µM	20.98 $_{e}$	34.71 $_{bc}$	40.94 $_{b}$	97.39 $_{de}$	136.03 $_{bc}$	152.95 $_{b}$
Salicylic acid	0µM	20.21 $_{d}$	20.61 $_{d}$	34.79 $_{ab}$	93.67 $_{d}$	94.52 $_{d}$	135.77 $_{bc}$
	50µM	24.45 $_{bcd}$	28.74 $_{bc}$	36.07 $_{ab}$	103.48 $_{cd}$	120.53 $_{bcd}$	143.20 $_{b}$
	100µM	30.71 $_{ab}$	32.57 $_{ab}$	40.78 $_{a}$	123.16 $_{bcd}$	133.38 $_{bc}$	151.06 $_{b}$
	200µM	32.67 $_{ab}$	26.61 $_{bcd}$	43.11 $_{a}$	134.79 $_{bc}$	112.91 $_{cd}$	173.06 $_{a}$
Phenylalanine	0 mg/l	20.21 $_{e}$	20.61 $_{e}$	34.79 $_{cde}$	93.67 $_{e}$	94.52 $_{e}$	135.77 $_{cde}$
	5 mg/l	32.18 $_{cde}$	37.98 $_{cd}$	49.73 $_{bc}$	132.43 $_{cde}$	144.72 $_{cd}$	183.63 $_{bc}$
	10 mg/l	39.56 $_{cd}$	46.08 $_{bc}$	70.07 $_{a}$	148.07 $_{cd}$	173.77 $_{bc}$	212.40 $_{a}$
	20 mg/l	45.69 $_{bc}$	57.64 $_{b}$	59.83 $_{b}$	172.97 $_{bc}$	196.15 $_{ab}$	199.83 $_{ab}$

* Values for total phenolic content expressed as µg gallic acid equivalent (GAE) per 10 g fresh weight samples (FW).
* Values for total flavonoid content expressed as µg rutin equivalent (RE) per 10 g fresh weight samples (FW).
* Values expressed as means of five biological replicated experiments and each biological replicate consisted of three technical replicates.
* Different letters within row and column (lowercase) indicate a significant difference ($p<0.05$) according to Duncan's multiple range test.

Despite this interesting observation, there were limitations seen in this study. According to Namdeo (2007), it was noted that a high precursor dosage could induce a hypersensitivity reaction that led to lower production of metabolites, whereas an optimum precursor level was required for metabolite induction. This statement was supported by the findings in this study, in which 20 mg/l of Phe produced a lower level of phenolic (59.83 µg/10g FW) and flavonoid (199.83 µg/10g FW) compounds in dark-induced callus cultures at the third week of inoculation, as compared to 10 mg/l of Phe (Table 6.5). Similar observations were noted in hairy root cultures of yew plants, where the addition of 1 µM Phe to the MS medium resulted in increased paclitaxel content (1.88 fold), when compared to 100 µM of Phe (Syklowska-Baranek *et al*., 2009). Again, this study demonstrates that the 'overloading' of the precursor can have adverse effects, as explained in Section 6.2.1.2.

The above mentioned trend was similarly seen in MJ-elicited callus cultures, where a higher concentration (200 µM) of MJ led to lower production of phenolic (40.94 µg/10g FW) and flavonoid (152.95 µg/10g FW) compounds. Similar observation was also found in the production of taxol from cell suspension cultures of *Taxus chinensis,* whereby MJ at 200 µM concentration led to lower taxol production (3.58 fold), when compared to 100 µM of MJ concentration (Shinde *et al*., 2009b). The authors from the same study hypothesized that the combination of MJ molecules with its receptor played an important role in activating the signal transduction cascade for taxol production. Generally, the binding of elicitor molecules with its receptors may form an equilibrium state to activate signal transduction cascade at the optimal concentration of elicitor. When MJ concentration was greater than 100 µM, a large number of MJ molecules competitively bound with the limited number of receptor molecules, interfering with signal transduction cascade and thus inhibiting taxol production (Shinde *et al*., 2009b). Hence, appropriate dosage of elicitor and exposure time need to be taken into consideration as a fundamental factor for successful elicitation.

The analysis of variance exhibited lower total phenolic and total flavonoid contents in dark-induced callus cultures after the addition of MJ and SA at all levels of concentration and duration of contact ($p>0.05$) (Table 6.5). The reason for this phenomenon is similar as explained in

Section 6.3.1.1 and 6.3.1.3. Thus, Phe again remained the most desirable precursor in enhancing the production of phenolic and flavonoid compounds for dark-induced callus cultures of sweet shoot.

In this study, the highest concentration of phenolic and flavonoid compounds produced from dark-induced callus culture (70.07 µg/10g FW and 212.40 µg/10g FW, respectively) was substantially lower than those induced in light-induced callus cultures of sweet shoot (246.62 µg/10g FW and 636.26 µg/10g FW, respectively). The total phenolic and total flavonoid contents of dark-induced callus cultures were lower by around 32% to 34% when compared to light-induced callus cultures. The differences seen may be due to excessive light energy leading to higher stress levels placed on light-induced callus cultures, via its effects on photosynthesis (Chew *et al*., 2011). The chlorophylls may undergo photosensitization process (process of transferring absorbed light energy), which triggered the production of highly reactive oxygen species at a cellular level, as evidenced by light-induced callus cultures of *Piper imperial* (Diaz *et al*., 2012), *Leguminosae* family (Chew *et al*., 2011) and many other medicinal plants (Bhattacharya *et al*., 2009). Light-induced callus is therefore required to generate highly effective antioxidants and free radical scavengers to remove reactive oxygen species (ROS) and to minimize the photosensitization induced oxidative damage. The concept mentioned above has been reported by Shinde *et al*. (2010) that callus cultures of *Psoralea coraylifolia* grown under continuous illumination conditions produced higher multiplications of daidzein (2.02 fold), genistein (1.5 fold) and total phenolic content (1.27 fold) than that of dark-induced callus cultures. Similarly, callus cultures of *Genista tinctoria* showed enhanced levels of isoflavones (1.30 fold) production when grown in light compared to dark (Luczkiewicz and Glold, 2003). Thus, light irradiation played an important role in controlling the expression of specific genes in secondary metabolism of plants.

6.3.1.5 Somatic embryo culture of sweet shoot

In this study, an average total phenolic content of 36.98 µg/10g FW and average total flavonoid content of 143.77 µg/10g FW were recorded in untreated somatic embryos of sweet shoot harvested at week 0 (baseline). Hormone-free MS medium supplemented with Phe had a beneficial influence on phenolic and flavonoid accumulation in somatic

embryos of sweet shoot ($p<0.05$) (Table 6.6). Based on the results shown in Table 6.6, Phe at 20 mg/l concentration had stimulatory effects on phenolic (98.51 µg/10g FW) and flavonoid (314.21 µg/10g FW) production after 3 weeks of precursor treatment, which were 2.66 and 2.19 times increased from baseline respectively. Besides that, the effect of precursor feeding was also dependent on the contact time between precursor and cultures of sweet shoot. As the precursor contact time increased from week 0 to week 3, the production of phenolic and flavonoid compounds of sweet shoot were significantly enhanced in MS medium fortified with 20 mg/l of Phe at week 3 ($p<0.05$). Compared with untreated cultures at week 3, this represented a 2.42 and 2.06 folds higher concentration in production of phenolic and flavonoid compounds. The results obtained in this study were in accordance with a report on *Lithospermum erythorohizon*, which showed that a higher concentration of Phe (0.01 M) led to an increase in amounts of shikonin (3.68 fold) content (Mizukami *et al*., 1977; Okamoto *et al*., 1995).

Table 6.6. Comparison of total phenolic and total flavonoid contents in somatic embryos of sweet shoot treated with methyl jasmonate, salicylic acid and phenylalanine, after 21 weeks of elicitor treatment.

Types of elicitors/precursors	Concentration	Total Phenolic Content (µg GAE/10 g FW)			Total Flavonoid Content (µg RE/10 g FW)		
		week 1	week 2	week 3	week 1	week 2	week 3
Methyl jasmonate	0µM	28.67 $_{bcd}$	43.71 $_{b}$	40.63 $_{bc}$	119.28 $_{cd}$	173.11 $_{a}$	152.63 $_{b}$
	50µM	26.03 $_{d}$	28.87 $_{bcd}$	51.97 $_{a}$	112.20 $_{d}$	123.20 $_{c}$	271.15 $_{a}$
	100µM	30.95 $_{bcd}$	43.72 $_{b}$	38.35 $_{bc}$	126.15 $_{c}$	172.01 $_{a}$	146.61 $_{b}$
	200µM	27.83 $_{d}$	36.71 $_{bc}$	37.07 $_{bc}$	117.23 $_{cd}$	143.63 $_{b}$	145.45 $_{b}$
Salicylic acid	0µM	28.67 $_{cd}$	43.71 $_{a}$	40.63 $_{ab}$	119.28 $_{cd}$	173.11 $_{a}$	152.63 $_{ab}$
	50µM	30.03 $_{bcd}$	25.19 $_{d}$	41.19 $_{ab}$	117.56 $_{cd}$	103.28 $_{d}$	153.35 $_{ab}$
	100µM	30.64 $_{bcd}$	29.21 $_{cd}$	44.75 $_{a}$	120.16 $_{cd}$	121.80 $_{cd}$	173.97 $_{a}$
	200µM	28.13 $_{cd}$	34.96 $_{bc}$	37.81 $_{bc}$	117.12 $_{cd}$	136.05 $_{bc}$	143.81 $_{bc}$
Phenylalanine	0 mg/l	28.67 $_{e}$	43.71 $_{cd}$	40.63 $_{cde}$	119.28 $_{e}$	173.11 $_{cd}$	152.63 $_{cde}$
	5 mg/l	42.14 $_{cd}$	47.03 $_{cd}$	65.77 $_{bc}$	163.11 $_{cde}$	177.99 $_{cd}$	199.10 $_{c}$
	10 mg/l	46.95 $_{cd}$	62.88 $_{bc}$	79.39 $_{b}$	177.55 $_{cd}$	196.36 $_{c}$	256.96 $_{b}$
	20 mg/l	34.52 $_{de}$	57.65 $_{bcd}$	98.51 $_{a}$	135.85 $_{de}$	184.27 $_{cd}$	314.21 $_{a}$

* Values for total phenolic content expressed as µg gallic acid equivalent (GAE) per 10 g fresh weight samples (FW).
* Values for total flavonoid content expressed as µg rutin equivalent (RE) per 10 g fresh weight samples (FW).
* Values expressed as means of five biological replicated experiments and each biological replicate consisted of three technical replicates.
* Different letters within row and column (lowercase) indicate a significant difference ($p<0.05$) according to Duncan's multiple range test.

Similarities were seen between dark-induced callus cultures and somatic embryo cultures when it came to yield enhancement of phenolic and flavonoid compounds with MJ and SA as there was no significant increase in the production of both phenolics and flavonoids ($p>0.05$). This finding is converse to that found in shadehouse-grown plant, shoot culture and light-induced callus of sweet shoot. Thus, different types of plant cultures responded differently to the elicitors, which was similarly reported in cultured tissues of *Centella asiatica* by Ong *et al*. (2002). It was also documented that some elicitors induced metabolic routes that differ from the targeted one. For example, Sevon *et al*. (1992) found that hyoscyamine content in hairy root cultures of *Hyoscyamus muticus* increased by 5 folds with the use of chitosan. On the other hand, Ballica *et al*. (1993) failed to induce the production of hyoscyamine in cell cultures of *Datura stramonium* using chitosan. Hence, the use of elicitors, which are not specific to the tissue culture system, may cause ineffective elicitation.

Based on the results from colourimetric analysis, the inoculation of exogenous Phe for the production of phenolics and flavonoids markedly increased in somatic embryos, as compared to dark-induced callus cultures of sweet shoot. This may be due to the morphological differentiation processes occurring in each individual cultured tissue (Agarwal and Kamal, 2007). For some plants, the formation of secondary metabolites is integrated into the differentiation process, as previously explained in Section 6.3.1.3. In this study, the morphological structures of somatic embryos are more differentiated than dark-induced callus cultures of sweet shoot, as shown in Section 5.3.3. Well defined embryogenic characteristics such as starch grains, nuclei, procambium, cytoplasmic structures and meristematic tissues were observed in somatic embryos of sweet shoot. Therefore, the phenolic and flavonoid compounds were synthesized in greater quantity in somatic embryos than dark-induced callus of sweet shoot, as evidenced by Agarwal and Kamal (2007) in *in vitro* cultures of *Momordica charantia*. However, the total phenolic and total flavonoid values obtained from somatic embryos were still comparatively lower than those obtained from shadehouse-grown plants, shoot cultures and light-induced callus cultures of sweet shoot. It could be attributed to the differences in incubation conditions as somatic embryos of sweet shoot were incubated in the absence of light. Hence,

similar to dark-induced callus cultures, the embryos were not required to generate antioxidants against the photosensitization induced oxidative damage, as previously mentioned in Section 6.3.1.4.

6.3.2 Determination of DPPH and FRAP antioxidant activity

6.3.2.1 Shadehouse-grown sweet shoot

Analysis of variance was used to determine the effects of elicitor and precursor treatments on antioxidant activity of cultured tissues. Untreated shadehouse-grown plants of sweet shoot at week 0 had an antioxidant activity averaging 54.03% for DPPH assay and 397.56 µg/10g FW for FRAP assay (baseline). In Table 6.7, the overall picture showed significant effects of elicitor and precursor treatments in improving the antioxidant capacity of shadehouse-grown sweet shoot, and Phe was found to be the most effective ($p<0.05$). The highest antioxidant activity of DPPH and FRAP were recorded at 93.45% and 3244.79 µg/10g FW at concentrations of 20 mg/l and 10 mg/l Phe, respectively, after three weeks of precursor treatment. This corresponds to a 1.73 and 8.16 fold increase in antioxidant activity compared to baseline, and 1.29 and 5.66 fold rise compared to untreated plants after 3 weeks, respectively. A similar observation was obtained in lentil sprout, in which DPPH and FRAP antioxidant activities were increased by 1.29 and 1.34 fold respectively, after the addition of 0.1 mM of Phe (Swieca *et al*., 2014). The promoting effects of Phe in shadehouse-grown plants may be due to the activation of specific enzymes such as PAL and CHS, as discussed earlier in Section 6.3.1.1. These results were in line with Wang *et al*. (2009), Ghasemzadeh *et al*. (2012) and Mendhulkar and Vakil (2013) for Chinese bayberries, *Zingiber officinale* and *Andrographis paniculata*.

Table 6.7. Effect of methyl jasmonate, salicylic acid and phenylalanine on the DPPH radical scavenging activity and FRAP reducing activity of shadehouse-grown sweet shoot, after 3 weeks of elicitor treatment.

Types of elicitors/precursors	Concentration	DPPH radical scavenging activity (%)			FRAP assay (µg $FeSO_4$/10 g FW)		
		week 1	week 2	week 3	week 1	week 2	week 3
Methyl jasmonate	0µM	60.15 $_{c}$	61.66 $_{c}$	72.45 $_{bc}$	430.61 $_{e}$	457.05 $_{de}$	573.45 $_{bc}$
	50µM	60.69 $_{c}$	61.10 $_{c}$	80.32 $_{ab}$	432.93 $_{e}$	460.40 $_{de}$	598.21 $_{bc}$
	100µM	62.03 $_{c}$	69.91 $_{bc}$	84.68 $_{a}$	459.67 $_{de}$	510.75 $_{cd}$	630.01 $_{b}$
	200µM	69.09 $_{bc}$	70.69 $_{bc}$	82.51 $_{ab}$	530.36 $_{bcd}$	546.45 $_{bcd}$	667.29 $_{a}$
Salicylic acid	0µM	60.15 $_{cd}$	61.66 $_{cd}$	72.45 $_{ab}$	430.61 $_{cde}$	457.05 $_{cd}$	573.45 $_{ab}$
	50µM	58.59 $_{d}$	60.19 $_{cd}$	61.28 $_{cd}$	407.09 $_{e}$	433.25 $_{cde}$	457.21 $_{cd}$
	100µM	67.30 $_{bc}$	62.24 $_{cd}$	76.68 $_{a}$	496.89 $_{bc}$	530.72 $_{bc}$	580.06 $_{ab}$
	200µM	68.05 $_{bc}$	74.36 $_{ab}$	76.14 $_{a}$	480.69 $_{bcd}$	451.79 $_{cd}$	619.88 $_{a}$
Phenylalanine	0 mg/l	60.15 $_{d}$	61.66 $_{d}$	72.45 $_{cd}$	430.61 $_{e}$	457.05 $_{de}$	573.45 $_{de}$
	5 mg/l	75.10 $_{cd}$	80.12 $_{bc}$	87.82 $_{ab}$	583.32 $_{de}$	608.97 $_{d}$	1239.41 $_{c}$
	10 mg/l	79.47 $_{bc}$	82.57 $_{bc}$	91.31 $_{ab}$	590.43 $_{de}$	829.01 $_{cd}$	3244.79 $_{a}$
	20 mg/l	81.93 $_{bc}$	88.93 $_{ab}$	93.54 $_{a}$	650.51 $_{cd}$	1917.11 $_{bc}$	2211.97 $_{b}$

* Values for FRAP assay expressed as µg $FeSO_4$ equivalent per 10 g fresh weight samples (FW).
* Values expressed as means of five biological replicated experiments and each biological replicate consisted of three technical replicates.
* Different letters within row and column (lowercase) indicate a significant difference ($p<0.05$) according to Duncan's multiple range test.

There was slight difference in the optimal Phe concentration for both antioxidant assays. In DPPH assay, 20 mg/l of Phe produced the best result but for FRAP assay, the highest antioxidant activity was at 10 mg/l of Phe. This observation contradicts with the results seen in section 6.3.1.1, which showed the highest phenolic and flavonoid contents were at 20 mg/l of Phe harvested during week three. The possible contributory factors to this could be ascribed to the availability of different bioactive phytochemicals which had antioxidant activities (Hsu *et al*., 2005). Preliminary phytochemical screening indicated the presence of alkaloids, glycosides, saponins, sterols and steroids in shadehouse-grown plants of sweet shoot treated with Phe (Appendix Table 13). These bioactive phytochemicals together with phenolics and flavonoids may provide synergistic actions leading to a higher antioxidant capacity (Cetkovic *et al*., 2004). All these bioactive phytochemicals were most probably induced at lower concentrations of Phe, in this study probably at 10 mg/l. Moreover, the Folin-Ciocalteau method and aluminium chloride colourimetric assay are not an absolute measurement for the amount of phenolic and flavonoid compounds (Wong *et al*., 2006) as different types of phenolic and flavonoid compounds have differing antioxidant activities, which were dependant on their structure (Sengul *et al*., 2009).

Numerous studies, including those by Wong *et al*. (2006), Maisuthisakul *et al*. (2008) and Andarwulan *et al*. (2010), reported that the antioxidant capacity of plant extracts could be attributed to the total phenolic and total flavonoid contents of sweet shoot. To analyze the correlative relationships amongst the total antioxidant activity (DPPH and FRAP), total phenolic content, and total flavonoid content of sweet shoot, a Pearson correlation coefficient analysis was conducted in this study (Table 6.8). In this study, the DPPH values were closely correlated to the total phenolic and total flavonoid contents of sweet shoot with $R=0.88$ ($p<0.05$) and 0.87 ($p<0.05$), respectively. Furthermore, the reducing potential of antioxidant components is strongly associated with the phenolic ($R=0.84$, $p<0.05$) and flavonoid ($R=0.87$, $p<0.05$) contents of sweet shoot treated with Phe. These results indicated that in sweet shoot plant extracts with higher levels of phenolic and flavanoids, greater scavenging activity and reducing power were observed, similar to reports shown in *Moringa Oleifera* (Siddhuraju and Becker, 2003), wheat (Chen *et al.*, 2006b) and *Azadirachta indica* (Sultana *et al.*, 2007). In between the two antioxidant

activity assay results (DPPH and FRAP assay), significant correlations were noted, suggesting that both of these antioxidant activity assays were reliable and interchangeable. DPPH correlated well with FRAP ($R=0.74$, $p<0.05$), and this could be explained from the basic concept that antioxidants are reducing agents. These results were supported by the works done for *Centella asiatica* (Wong *et al*., 2006), *Salvia miltiorrhiza* (Dong *et al*., 2010) and *Zingiber officinale* (Ghasemzadeh *et al*., 2012).

Table 6.8. Correlation between measured parameters in shadehouse-grown plants of sweet shoot.

	DPPH radical scavenging activity	**FRAP reducing activity**
Total phenolic content	0.8858	0.8406
Total flavonoid content	0.8743	0.8699
DPPH radical scavenging activity		0.7412

6.3.2.2 *In vitro* shoot culture of sweet shoot

The effects of MJ, SA and Phe on the antioxidant activity of *in vitro* shoot cultures varied according to the elicitor concentration and contact duration tested in the present study. At week 0, untreated shoot cultures displayed the ability to scavenge 58.02% of DPPH free radicals and could reduce ferric ions at a concentration of 388.49 µg/10g FW (baseline). The addition of Phe to shoot cultures significantly increased the antioxidant activities in sweet shoot, followed by those treated with MJ and SA (Table 6.9) ($p<0.05$), with the maximum scavenging of DPPH free radicals (84.53%) seen in week 3 with Phe concentration of 10 mg/l, corresponding to 1.45 fold greater than baseline. It can be suggested that for large-scale production of antioxidants from shoot cultures of sweet shoot, Phe at concentration of 10 mg/l would be more economical based on the above results, as the phenolic and flavonoid contents were also produced maximally at this similar concentration (mentioned earlier in Section 6.3.1.2).

Table 6.9. Effect of methyl jasmonate, salicylic acid and phenylalanine on the DPPH radical scavenging activity and FRAP reducing activity of shoot cultures of sweet shoot, after 21 days of elicitor treatment.

Types of elicitors/precursors	Concentration	DPPH radical scavenging activity (%)			FRAP assay (µg $FeSO_4$/10 g FW)		
		week 1	week 2	week 3	week 1	week 2	week 3
Methyl jasmonate	0µM	66.07 $_{bcd}$	67.14 $_{bc}$	72.25 $_{b}$	567.64 $_{cd}$	581.55 $_{cd}$	606.87 $_{c}$
	50µM	54.41 $_{d}$	55.76 $_{d}$	73.50 $_{b}$	464.29 $_{d}$	474.21 $_{d}$	583.09 $_{cd}$
	100µM	65.85 $_{bcd}$	54.41 $_{d}$	79.53 $_{a}$	566.33 $_{cd}$	575.34 $_{cd}$	724.11 $_{b}$
	200µM	61.26 $_{cd}$	64.84 $_{bcd}$	69.82 $_{bc}$	583.96 $_{cd}$	611.11 $_{c}$	1998.76 $_{a}$
Salicylic acid	0µM	66.07 $_{bc}$	67.14 $_{bc}$	72.25 $_{ab}$	567.64 $_{bc}$	581.55 $_{bc}$	606.87 $_{b}$
	50µM	51.55 $_{d}$	59.70 $_{bcd}$	69.30 $_{bc}$	445.23 $_{e}$	508.07 $_{cd}$	566.80 $_{bc}$
	100µM	57.86 $_{bcd}$	64.46 $_{bc}$	74.65 $_{a}$	451.47 $_{e}$	666.69 $_{a}$	579.97 $_{bc}$
	200µM	51.89 $_{d}$	57.57 $_{bcd}$	72.73 $_{a}$	485.44 $_{cde}$	475.69 $_{cde}$	644.70 $_{ab}$
Phenylalanine	0 mg/l	66.07 $_{c}$	67.14 $_{c}$	72.25 $_{bc}$	567.64 $_{g}$	581.55 $_{g}$	606.87 $_{fg}$
	5 mg/l	66.99 $_{c}$	73.85 $_{bc}$	79.15 $_{ab}$	579.69 $_{g}$	640.93 $_{f}$	2047.99 $_{c}$
	10 mg/l	72.86 $_{bc}$	78.70 $_{ab}$	84.53 $_{a}$	630.96 $_{f}$	1354.37 $_{d}$	2986.17 $_{b}$
	20 mg/l	77.31 $_{ab}$	82.44 $_{a}$	82.71 $_{a}$	1053.03 $_{e}$	2398.07 $_{bc}$	4652.09 $_{a}$

* Values for FRAP assay expressed as µg $FeSO_4$ equivalent per 10 g fresh weight samples (FW).
* Values expressed as means of five biological replicated experiments and each biological replicate consisted of three technical replicates.
* Different letters within row and column (lowercase) indicate a significant difference ($p<0.05$) according to Duncan's multiple range test.

FRAP assay shown in Table 6.9 exhibited the highest ferric ion reducing capacity (4652.09 µg/10g FW) in shoot cultures treated with 20 mg/l of Phe by week 3 (Table 6.9). Again, the difference seen in this study may be due to the presence of other bioactive phytochemical compounds, as mentioned in Section 6.3.2.1, which was also seen during preliminary phytochemical screenings in *in vitro* shoot cultures of sweet shoot treated with Phe (Appendix Table 14).

In this study, an inhibitory effect on the DPPH antioxidant activity was seen at the highest concentrations of both MJ and SA-treated shoot cultures (200 µM). The highest DPPH antioxidant activity for both MJ and SA were in fact at moderate concentrations of 100 µM. This can be explained from the perspective of feedback inhibition, which occurs in the metabolic pathway as cited in Section 6.3.1.2 (Zia *et al*., 2007). In FRAP assay, however, the highest concentration of MJ (200 µM) revealed a higher ferric ion reducing capability. Despite this, it was still significantly weaker than those cultured in any concentration of Phe at week 3.

Pearson correlation coefficient analysis, as exhibited in Table 6.10, revealed that significant correlations were achieved amongst the total phenolic content, total flavonoid content and antioxidant activity of *in vitro* shoot cultures of sweet shoot. The strongest correlation was found between total phenolic content and DPPH free radical scavenging activity ($R=0.90$, $p<0.05$), suggesting that the scavenging ability of sweet shoot was probably contributed by polyphenols present in shoot cultures. Furthermore, excellent correlationship also existed between total flavonoid content and DPPH free radical scavenging activity ($R=0.87$, $p<0.05$). Upon examining FRAP reducing activity, good correlations were also found with total phenolic content ($R=0.83$, $p<0.05$) and total flavonoid content ($R=0.84$, $p<0.05$) respectively. In correlating between FRAP and DPPH activity, the coefficient was high at 0.81 ($p<0.05$). The strongest free radical scavenging activity and reducing power was detected in shoot cultures fed with Phe. These results suggested that the high phenolic and flavonoid contents were most probably responsible for the antioxidant activity and reducing power of the *in vitro* shoot cultures. Similar findings were also seen in shadehouse-grown plants of sweet shoot treated with Phe, as reported earlier in Section 6.3.2.1.

Table 6.10. Correlation between measured parameters in *in vitro* shoot culture of sweet shoot.

	DPPH radical scavenging activity	FRAP reducing activity
Total phenolic content	0.8999	0.8250
Total flavonoid content	0.8706	0.8437
DPPH radical scavenging activity		0.8068

6.3.2.3 Light-induced callus culture of sweet shoot

Elicitor and precursor feeding of light-induced callus were carried out by adding MJ, SA and Phe individually in hormone-free MS medium for an elicitation period of 3 weeks. At week 0, the untreated light-induced callus cultures of sweet shoot showed antioxidant activities with a mean value of 49.15% for DPPH assay and a mean value of 334.96 µg/10g FW for FRAP assay at baseline.

The addition of elicitors and precursor significantly enhanced the ability for antioxidant production in light-induced callus cultures at all levels of concentrations and contact durations ($p<0.05$) (Table 6.11), with the most potent being Phe at a concentration of 20 mg/l of Phe as the reduction capacity of ferric ions (5941.66 µg/10g FW) and scavenging activity of DPPH radicals (97.35%) were the highest, after three weeks of precursor feeding. These results equal to an increase of 1.98 fold and 17.74 fold from baseline, and higher by 1.19 and 8.01 folds in both DPPH and FRAP antioxidant activities, when compared to untreated light-induced callus cultures at third week. As seen in Section 6.3.1.3, this Phe concentration (20 mg/l) also produced the highest phenolic and flavonoid contents in light-induced callus harvested at week 3. These combination of results suggested that Phe at this concentration (20 mg/l) would be the most ideal for production of biochemically active antioxidant metabolites in light-induced callus cultures of sweet shoot. The reasons for this analogous result were discussed in detail at Section 6.1 and Section 6.3.1.1, in which Phe served as an upstream precursor in phenylpropanoid pathway for the production of antioxidant metabolites. These results were also in agreement with the study done by Bemani *et al.* (2013) in cell suspension cultures of *Corylus avellana*, where the antioxidant activities were substantially enhanced by 1.5 fold for DPPH assay and 1.3 fold for FRAP assay, after the addition of Phe at a concentration of 6 mM.

Table 6.11. Effect of methyl jasmonate, salicylic acid and phenylalanine on the DPPH radical scavenging activity and FRAP reducing activity of light-induced callus cultures of sweet shoot, after a treatment period of 3 weeks.

Types of elicitors/precursors	Concentration	DPPH radical scavenging activity (%)			FRAP assay (µg $FeSO_4$/10 g FW)		
		week 1	week 2	week 3	week 1	week 2	week 3
Methyl jasmonate	0µM	67.80 $_{e}$	79.97 $_{cd}$	82.06 $_{bc}$	476.87 $_{e}$	583.19 $_{d}$	742.03 $_{cd}$
	50µM	70.95 $_{d}$	79.65 $_{cd}$	87.60 $_{ab}$	527.23 $_{de}$	533.12 $_{de}$	1024.99 $_{c}$
	100µM	79.12 $_{bcd}$	83.71 $_{bc}$	90.44 $_{ab}$	581.03 $_{d}$	699.21 $_{cd}$	2131.59 $_{b}$
	200µM	82.98 $_{bc}$	85.67 $_{abc}$	94.72 $_{a}$	596.45 $_{d}$	934.49 $_{c}$	3865.47 $_{a}$
Salicylic acid	0µM	67.80 $_{cd}$	79.97 $_{ab}$	76.99 $_{bc}$	476.87 $_{cd}$	583.19 $_{bc}$	742.03 $_{bc}$
	50µM	68.08 $_{cd}$	74.84 $_{bc}$	80.88 $_{ab}$	462.53 $_{d}$	566.70 $_{bc}$	640.13 $_{ab}$
	100µM	72.36 $_{bcd}$	82.62 $_{a}$	82.06 $_{a}$	565.62 $_{bc}$	742.03 $_{a}$	645.96 $_{ab}$
	200µM	64.04 $_{d}$	67.52 $_{cd}$	76.26 $_{bc}$	479.18 $_{cd}$	576.26 $_{bc}$	478.13 $_{cd}$
Phenylalanine	0 mg/l	67.80 $_{d}$	79.97 $_{cd}$	82.06 $_{cd}$	476.87 $_{g}$	583.19 $_{fg}$	742.03 $_{f}$
	5 mg/l	80.43 $_{cd}$	81.40 $_{cd}$	90.51 $_{bc}$	593.61 $_{fg}$	743.76 $_{f}$	2541.89 $_{d}$
	10 mg/l	82.29 $_{cd}$	88.93 $_{bc}$	94.12 $_{b}$	709.59 $_{f}$	2504.55 $_{d}$	3116.88 $_{c}$
	20 mg/l	89.17 $_{bc}$	92.75 $_{b}$	97.35 $_{a}$	1903.05 $_{e}$	4300.93 $_{b}$	5941.66 $_{a}$

* Values for FRAP assay expressed as µg $FeSO_4$ equivalent per 10 g fresh weight samples (FW).
* Values expressed as means of five biological replicated experiments and each biological replicate consisted of three technical replicates.
* Different letters within row and column (lowercase) indicate a significant difference ($p<0.05$) according to Duncan's multiple range test.

Amongst the two elicitors tested, the DPPH and FRAP antioxidant activities for light-induced cultures treated with MJ were significantly higher than those treated with SA ($p<0.05$) (Table 6.11). The DPPH scavenging activity of MJ-treated callus cultures ranged from 70.95% to 94.72%, whereas it ranged from 68.08% to 82.06% for SA-treated callus cultures. For FRAP assay, an antioxidant activity range of 527.23 µg/10g FW to 3865.47 µg/10g FW was detected in MJ-elicited callus cultures, whereas the ferric reducing activity of SA-elicited callus cultures ranged from 462.53 µg/10g FW to 742.03 µg/10g FW. The superior effects of MJ elicitation for improvement of antioxidant capacity may be due to the activation of a higher number of defense-related genes in light-induced callus cultures of sweet shoot (Hendrawati *et al*., 2006) when compared to SA. Similar phenomenon was observed in *Arabidopsis* plants treated with MJ, which was previously discussed in detail in Section 6.3.1.1.

With reference to Table 6.12, Pearson correlation coefficient showed positive linear correlation of total phenolic content against the antioxidant activities based on DPPH ($R=0.84$, $p<0.05$) and FRAP ($R=0.95$, $p<0.05$) assays. Predictably, the antioxidant activity of light-induced callus also showed strong correlationship with total flavonoid content of sweet shoot ($R_{DPPH}=0.87$ and $R_{FRAP}=0.96$, $p<0.05$). The increased antioxidant activity could be attributed to the presence of flavonoid and phenolic compounds in light-induced callus treated with Phe, which strengthens the results shown in both shadehouse-grown plants and shoot cultures. Positive correlation was again seen in DPPH and FRAP assay ($R=0.85$, $p<0.05$), contributing to the significant results of this study thus far. The results obtained in this study were further supported by previous reports on callus cultures of *Phalaenopsis* orchid (Ali *et al*., 2006) and *Raphanus sativus* (Kim *et al*., 2006).

Table 6.12. Correlation between measured parameters in light-induced callus culture of sweet shoot.

	DPPH radical scavenging activity	**FRAP reducing activity**
Total phenolic content	0.8356	0.9485
Total flavonoid content	0.8732	0.9579
DPPH radical scavenging activity		0.8471

6.3.2.4 Dark-induced callus culture of sweet shoot

Data shown in Table 6.13, displayed levels of DPPH and FRAP antioxidant activities of untreated dark-induced callus cultures of 49.65% and 328.38 µg/10g FW, respectively (baseline). The addition of MJ, SA and Phe were shown, statistically, to be effective in improving the antioxidant capacity of dark-induced callus cultures of sweet shoot ($p<0.05$). Amongst the tested treatments, Phe-treated cultures showed the greatest increase in antioxidant activity, followed by cultures elicited by MJ and SA (Table 6.13). The highest DPPH (83.47%) and FRAP antioxidant activity (825.04 µg/10g FW) was at Phe concentration of 10 mg/l. This was 1.68 and 2.51 folds higher than baseline respectively (Table 6.13). Upon comparing to untreated callus cultures at week 3, these maximal levels of DPPH and FRAP antioxidant activities were 1.32 and 1.83 folds higher, respectively. In comparison with similar dark-induced callus cultures in Section 6.3.1.4, the highest phenolic and flavonoid contents were again in Phe concentration of 10 mg/l at week 3. It can be conceived that Phe at 10 mg/l is the most ideal precursor for enhancing antioxidant production of biochemically active metabolites in dark-induced callus cultures of sweet shoot. Higher concentrations of Phe (20 mg/l), however, had adverse outcomes, as the DPPH and FRAP activities were much lower. The explanation for this occurrence could be due to feedback inhibition in the metabolic pathway, as explained in the similar section as mentioned above.

Table 6.13. Effect of methyl jasmonate, salicylic acid and phenylalanine on the DPPH radical scavenging activity and FRAP reducing activity of dark-induced callus cultures of sweet shoot, after a treatment period of 21 days.

Types of elicitors/precursors	Concentration	DPPH radical scavenging activity (%)			FRAP assay (µg $FeSO_4$/10 g FW)		
		week 1	week 2	week 3	week 1	week 2	week 3
Methyl jasmonate	0µM	50.91 $_{cde}$	51.45 $_{cde}$	63.23 $_{bc}$	352.41 $_{de}$	359.56 $_{de}$	451.03 $_{bc}$
	50µM	45.01 $_{e}$	52.39 $_{cde}$	66.09 $_{b}$	339.78 $_{e}$	429.65 $_{cd}$	479.01 $_{b}$
	100µM	57.17 $_{cd}$	68.92 $_{b}$	75.38 $_{a}$	431.59 $_{cd}$	539.11 $_{a}$	480.37 $_{b}$
	200µM	52.29 $_{cde}$	62.13 $_{bc}$	54.27 $_{cde}$	407.57 $_{cd}$	473.63 $_{b}$	553.35 $_{a}$
Salicylic acid	0µM	50.91 $_{d}$	51.45 $_{d}$	63.23 $_{ab}$	352.41 $_{e}$	359.56 $_{e}$	451.03 $_{bc}$
	50µM	56.23 $_{cd}$	59.97 $_{bc}$	65.46 $_{ab}$	382.11 $_{de}$	419.69 $_{cd}$	473.31 $_{b}$
	100µM	60.70 $_{bc}$	61.02 $_{bc}$	68.46 $_{a}$	429.70 $_{bc}$	438.91 $_{bc}$	483.50 $_{b}$
	200µM	61.63 $_{bc}$	58.03 $_{bc}$	70.02 $_{a}$	400.51 $_{cd}$	444.86 $_{bc}$	533.45 $_{a}$
Phenylalanine	0 mg/l	50.91 $_{e}$	51.45 $_{e}$	63.23 $_{cd}$	352.41 $_{f}$	359.56 $_{f}$	451.03 $_{e}$
	5 mg/l	61.96 $_{cd}$	65.67 $_{cd}$	77.11 $_{abc}$	433.49 $_{e}$	473.67 $_{d}$	589.41 $_{bc}$
	10 mg/l	74.02 $_{bc}$	74.93 $_{bc}$	83.47 $_{a}$	547.56 $_{bcd}$	717.22 $_{ab}$	825.04 $_{a}$
	20 mg/l	67.51 $_{cd}$	80.33 $_{ab}$	82.29 $_{a}$	479.89 $_{d}$	658.23 $_{abc}$	565.26 $_{bcd}$

* Values for FRAP assay expressed as µg $FeSO_4$ equivalent per 10 g fresh weight samples (FW).
* Values expressed as means of five biological replicated experiments and each biological replicate consisted of three technical replicates.
* Different letters within row and column (lowercase) indicate a significant difference ($p<0.05$) according to Duncan's multiple range test.

The overall values for antioxidant activities obtained from dark-induced callus cultures (83.47% for DPPH assay and 825.04 µg/10g FW for FRAP assay) were significantly lower when compared to light-induced callus cultures (97.35% for DPPH assay and 5941.66 µg/10g FW for FRAP assay) ($p<0.05$). According to Jacob and Malpathak (2004), plant cell cultures grown under illuminated conditions enhanced the levels of photosynthesis, which can generate intermediate compounds not included in the culture media, but were needed for improvement of primary and secondary metabolism in these cultures. This phenomenon may explain the higher antioxidant activity which was observed in light-induced callus cultures of sweet shoot. Additionally, cultures induced under continuous illumination also produced higher total phenolic (246.62 µg/10g FW) and flavonoid (636.26 µg/10g FW) content than those incubated in the dark (70.07 µg/10g FW for phenolic content and 212.40 µg/10g FW for flavonoid content). More detailed discussions were noted in the last paragraph of section 6.3.1.4.

The correlations of antioxidant activities (DPPH and FRAP assays) with total phenolic and total flavonoid contents of dark-induced callus were shown in Table 6.14. Pearson correlation coefficient analysis revealed that DPPH free radical scavenging activity and FRAP reducing power positively correlated to total phenolic ($R_{DPPH}=0.95$ and $R_{FRAP}=0.96$, $p<0.05$) and total flavonoid ($R_{DPPH}=0.88$ and $R_{FRAP}=0.86$, $p<0.05$) contents of sweet shoot dark-induced callus cultures fed with 10 mg/l of Phe. These results further strengthen the proposition made in previous sections, whereby sweet shoot treated with Phe, which had strong antioxidant activity, had high total phenolic and total flavonoid contents. Similar observations were made by Arnous *et al*. (2000) and Pulido *et al.* (2000), whereby a strong correlation between DPPH and FRAP assay was observed in red and white wine analysis.

Table 6.14. Correlation between measured parameters in dark-induced callus culture of sweet shoot.

	DPPH radical scavenging activity	**FRAP reducing activity**
Total phenolic content	0.9547	0.8837
Total flavonoid content	0.9645	0.8645
DPPH radical scavenging activity		0.8796

6.3.2.5 Somatic embryo culture of sweet shoot

In this study, the exogenous application of elicitors (SA and MJ) and precursor (Phe) to hormone-free MS medium played a major role in enhancing DPPH scavenging activity and FRAP reducing activity of somatic embryos in sweet shoot. The antioxidant activity in untreated somatic embryos at week 0, averaged at 55.38% for DPPH assay and 351.85 µg/10g FW for FRAP assay (baseline). An increment in DPPH and FRAP antioxidant activities were observed in somatic embryos fed with Phe, followed by those treated with MJ and SA ($p<0.05$) (Table 6.15). The highest DPPH free radical scavenging activity (90.71%) was obtained on MS medium fortified with 20 mg/l of Phe, after three weeks of precursor treatment. This corresponds to a 1.64 fold rise in DPPH antioxidant activity compared to baseline. In contrast, the strongest FRAP reducing ability (1322.91 µg/10g FW) was obtained in somatic embryos treated with 10 mg/l of Phe, which was 3.76 fold higher than baseline. The differences in concentration of Phe in producing maximum DPPH and FRAP activities may be due to the presence of other biochemically active compounds in somatic embryo extracts, such as alkaloids, glycosides, saponins, sterols and steroids (Appendix Table 15). This observation was similar to Sections 6.3.2.1 and 6.3.2.2 in shadehouse-grown plants and *in vitro* shoot cultures of sweet shoot.

Table 6.15. Effect of methyl jasmonate, salicylic acid and phenylalanine on the DPPH radical scavenging activity and FRAP reducing activity of somatic embryos of sweet shoot, after 3 weeks of elicitor treatment.

Types of elicitors/precursors	Concentration	DPPH radical scavenging activity (%)			FRAP assay (µg $FeSO_4$/10 g FW)		
		week 1	week 2	week 3	week 1	week 2	week 3
Methyl jasmonate	0µM	58.96 $_{c}$	71.69 $_{b}$	67.83 $_{bc}$	413.34 $_{de}$	565.41 $_{b}$	485.37 $_{c}$
	50µM	57.44 $_{c}$	59.84 $_{c}$	79.08 $_{a}$	361.57 $_{e}$	375.97 $_{e}$	621.63 $_{a}$
	100µM	67.34 $_{bc}$	71.70 $_{b}$	66.40 $_{bc}$	366.75 $_{e}$	364.95 $_{e}$	486.79 $_{c}$
	200µM	58.80 $_{c}$	65.86 $_{bc}$	65.48 $_{bc}$	386.32 $_{e}$	454.67 $_{d}$	474.26 $_{c}$
Salicylic acid	0µM	58.96 $_{cd}$	71.69 $_{ab}$	67.83 $_{bc}$	413.34 $_{d}$	565.41 $_{a}$	485.37 $_{b}$
	50µM	57.54 $_{cd}$	59.36 $_{cd}$	69.03 $_{ab}$	410.25 $_{d}$	426.79 $_{cd}$	493.39 $_{b}$
	100µM	60.48 $_{cd}$	60.53 $_{cd}$	73.02 $_{a}$	415.54 $_{d}$	460.85 $_{bc}$	565.99 $_{a}$
	200µM	58.71 $_{cd}$	64.60 $_{bc}$	65.34 $_{bc}$	436.19 $_{cd}$	454.58 $_{bc}$	470.04 $_{bc}$
Phenylalanine	0 mg/l	58.96 $_{e}$	71.69 $_{d}$	67.83 $_{de}$	413.34 $_{f}$	565.41 $_{def}$	485.37 $_{ef}$
	5 mg/l	69.47 $_{d}$	76.04 $_{cd}$	83.78 $_{bc}$	449.59 $_{f}$	579.25 $_{de}$	692.09 $_{c}$
	10 mg/l	75.83 $_{cd}$	80.26 $_{bcd}$	87.33 $_{ab}$	523.27 $_{ef}$	663.08 $_{cd}$	1322.91 $_{a}$
	20 mg/l	61.92 $_{e}$	82.77 $_{bc}$	90.71 $_{a}$	575.65 $_{de}$	580.22 $_{de}$	967.10 $_{b}$

* Values for FRAP assay expressed as µg $FeSO_4$ equivalent per 10 g fresh weight samples (FW).
* Values expressed as means of five biological replicated experiments and each biological replicate consisted of three technical replicates.
* Different letters within row and column (lowercase) indicate a significant difference ($p<0.05$) according to Duncan's multiple range test.

Amongst the two tested elicitors (MJ and SA), the influence of elicitors on both antioxidant activities depended on its concentrations and contact durations ($p<0.05$). In MJ-elicited somatic embryos, the optimal concentration was a lowly 50 µM as that produced the highest antioxidant activity in both DPPH (79.08%) and FRAP (621.63 µg/10g FW) assays. Higher MJ concentrations (100 and 200 µM) led to lower antioxidant activity. Meanwhile, in SA-elicited somatic embryos, the best antioxidant activity in both DPPH (73.02%) and FRAP (565.99 µg/10g FW) assays, was seen in concentrations of 100 µM. Subsequent increment in SA concentration (200 µM) produced lower antioxidant activity. These two elicitors appear to amplify the antioxidant levels at lower concentration and may be due to the positive effects on plant defense signaling pathway. However, at higher concentrations, the elicitors may have inhibitory effects leading to lower antioxidants assay levels. This has been highlighted before in Sections 6.3.1.3 and 6.3.1.4.

The overall correlations between DPPH and FRAP assays, and phenolic and flavonoid contents in somatic embryos were positive in a linear manner (Table 6.16). The best correlation was seen between total phenolic content and DPPH radical scavenging activity ($R=0.93$, $p<0.05$); as phenolic compounds reflect the antioxidant activity in stressed cultures, this may contribute to cell protection from free radical oxidative damage and leads to reduction in its toxicity on cytoplasmic structures (Mittler, 2002; Wahid and Ghazanfar, 2006). For total flavonoid content, the DPPH ($R=0.89$, $p<0.05$) and FRAP ($R=0.82$, $p<0.05$) antioxidant activities of somatic embryos fed with Phe were also correlated with a positive regression coefficient (Table 6.16). Besides that, positive linear correlation ($R=0.73$, $p<0.05$) was observed between DPPH and FRAP assay in cultures elicited with Phe. These findings were in complete coherence with previous experiments on shadehouse-grown plants and other cultured tissues (shoot cultures, light and dark-induced callus cultures), suggesting that infusion of Phe into sweet shoot boosted the production of bioactive metabolites required for their antioxidant activities.

Table 6.16. Correlation between measured parameters in somatic embryo cultures of sweet shoot.

	DPPH radical scavenging activity	FRAP reducing activity
Total phenolic content	0.9317	0.8158
Total flavonoid content	0.8947	0.8198
DPPH radical scavenging activity		0.7292

6.3.3 Determination of PAL and CHS enzymatic activity

In the past decade, several important pathways are associated in the production of flavonoids and phenolics, such as shikimate pathway, phenylpropanoid pathway and flavonoid biosynthetic pathway, as reported in model plants like *Arabidopsis* and tobacco (Petersen and Simmonds, 2003; Fraser and Chapple, 2011). A large number of enzymes and genes are involved in these metabolic pathways and according to Winkel-Shirley (2001) and Ghasemzadeh *et al.* (2012), regulatory enzymes such as PAL and CHS are the key entry enzymes committed to the production of antioxidants in plants. It was also mentioned in the report by Fraser and Chapple (2011) that PAL and CHS enzyme activities could be induced by environmental stresses such as nutrient deficiency, prolonged exposure to UV light and extreme weather and pathogen attack.

A study done in tobacco plant showed that the suppression of PAL enzyme led to the formation of abnormal phenotypes, such as altered leaf shape, abnormal flower morphology, localized fluorescent lesions and reduced pollen viability (Elkind *et al*., 1990). As such, these enzymes were important in plant defense mechanism, in order to have a better adaptation to the environmental stresses. Since both of these enzymes are developmentally and tissue specifically regulated, many scientists attempted to enhance PAL and CHS enzymatic activities in cultured tissues via precursor, abiotic and biotic elicitor treatment. Examples of studies done were in parsley (SA treatment), citrus (chilling injury treatment), grapes (MJ treatment) and beans (fungal treatment) (Thukle and Conrath, 1998; Campos *et al*., 2003; Lafuente *et al*., 1997; Chen *et al*., 2006b), which resulted in the accumulation of secondary metabolites and subsequent increase in antioxidant activities.

It has been shown from the above experiments that light-induced callus cultures of sweet shoot produced the most promising levels of

phenolic (246.62 µg/10g FW) and flavonoid (636.26 µg/10g FW) contents in addition with higher antioxidant activities (97.35% for DPPH and 5941.66 µg/10g FW for FRAP assay). Therefore, it is with relative ease to choose this data for further analysis for PAL and CHS enzymatic activity in comparison with the secondary metabolites and antioxidant activities in light-induced callus culture of sweet shoot.

Based on the results shown in Table 6.17, the exogenous incorporation of MJ and SA treatments significantly enhanced the enzymatic activities in light-induced callus culture of sweet shoot, however, the levels found in Phe treated cultures were significantly higher than the two elicitors ($p<0.05$) (Table 6.17). Untreated callus cultures of sweet shoot at week 0 had enzymatic activities averaging 11.86 mmol CA/g FW for PAL assay and 1.82 nkat/mg protein for CHS assay (baseline). In this study, the highest PAL (101.18 mmol CA/g FW) and CHS (14.49 nkat/mg protein) enzymatic activities were attained in light-induced callus cultures fed with 20 mg/l of Phe at week 3, which was 8.53 and 7.96 folds higher than baseline, respectively (Table 6.17). Furthermore, it was also higher by 2.51 and 2.44 folds in both PAL and CHS activities respectively, when compared to the untreated cultures at third week. Similar results were reported by Beaudoin-Eagan and Thorpe (1985) in cell suspension cultures of tobacco, in which PAL activity was increased by 2.67 fold after the addition of 0.37 mBq [U-'4C] Phe. These findings were also seen in cell suspension cultures of *Arnebia euchroma* treated with 0.01 mM Phe (Syklowska-Baranek *et al*., 2012), where the PAL activity was increased from 0.3 U/mg protein to 1.7 U/mg protein.

Table 6.17. Comparison of PAL and CHS enzymatic activities in light-induced callus cultures of sweet shoot treated with methyl jasmonate, salicylic acid and phenylalanine, after 3 weeks of elicitor treatment.

Types of elicitors/ precursor	Concentration	PAL enzymatic activity (mmol CA/g FW)			CHS enzymatic activity (nkat/mg protein)		
		week 1	week 2	week 3	week 1	week 2	week 3
Methyl jasmonate	0µM	21.06 $_{e}$	31.34 $_{de}$	40.30 $_{cd}$	2.12 $_{e}$	4.04 $_{cd}$	5.94 $_{bc}$
	50µM	33.86 $_{de}$	41.68 $_{cd}$	51.52 $_{bc}$	2.29 $_{e}$	4.96 $_{bcd}$	6.34 $_{bc}$
	100µM	49.34 $_{bc}$	52.84 $_{bc}$	60.46 $_{b}$	3.63 $_{de}$	5.73 $_{bc}$	8.95 $_{ab}$
	200µM	56.30 $_{b}$	56.10 $_{b}$	76.90 $_{a}$	4.15 $_{cd}$	7.10 $_{b}$	10.42 $_{a}$
Salicylic acid	0µM	21.06 $_{d}$	31.34 $_{cd}$	40.30 $_{bcd}$	2.12 $_{d}$	4.04 $_{cd}$	5.94 $_{bc}$
	50µM	23.80 $_{d}$	30.22 $_{cd}$	44.74 $_{bc}$	2.20 $_{d}$	4.28 $_{cd}$	5.38 $_{bcd}$
	100µM	29.44 $_{cd}$	39.70 $_{bcd}$	61.04 $_{a}$	2.58 $_{d}$	5.75 $_{bc}$	8.12 $_{a}$
	200µM	37.48 $_{bcd}$	47.96 $_{bc}$	56.40 $_{ab}$	3.58 $_{cd}$	6.32 $_{b}$	7.54 $_{a}$
Phenylalanine	0 mg/l	21.06 $_{e}$	31.34 $_{e}$	40.30 $_{de}$	2.12 $_{e}$	4.04 $_{de}$	5.94 $_{cd}$
	5 mg/l	49.22 $_{de}$	57.86 $_{cd}$	58.44 $_{cd}$	3.29 $_{de}$	6.77 $_{cd}$	8.65 $_{bcd}$
	10 mg/l	58.16 $_{cd}$	70.24 $_{bc}$	87.74 $_{b}$	7.92 $_{bcd}$	8.60 $_{bcd}$	11.66 $_{b}$
	20 mg/l	62.88 $_{bcd}$	77.56 $_{bc}$	101.18 $_{a}$	5.19 $_{cde}$	9.35 $_{bc}$	14.49 $_{a}$

* Values for PAL assay expressed as mmol of cinnamic acid (CA) equivalent per g fresh weight samples (FW).
* Values for CHS assay expressed as nanokatal (nkat) per mg protein.
* Values expressed as means of five biological replicated experiments and each biological replicate consisted of three technical replicates.
* Different letters within row and column (lowercase) indicate a significant difference ($p<0.05$) according to Duncan's multiple range test.

The analysis of variance showed that the enzymatic (PAL and CHS) activities of light-induced callus culture were strongly influenced by both increased concentrations and contact durations of elicitors and precursor ($p<0.05$). When the Phe concentration increased from 5 to 20 mg/l, the enzymatic activities of sweet shoot increased significantly in all tested callus cultures ($p<0.05$). Moreover, these enzymatic activities also had a steady rise from week 0 to week 3 after Phe inoculation. These observations concurred with studies done by Yuan *et al*. (2002), Dong *et al.* (2010) and Ghasemzadeh *et al.* (2012) illustrating that both elicitor dosages and contact durations significantly improved the levels of PAL and CHS activity in cell cultures of *Salvia miltiorrhiza*, *Taxus chinensis* and *Zingiber officinale*.

Recently, many molecular biology experiments have been carried out for the characterization and expression of enzymatic genes involved in phenylpropanoid pathway and flavonoid biosynthetic pathway, especially on the *PAL* and *CHS* genes. In 1997, the pattern for mRNA accumulation was found to parallel that of PAL enzyme activity in cell cultures of *Lithospermum erythrorhizon (*Yazaki *et al., 1997).* The authors stated that PAL activity of cell cultures markedly increased after the addition of elicitor and gradually decreased towards the end of culture period in *Lithospermum erythrorhizon*. Similar findings were also reported by MacDonald and D'Cunha (2007), Sullivan (2009) and Zhang *et al*. (2010), whereby the mRNA expression of *PAL* gene were greatly increased in the cell cultures of *Lithospermum erythrorhizon* treated with Phe, fungal elicitor and MJ. Additionally, northern blot analysis also revealed that the elevation of phenolic and flavonoid content in MJ-elicited sweet basil could be due to the expression of *PAL* gene and other enzymatic genes, such as *cinnamate-4-hydroxylase* (*C4H*) gene, which converted cinnamic acid into coumaric acid (Li *et al*., 2007). This result proved that different enzymes besides PAL were also involved in the production of flavonoids and phenolics.

The recent advances in molecular biology techniques had also helped Xu *et al*. (2008) to isolate the full length of cDNA and genomic DNA of *PAL* genes (*GbPAL*) from *Ginkgo biloba* (GenBank accession number of EU071050). The temporal expression profiling analysis showed that the transcription levels of *GbPAL* were significantly correlated with flavonoid

accumulation in *Ginkgo biloba* treated with SA. These findings suggested that *GbPAL* may play a regulatory role in the biosynthesis of flavonoids in *Ginkgo biloba* at the transcriptional level (Wen *et al.*, 2005). Furthermore, the importance of *CHS* gene in regulating the production of flavonoids were also reported by Obinata *et al*. (2003) and Ghasemzadeh *et al*. (2012) in cultured grape cells and *Zingiber officinale* treated with SA. The authors pointed out that CHS activity correlated with the accumulation of flavonoids in cultured tissues. Similarly, a positive correlation ($R=0.9223$, $p<0.05$) between PAL and CHS enzymatic activity was noted in this study. Hence, PAL and CHS may be the key enzymes which were responsible for the phenylpropanoid metabolism and flavonoid metabolism in light-induced callus cultures of sweet shoot.

To strengthen the findings discussed earlier, Pearson correlation coefficient analysis was performed on all the above mentioned parameters, namely total phenolic and total flavonoid contents, DPPH and FRAP antioxidant activities and PAL and CHS enzymatic activities in light-induced callus cultures of sweet shoot. The results, as shown in Table 6.18, displayed staggering positivity in relation to all these parameters suggesting higher phenolic and flavonoid contents of sweet shoot led to the multiplication of antioxidant activities via enhanced enzymatic activities. These results also revealed that Phe, MJ and SA could acts as signaling molecules that cause changes in gene expression levels that activate PAL and CHS enzyme in phenylpropanoid pathway and flavonoid biosynthetic pathway. Similar observations were also documented in cultured tissues of *Panax ginseng*, *Hypericum perforatum*, sweet basil and radish sprout, whereby the addition of elicitors enhanced the PAL and CHS activities, which in turn led to the accumulation of phenolic and flavonoid compounds (Kim *et al*., 2005; Conceicao *et al.,* 2006; Kim *et al*., 2006; Koca and Karamam, 2014). The results obtained in this study indicated that hormone-free MS medium supplemented with Phe, stimulated enzymatic activities of sweet shoot and could be used as a substrate for the initial step of phenylpropanoid metabolism and flavonoid biosynthetic pathway.

Table 6.18. Correlation between measured parameters in light-induced callus culture of sweet shoot.

	PAL enzymatic activity	CHS enzymatic Activity
Total phenolic content	0.8899	0.8966
Total flavonoid content	0.9086	0.9012
DDPH radical scavenging activity	0.9198	0.8691
FRAP reducing activity	0.8695	0.8650
PAL enzymatic activity		0.9223

6.4 CONCLUSION

An optimized protocol for sustainable metabolite production and antioxidant capacity was successfully established for shadehouse-grown plants and cultured tissues of sweet shoot. The present study showed that shadehouse-grown plants of sweet shoot have the ability to produce secondary metabolites such as phenolic and flavonoid compounds and to neutralize the free radicals generated by oxidative stresses. The best of all plant materials were light-induced callus cultures of sweet shoot, especially those cultured in hormone-free MS medium supplemented with 20 mg/l of Phe. It showed the highest level of phenolic (246.62 µg/10g FW) and flavonoid (636.26 µg/10g FW) compounds, antioxidant activities (97.35% for DPPH and 5941.66 µg/10g FW for FRAP assay) and enzymatic activities (101.18 CA/g FW for PAL and 14.49 nkat/mg protein for CHS assay), when compared to shadehouse-grown plants and other cultured tissues treated with Phe. This result was significantly higher by 6.99 fold for total phenolic content, 4.64 fold for total flavonoid content, 1.98 fold for DPPH free radical scavenging activity, 17.74 fold for FRAP reducing activity, 8.53 fold for PAL enzymatic activity and 7.96 fold for CHS enzymatic activity when compared to untreated light-induced callus cultures of sweet shoot.

Furthermore, Pearson's correlation coefficient analysis also showed positive correlations amongst the total phenolic content, total flavonoid content, antioxidant activities and enzymatic activities of light-induced callus cultures of sweet shoot treated with Phe. These findings suggested that the exogenous application of Phe into culture medium boosted the production of metabolite production which was responsible for the antioxidant activities in light-induced callus cultures of sweet shoot. Similarly, PAL and CHS enzymatic activities were also strongly correlated with the production of metabolite in light-induced callus cultures,

signifying that Phe could be the ideal signaling molecule in activating the enzymatic gene expression of *PAL* and *CHS* in phenylpropanoid pathway and flavonoid biosynthetic pathway. In conclusion, this is the first study to exhibit enhancements in phenolic and flavonoid production, including elevation of antioxidant and enzymatic activities, in cultured tissues of sweet shoot utilizing Phe, MJ and SA as precursor and elicitors.

CHAPTER 7: ANALYSIS OF ANTIOXIDANTS AND PAPAVERINE IN ELICITED AND NON-ELICITED CULTURES OF *Sauropus androgynus* USING REVERSED-PHASE HPLC METHOD

7.1 INTRODUCTION

Flavonoids are widely distributed in woody plant species and have many metabolic functions, which are responsible for the plant's own defense, growth and development (Wink, 2010; Ferreyra *et al*., 2012). All flavonoid compounds share the basic C6-C3-C6 structural skeleton, containing two C6 aromatic rings (A and B) and a heterocylic ring (C) (Figure 7.1) (Kumar and Pandey, 2013). From the variations in the heterocylic ring, flavonoid compounds can be subdivided into flavones (apigenin), flavonols (quercetin and kaempferol), flavanones (naringenin), isoflavones (daidzein and genistein) and anthocyanidin (Seigler, 1998; Ren *et al*., 2003; Stalikas, 2007).

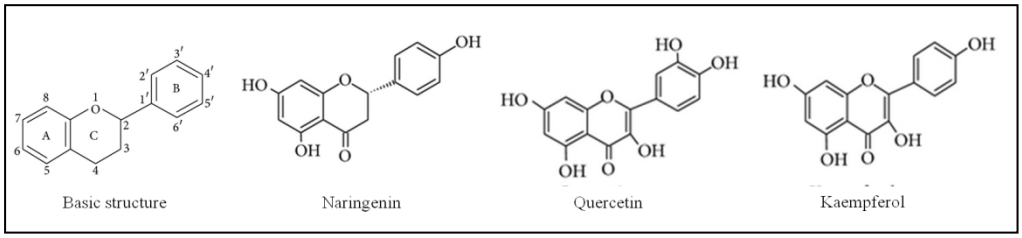


Figure 7.1. Basic chemical structures of naringenin, quercetin and kaempferol (Kumar and Pandey, 2013).

Recently, plant flavonoids have been studied extensively for its biological activities including antioxidative activity, free radical scavenging capacity, anticancer activity and anti-human immunodeficiency virus activity (Yao *et al*., 2004). Flavonoids especially naringenin, quercetin and kaempferol are valuable sources for industrially important pharmaceuticals, as these compounds have been shown to inhibit the growth of cancer cells in breast cancer, colon cancer, ovarian cancer, colorectal cancer, pancreatic cancer, prostate cancer and to induce apoptosis in cells of squamous cell carcinoma and promyelocytic leukemia (Wenzel *et al*., 2004; Bandyopadhyay *et al*., 2008; Zhang *et al*., 2008).

Furthermore, naringenin, quercetin and kaempferol are also an integral part of the human diet (Bravo, 1998; Halliwell and Gutteridge, 1998) and these flavonoids are renowned for its health benefits with

scientific evidence supporting that regular intake of dietary flavonoids reduces the risk of age-associated diseases, such as arthritis, diabetes, hypertension and Alzheimer's disease (Nijveldt *et al*., 2001; Schroeter *et al*., 2006; Tapas *et al*., 2008; Kumar and Pandey, 2013). The absorption of these flavonoids by the human body depends on its chemical structures and physiochemical properties, such as molecular size, configuration, lipophilicity, solubility and acid dissociation constant (pKa) (Havsteen, 2002). According to Pretorius (2003), most of these flavonoids can be present in the form of either glycoside conjugates or aglycones. Flavonoid aglycones such as naringenin, quercetin and kaempferol (Figure 7.1) are found to be easily absorbed by the human body, whereas for flavonoid glycosides, there is a need to convert the glycosides into an aglycan form for better human gut absorption (Hollman *et al.,* 1999). Moreover, Havsteen (2002) reported that flavonoid aglycones were more potent antioxidants than their corresponding glycoside family. This observation was also comparable with the experiment done by Ratty and Das (1988), where the antioxidant properties of flavonol glycosides extracted from tea declined as the number of glycosidic moieties increased. Hence, flavonoid aglycones of naringenin, quercetin and kaempferol were chosen to be extracted from sweet shoot, due to its medicinal benefits as mentioned above.

Besides that, the diagram shown in Figure 7.2 revealed that the production of naringenin, quercetin and kaempferol shared the similar biosynthetic pathways (shikimate pathway, phenylpropanoid pathway and flavonoid biosynthetic pathway) in maize (Ferreyra *et al*., 2012). As previously mentioned in Chapter 6, the flavonoid biosynthetic pathway begins with the deamination of phenylalanine, producing trans-cinnamic acid and subsequently Coumaroyl CoA via PAL enzyme. Coumaroyl CoA converts into naringenin via the activation of CHS and chalcone isomerase (CHI) enzyme activities. Following this, both quercetin and kaempferol are synthesized from dihydroflavonols via flavonol synthase (FLS) enzyme. Since all three flavonoids were hypothesized to share the similar biosynthetic pathway, Pearson's correlation coefficient analysis was carried out in this study, in order to determine the associations between enzymatic activities (PAL and CHS assay) and flavonoid contents in all tested sweet shoot.

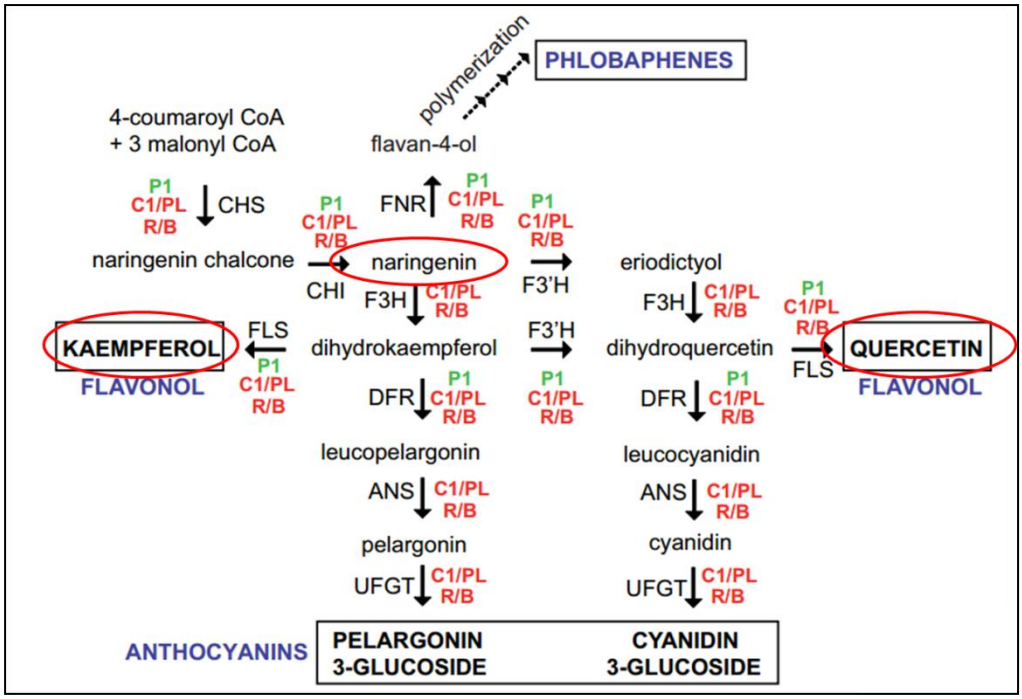


Figure 7.2. Flavonoid biosynthetic pathway of maize (Ferreyra *et al*., 2012).

With the qualitative detection of flavonoid content obtained in sweet shoot (Chapter 6), this study aims to extract specific flavonoids that were deem beneficial and of high economical significance for the pharmaceutical industry. Several reports showed successful isolation of quercetin and kaempferol from the field-grown plants of sweet shoot (Wang and Lee, 1997; Koo and Mohamed, 2001; Andarwulan *et al*., 2012). However, the production of these flavonoid aglycones was often low with less than 1% dry weight of sweet shoot (Selvi and Basker, 2012). Ferreyra *et al*. (2012) reported that the accumulation of these flavonoids in maize was closely associated with the stresses induced by different types of elicitors and precursors. Hence, elicitor and precursor treatments were added to shadehouse-grown plants and cultured tissues, in order to increase the production and to induce *de novo* biosynthesis of these flavonoid aglycones in sweet shoot.

The benzylisoquinoline alkaloid (BIA) papaverine is commonly used as a vasodilator, muscle relaxant and antispasmodic and it is primarily synthesized in opium poppy (*Papaver somniferum*), as mentioned earlier in Chapter 2 (Desgagne-Penix and Facchini, 2012). However, there were several case studies reporting that papaverine, via the over-consumption of sweet shoot leaves, may cause the development of a severe lung

disease called obliterative bronchiolitis (Lin *et al*., 1996; Ger *et al*., 1997; Yu *et al.*, 2007a). Thus, it is essemtial to determine the amount of papaverine present in these elicitor/precursor-treated cultures. If the papaverine production is enhanced by the elicitor and precursor treatments, this may be an unwanted effect that needs to be rectified, even though the levels of flavonoid compounds were elevated.

Therefore, this is the first paper describing a comparative analysis of naringenin, quercetin and kaempferol content between shadehouse-grown plants and cultured tissues of sweet shoot, and also to study the effects of elicitation and precursor feeding on these flavonoid productions. This study also aims to dispel any concerns that papaverine production may be enhanced after elicitation to ensure safe utilization for commercial production of flavonoids from sweet shoot. Once optimization is achieved, the elicitor/precursor treatment protocol can provide an alternative method in enhancing the yield of bioactive flavonoids on a sustainable basis.

7.2 MATERIALS AND METHODS

7.2.1 Plant materials

In this study, six-month-old shadehouse-grown plants and cultured tissues (*in vitro* shoot cultures, light-induced callus cultures, dark-induced callus cultures and somatic embryos) of sweet shoot were used as plant materials to study the effects of precursor (phenylalanine) and elicitors (methyl jasmonate and salicylic acid) on the biosynthesis of naringenin, quercetin, kaempferol and papaverine. The establishment of shadehouse-grown plants and cultured tissues of sweet shoot were discussed in great detail in Chapter 6 (Section 6.2.2.1 and 6.2.2.2).

All these plant materials were individually supplemented with different concentrations of methyl jasmonate (MJ) (0, 50, 100 and 200 µM), salicylic acid (SA) (0, 50, 100 and 200 µM) or phenylalanine (Phe) (0, 5, 10 and 20 mg/l) for a period of three weeks (week 0, 1, 2 and 3). The methods of precursor feeding and elicitor inoculation in sweet shoot were done similarly to the protocols described in Chapter 6 (Section 6.2.3 and 6.2.4).

7.2.2 Extraction of flavonoid aglycones

The extraction procedure used for acid hydrolysis of flavonoids in plant samples of sweet shoot were mentioned earlier in Chapter 6 (Section 6.2.5). Secondary metabolites from sweet shoot samples, like naringenin, quercetin, kaempferol and papaverine were extracted using 25 ml of 62.5% (v/v) acidified methanol (HPLC grade) (Fisher Scientific, USA) containing 1.2 M hydrochloric acid (HCl) (Fisher Scientific, USA) and 2.0 g/l tert-butylhydroquinone (TBHQ) (Sigma-Aldrich, USA). Basic reflux system was adopted by heating the plant samples at 90^{o}C for 2 hours and allowed to cool on ice prior to HPLC analysis. The whole process was repeated thrice to ensure complete extraction of flavonoid aglycones from sweet shoot samples. Each extract was filtered through Whatman filter paper (Whatman, England) and sonicated for 3 minutes to remove oxygen and bubbles from the crude extract, as these substances can produce spikes and baseline noise on the chromatograms (Dong, 2006). The sonicated extract (3 ml) was filtered through a 17 mm, 0.45 µm nylon syringe filter (Titan, USA) into HPLC amber borosilicate glass vials before injecting into the HPLC machine.

7.2.3 Preparation of flavonoid standards

Commercially available standards of naringenin, quercetin, kaempferol and papaverine were purchased from Sigma-Aldrich, USA, as these compounds were frequently found in many woody plant species (Hertog *et al*., 1992; Meyer *et al*., 1998; Matkowski, 2008), and were also used as a comparison for this study. The standard stock solutions were dissolved in 62.5% (v/v) of acidified methanol and sterilized using 0.2 µm syringe filter (Minisart, Sartorius) in laminar flow cabinet. The stock solutions were stored at 4^{o}C for further use.

For the calibration curves, five additional concentrations (50, 100, 150, 200 and 250 ppm) of each individual standard were prepared by diluting the stock solutions with 62.5% (v/v) of acidified methanol. These diluted standards were then syringe filtered through a 17 mm, 0.45 µm nylon membrane filter (Titan, USA) prior to 20 µl injection into the HPLC system.

7.2.4 Reversed-phase high performance liquid chromatography

Secondary metabolites of sweet shoot such as naringenin, papaverine, quercetin and kaempferol were quantified using Perkin Elmer Series 200 HPLC system comprising Series 200 analytical pump, Series 200 autosampler, 600 Series LINK and Series 200 UV/Vis detector (Perkin Elmer Inc, USA) (Figure 7.3). Reversed-phase separations were carried out using a Hypersil Gold Silica C18 endcapped column (250 mm x 4.6 mm I.D., 5 µm particle size) as a stationary phase and a C18 guard column (Thermo Scientific, USA). Mobile phases were filtered twice under vacuum through a 47 mm, 0.45 µm nylon membrane filter (Titan, USA) and degassed via sonication. The solvent system consisted of (A) acetonitrile-0.025M KH_2PO_4 (25:75; v/v) (pH 2.5) (Fisher Scientific, USA), (B) 0.025M KH_2PO_4 (pH 2.5) (Fisher Scientific, USA) and (C) acetonitrile (Fisher Scientific, USA). The acidic solvents at pH 2.5 were adjusted using phosphoric acid (Fisher Scientific, USA). All of the organic solvents used were of HPLC grade. The linear gradient elution was performed as follows: 0-18 min, 100:0:0; 18-30 min, 0:60:40; and 30-40 min, 100:0:0. The C18 column was flushed overnight with a gradient system from 5% acetonitrile / 95% deionised water (18 MΩ Milli-Q type) to 95% acetonitrile / 5% deionised water (18 MΩ Milli-Q type) at 0.1 ml/min after completion of analysis for the day. This step was performed in order to avoid any precipitation of ions and buffers in the column which may cause blockages in the column frits. The C18 column was then washed with 20 column volumes (50 ml) of 40% deionised water (18 mΩ Milli-Q type) and 60% acetonitrile, prior to storage in room temperature with no buffers and ions present.

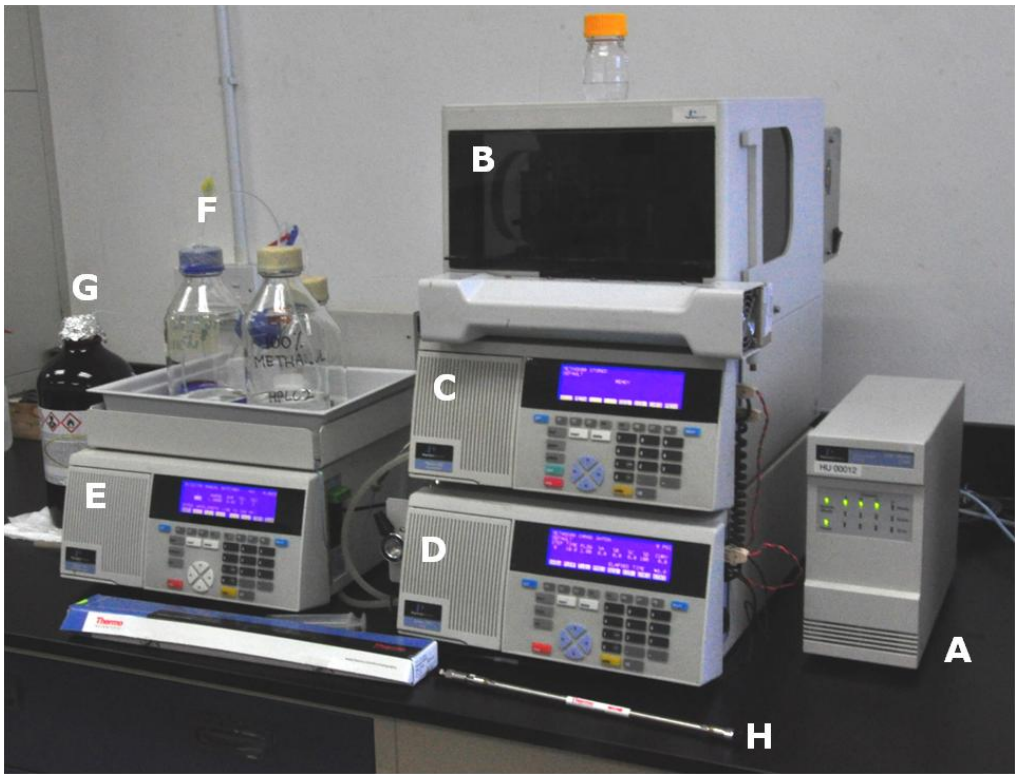


Figure 7.3. Perkin Elmer Series 200 HPLC system was used for flavonoid analysis. (A): Adapter to connect HPLC system to computer. (B): Series 200 autosampler. (C): 600 Series LINK. (D): Series 200 analytical pump. (E): Series 200 UV/Vis detector. (F): Solvent system. (G): Solvent waste bottle. (H): Hypersil Gold Silica C18 endcapped column (250 mm x 4.6 mm I.D., 5 µm particle size).

Standard solutions and crude extracts were syringe filtered through a 17 mm, 0.45 µm nylon membrane filter (Titan, USA) and degassed by sonicator prior to HPLC analysis. An injection volume of 20 µl of the extract was eluted at a flow rate of 1.0 ml/min in room temperature. The column head pressure was maintained between 1900 psi and 2000 psi, while the HPLC traces were plotted at an absorbance of 260 nm. The chromatography data of each flavonoid was assessed using PerkinElmer®'s TotalChrom® Chromatography Data System software. The identification of quercetin, kaempferol, naringenin and papaverine was achieved by comparison of both retention time (t_R) and the absorption spectra obtained for each eluted peak with those obtained for standards. Plant samples were spiked with standards to a final concentration of 100 ppm, in order to determine the recovery rate of standards from the acid extraction. Under optimal conditions, the detection limit values were 0.02 µg/g for papaverine, 0.03 µg/g for quercetin, 0.05 µg/g for kaempferol and 0.08 µg/g for naringenin.

Flavonoid and papaverine contents of elicited and non-elicited tissue cultures of sweet shoot were calculated based on the standard calibration curve and expressed as µg per 10 g fresh weight samples (FW). The standard curve regression equations were y = 67.45x – 21.75 (R^2 =0.9384) for papaverine, y = 124.53x – 7.50 (R^2 =0.9749) for quercetin, y = 1843.28x – 117.52 (R^2 =0.9604) for kaempferol and y = 9378.25x – 688.94 (R^2 =0.9099) for naringenin, where y axis was the detector response and x axis was the concentration of standards.

7.2.5 Statistical analysis

Statistical analysis was carried out on five biological replicates for each treatment with three technical replicates each. Each experiment was repeated twice. Statistical analysis, such as two-way analysis of variance (ANOVA) and Duncan's multiple range tests (DMRT) were performed with 5% level of significant using the SPSS statistical analysis software version 16. Associations between antioxidant activities (DPPH and FRAP assay), enzymatic activities (PAL and CHS assay) and flavonoid contents (naringenin, quercetin and kaempferol contents) in sweet shoot were determined using Pearson's correlation coefficient analysis.

7.3 RESULTS AND DISCUSSION

7.3.1 Quantification of flavonoid aglycones

The HPLC chromatogram of reference standards of naringenin, quercetin, kaempferol and papaverine is presented in Figure 7.4. The largest chromatographic peak in all reference standards at a retention time of 8.37 minutes was papaverine, followed by kaempferol at a retention time of 24.53 minutes. The third peak in the reference standard was quercetin, identified at a retention time of 16.01 minutes. Lastly, the chromatographic peak at a retention time of 6.25 minutes was naringenin.

The overall picture of all studied materials in both treated and untreated sweet shoot cultures showed that naringenin had the highest concentration than kaempferol and quercetin, respectively. This phenomenon is possibly due to the conversion of naringenin into different flavonoid compounds which share the similar biosynthetic pathway, such as isoflavones, flavanone, chalcones and anthocyanidins (shown in Figure 7.2). This observation concurs with the study performed by Skrzypczak-Pietraszek *et al*. (2014) in shoot cultures of *Exacum affine* and Giberti *et*

al. (2012) in cultured tissues of *Oryza sativa*, where the phenolic acid concentrations (chlorogenic acid and caffeic acid) decreased after three days of Phe treatment, due to the conversion of phenolic acids into different phenolic compounds by other enzymes involved in the same phenylpropanoid pathway.

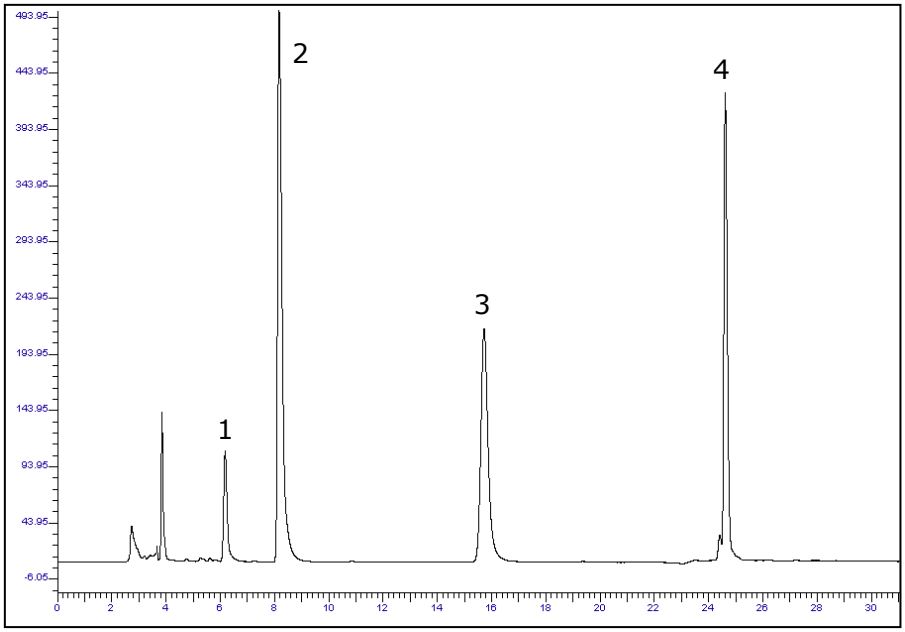


Figure 7.4. HPLC chromatogram of reference standards (100 ppm) in (1) naringenin (6.25 minutes), (2) papaverine (8.37 minutes), (3) quercetin (16.01 minutes) and (4) kaempferol (24.53 minutes).

7.3.1.1 Shadehouse-grown plants of sweet shoot

The results of HPLC analysis showed that the application of MJ, SA and Phe significantly induced the biosynthesis of naringenin, quercetin and kaempferol in shadehouse-grown plants of sweet shoot ($p<0.05$) (Table 7.1). The overall production of naringenin, quercetin and kaempferol responded well to an increasing elicitor and precursor concentration and contact duration in a linear manner ($p<0.05$). Each flavonoid content showed gradual increase in shadehouse-grown plants treated from the lowest to the highest concentration of MJ, SA and Phe. As contact time between elicitor/precursor and cultures increased, the concentration of each flavonoid also increased from week 0 to week 3.

Table 7.1. Comparison of three different flavonoids present in elicited and non-elicited shadehouse-grown plants of sweet shoot, after 3 weeks of treatments.

Types of elicitors/ precursors	Dosages	Types of secondary metabolites (µg/10 g FW)								
		Naringenin content			Quercetin content			Kaempferol content		
		week 1	week 2	week 3	week 1	week 2	week 3	week 1	week 2	week 3
Methyl jasmonate	0µM	178.39 $_{e}$	598.00 $_{de}$	1887.39 $_{cd}$	3.41 $_{e}$	3.85 $_{e}$	4.55 $_{e}$	372.23 $_{e}$	418.69 $_{e}$	472.75 $_{e}$
	50µM	511.86 $_{de}$	1621.68 $_{cd}$	2198.86 $_{bc}$	4.69 $_{e}$	6.89 $_{cde}$	12.34 $_{cd}$	468.65 $_{e}$	582.40 $_{d}$	930.59 $_{c}$
	100µM	1160.4 $_{cde}$	1752.87 $_{cd}$	2508.72 $_{b}$	7.33 $_{cde}$	15.15 $_{c}$	20.97 $_{b}$	645.11 $_{d}$	889.71 $_{c}$	1523.61 $_{b}$
	200µM	1591.31 $_{cd}$	2131.43 $_{bc}$	3254.03 $_{a}$	15.68 $_{c}$	23.73 $_{b}$	37.13 $_{a}$	837.26 $_{c}$	1268.17 $_{bc}$	1928.54 $_{a}$
Salicylic acid	0µM	178.39 $_{e}$	598.00 $_{d}$	1887.39 $_{b}$	3.41 $_{d}$	3.85 $_{d}$	4.55 $_{d}$	372.23 $_{d}$	418.69 $_{d}$	472.75 $_{d}$
	50µM	426.28 $_{d}$	750.95$_{cd}$	1939.56 $_{b}$	4.04 $_{d}$	5.53 $_{cd}$	7.04 $_{cd}$	443.09 $_{d}$	494.03 $_{d}$	562.87 $_{cd}$
	100µM	1023.37 $_{c}$	1606.23 $_{bc}$	2504.29 $_{a}$	5.91 $_{cd}$	12.35 $_{bc}$	16.49 $_{ab}$	568.45 $_{cd}$	827.44 $_{bc}$	1176.62 $_{a}$
	200µM	1196.67 $_{c}$	1766.49 $_{bc}$	2198.86 $_{ab}$	9.54 $_{bcd}$	16.78 $_{a}$	13.57 $_{bc}$	745.33 $_{bc}$	932.05 $_{bc}$	1047.49 $_{ab}$
Phenylalanine	0 mg/l	178.39 $_{g}$	598.00 $_{f}$	1887.39 $_{cde}$	3.41 $_{g}$	3.85 $_{g}$	4.55 $_{g}$	372.23 $_{e}$	418.69 $_{e}$	472.75 $_{e}$
	5 mg/l	669.33 $_{f}$	1996.13 $_{cde}$	2493.79 $_{c}$	6.69 $_{g}$	19.57 $_{f}$	31.54 $_{d}$	523.61 $_{e}$	1094.25 $_{d}$	1550.27 $_{cd}$
	10 mg/l	1729.47 $_{de}$	2262.91 $_{cd}$	3086.83 $_{b}$	16.95 $_{f}$	38.09 $_{c}$	53.11 $_{b}$	866.50 $_{d}$	1694.03 $_{cd}$	2333.65 $_{b}$
	20 mg/l	2116.49 $_{cd}$	2652.47 $_{c}$	3842.05 $_{a}$	25.72 $_{e}$	54.44 $_{b}$	68.90 $_{a}$	1397.92 $_{cd}$	2083.75 $_{bc}$	3195.72 $_{a}$

* Values for naringenin, quercetin and kaempferol content expressed as µg per 10 g fresh weight samples (FW).
* Values expressed as means of five biological replicated experiments and each biological replicate consisted of three technical replicates.
* Different letters within row and column (lowercase) indicate a significant difference ($p<0.05$) according to Duncan's multiple range test.

In this study, untreated shadehouse-grown plants of sweet shoot harvested at week 0 contained the lowest amount of flavonoids, with only 128.01 µg/10g FW in naringenin, 1.56 µg/10g FW in quercetin and 274.85 µg/10g FW in kaempferol (baseline). Shadehouse-grown plants treated with 20 mg/l of Phe significantly exhibited the highest production in naringenin (3842.05 µg/10g FW), quercetin (68.90 µg/10g FW) and kaempferol (3195.72 µg/10g FW), after three weeks of precursor treatment, when compared to those plants treated with MJ and SA (Table 7.1; Figure 7.5). These flavonoid concentrations increased approximately 30.01, 44.17 and 11.63 folds for naringenin, quercetin and kaempferol, respectively, when compared to untreated plants harvested at week 0. Besides that, the precursor dosage, 20 mg/l Phe, also significantly improved the production of naringenin, quercetin and kaempferol in shadehouse-grown plants by 2.04, 15.13 and 6.76 fold respectively, when compared to non-elicited shadehouse-grown plants harvested at week three. These results advocated Phe, at a concentration of 20 mg/l, as the most suitable precursor for the production of these flavonoids in shadehouse-grown plants of sweet shoot. This observation could be due to the role of Phe as an upstream biosynthetic precursor in phenylpropanoid pathway, which was then metabolized by shadehouse-grown sweet shoot into naringenin, quercetin and kaempferol via a series of enzymatic reactions such as PAL, CHS, chalcone isomerase (CHI) and flavonol synthase (FLS1), as previously mentioned in Section 7.1. Similar observations were also observed by Mathur and Goswami (2012) in *Maytenus emarginata*, whereby the addition of Phe (75 mg Phe/100 ml) significantly increased the concentration of kaempferol and quercetin by 1.28 and 1.33 fold, respectively. The beneficial effect of Phe was also reported by Fisher (1968) in *Fagopyrum tartaricum* and Moreno *et al*. (2010) in *Rubus idaeus* for yield enhancement of quercetin, naringenin and kaempferol.

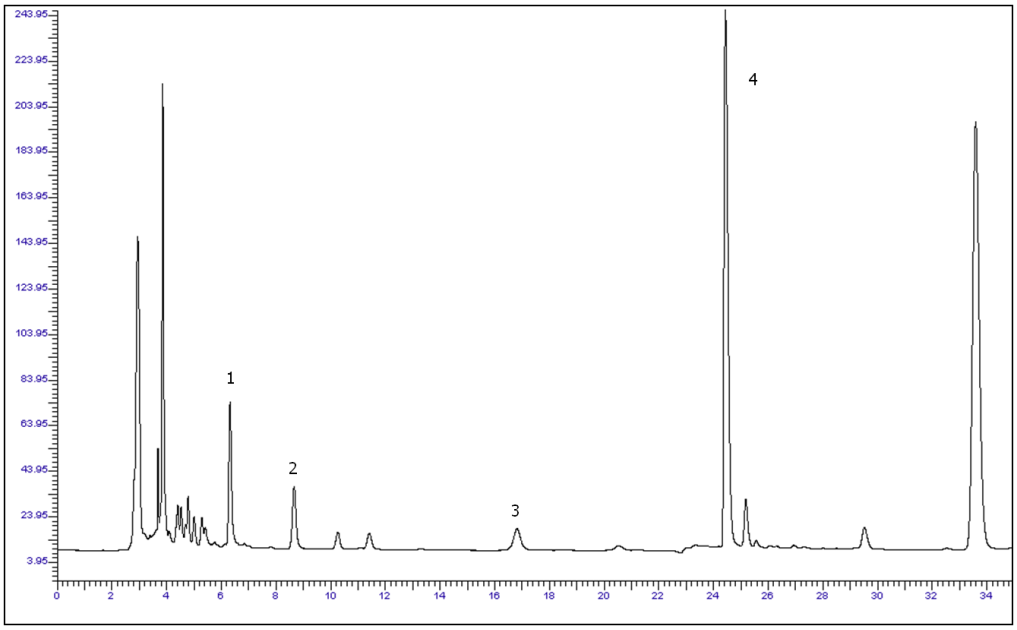


Figure 7.5. Chromatogram of shadehouse-grown plants of sweet shoot treated with 20 mg/l of Phe, after three weeks of precursor feeding. (1): Naringenin. (2): Papaverine. (3): Quercetin. (4): Kaempferol.

Among the two elicitors tested (MJ and SA), the production of naringenin, quercetin and kaempferol for those plants elicited with MJ were higher than those elicited with SA at all levels of concentration and contact duration. The naringenin, quercetin and kaempferol content of MJ-treated plants were between 61.01% and 78.64% more than SA-treated plants. The superior effects of MJ elicitation for yield enhancement of these flavonoids may be due to the activation of a higher number (18) of defense-related genes in shadehouse-grown plants of sweet shoot (Hendrawati *et al*., 2006) when compared to SA. Similar phenomenon was observed in *Arabidopsis* plants treated with MJ, which was previously discussed in Chapter 6 (Section 6.3.2.3). These findings were also seen in raspberry fruit treated with 0.1 mM MJ (Wang and Zheng, 2005; Moreno *et al*., 2010), in which the flavonol (quercetin, kaempferol and myricetin) contents were increased from 9.6 µg/g FW to 12.2 µg/g FW. These results were in agreement with the report done by Schreiner and Huyskens-Keil (2006), whereby the stimulatory effects of MJ were noted in flavonoid formation of banana, papaya and red raspberry. These results proved once again that the optimal conditions of elicitor and precursor treatment helped to enhance the yield of naringenin, quercetin and kaempferol in shadehouse-grown plants of sweet shoot.

With reference to Table 7.2, Pearson correlation coefficient analysis was carried out to analyze the correlative relationships amongst the naringenin content, quercetin content, kaempferol content, antioxidant activities (DPPH and FRAP assay) and enzymatic activities (PAL and CHS assay) of sweet shoot. The antioxidant activities based on DPPH and FRAP assays showed direct correlation to naringenin (R_{DPPH}=0.9298 and R_{FRAP}=0.7562, $p<0.05$), quercetin (R_{DPPH}=0.8834 and R_{FRAP}=0.8453, $p<0.05$) and kaempferol (R_{DPPH}=0.8822 and R_{FRAP}=0.8290, $p<0.05$) content. These results indicated that naringenin, quercetin and kaempferol may contribute to the free radical scavenging activity and ferric reducing activity in shadehouse-grown plants of sweet shoot treated with Phe, as supported by previous reports on *Zingiber officinale* (Ghasemzadeh *et al*., 2010), *Labisa pumila* (Karimi *et al*., 2011) and *Citrus aurantium* (Karimi *et al*., 2012).

Table 7.2. Correlation between measured parameters in shadehouse-grown plants of sweet shoot.

	Naringenin	**Quercetin**	**Kaempferol**
DPPH radical scavenging activity	0.9298	0.8834	0.8822
FRAP reducing activity	0.7562	0.8453	0.8290
PAL enzymatic activity	0.9552	0.9369	0.9404
CHS enzymatic activity	0.9472	0.8810	0.8972
Kaempferol	0.9208	0.9893	
Quercetin	0.9032		

Furthermore, enzymatic activities such as PAL and CHS enzymes also showed strong correlationship with naringenin (R_{PAL}=0.9552 and R_{CHS}=0.9472, $p<0.05$), quercetin (R_{PAL}=0.9369 and R_{CHS}=0.8810, $p<0.05$) and kaempferol (R_{PAL}=0.9404 and R_{CHS}=0.8972, $p<0.05$) content of sweet shoot. These findings revealed that PAL and CHS activities increased with a concomitant increase in flavonoid production (naringenin, quercetin and kaempferol) of shadehouse-grown plants treated with Phe. These results also suggested that the addition of Phe activated the PAL and CHS enzymes in phenylpropanoid pathway and flavonoid biosynthetic pathways for the production of naringenin, quercetin and kaempferol in sweet shoot, which was in agreement with the previous results discussed in Chapter 6 (Section 6.3.3). The activation of PAL and CHS enzymes resulted in enhancement of flavonoid compounds such as anthocyanin, quercetin, kaempferol, naringenin, rutin, stilbenes and myricetin, following the

exogenous application of elicitor and precursor, and this has been confirmed in many studies on *Arabidopsis* (Olsen *et al*., 2008), strawberry (Heredia and Cisneros-Zevallos, 2009) and *Ginkgo biloba* (Cheng *et al*., 2012).

Lastly, the overall correlation between naringenin, quercetin and kaempferol in sweet shoot were positive in a linear manner (Table 7.2). The strongest correlation was seen between quercetin and kaempferol ($R=0.9893$, $p<0.05$), followed by (1) naringenin and kaempferol ($R=0.9208$, $p<0.05$) and (2) naringenin and quercetin ($R=0.9032$, $p<0.05$) in sweet shoot. This study proved that the production of these flavonoids may have undergone the same flavonoid biosynthetic pathways via a series of enhanced enzymatic activities (PAL and CHS enzyme). Similar observations were also seen in Chapter 6 for the elevation of total phenolic and total flavonoid contents, antioxidant activities (DPPH and FRAP assay) and enzymatic activities (PAL and CHS assay) via elicitor and precursor treatments in shadehouse-grown plants of sweet shoot.

7.3.1.2 *In vitro* shoot cultures of sweet shoot

The results of quantitative analysis of flavonoids from *in vitro* shoot cultures of sweet shoot are shown in Table 7.3. The lowest concentration of naringenin (82.41 µg/10g FW), quercetin (1.23 µg/10g FW) and kaempferol (339.94 µg/10g FW) were isolated from the untreated shoot cultures harvested at week 0 (baseline). The exogenous application of Phe was the most effective in yield enhancement of naringenin, quercetin and kaempferol of sweet shoot, when compared to MJ and SA at all levels of concentration and contact duration ($p<0.05$). Hormone-free MS medium containing 10 mg/l of Phe had the most beneficial influence on naringenin (5284.71 µg/10g FW), quercetin (93.65 µg/10g FW) and kaempferol (4190.75 µg/10g FW) production in shoot cultures after three weeks of treatment (Figure 7.6; Table 7.3). This corresponds to a 64.12, 75.94 and 12.33 fold rise in naringenin, quercetin and kaempferol production, respectively, when compared to the baseline results. When comparing these maximal results to those from untreated shoot cultures at week three, a 2.87, 27.38 and 8.48 fold higher naringenin, quercetin and kaempferol production, respectively, was noted in sweet shoot. These results were comparable with a previous report for shoot cultures of *Exacum affine* (Skrzypczak-Pietraszek *et al*., 2014), in which the addition

of Phe (1.6 g/l) significantly enhanced the production of phenolic acids by 1.6 folds. Similar results were also reported by Rahimi *et al*. (2011) in *Silybum marianum*, whereby a significantly higher amount of naringenin (4.64 fold), silymarin (1.84 fold), taxifoline (4.16 fold), silydianin (2.44 fold) and silybin (1.58 fold) were induced by 100 µM of Phe after 72 hours of treatment, when compared to untreated shoot culture.

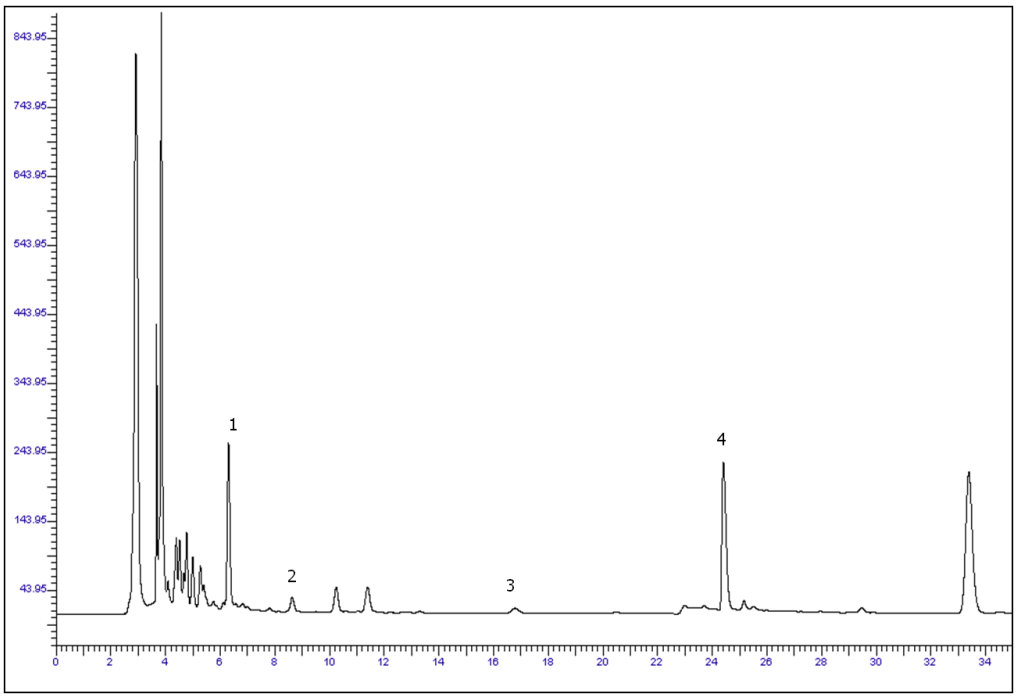


Figure 7.6. Chromatogram of *in vitro* shoot cultures of sweet shoot fed with 10 mg/l of Phe, after three weeks of precursor feeding. (1): Naringenin. (2): Papaverine. (3): Quercetin. (4): Kaempferol.

Table 7.3. Comparison of three different flavonoids present in elicited and non-elicited *in vitro* shoot cultures of sweet shoot, after 21 days of treatments.

Types of elicitors/ precursors	Dosages	Types of flavonoids (µg/10 g FW)								
		Naringenin content			Quercetin content			Kaempferol content		
		week 1	week 2	week 3	week 1	week 2	week 3	week 1	week 2	week 3
Methyl jasmonate	0µM	416.91 $_{f}$	750.95 $_{e}$	1842.05 $_{d}$	0.97 $_{g}$	2.08 $_{g}$	3.42 $_{f}$	387.81 $_{f}$	405.05 $_{f}$	494.03 $_{e}$
	50µM	539.55 $_{f}$	2161.68 $_{c}$	4145.93 $_{a}$	5.84 $_{ef}$	8.63 $_{e}$	28.17 $_{cd}$	503.93 $_{e}$	782.40 $_{de}$	1020.86 $_{c}$
	100µM	2212.69 $_{c}$	2732.02 $_{b}$	2968.23 $_{b}$	12.57 $_{de}$	23.71 $_{d}$	53.56 $_{a}$	914.16 $_{cd}$	1085.94 $_{c}$	2429.54 $_{a}$
	200µM	2641.49 $_{b}$	2650.10 $_{b}$	2825.07 $_{b}$	24.29 $_{d}$	31.74 $_{c}$	42.78 $_{b}$	1027.58 $_{c}$	1457.26 $_{b}$	1632.17 $_{b}$
Salicylic acid	0µM	416.91 $_{f}$	750.95 $_{e}$	1842.05 $_{d}$	0.97 $_{g}$	2.08 $_{g}$	3.42 $_{f}$	387.81 $_{e}$	405.05 $_{e}$	494.03 $_{de}$
	50µM	340.33 $_{f}$	1986.95 $_{d}$	2135.55 $_{cd}$	3.55 $_{f}$	5.51 $_{ef}$	8.28 $_{e}$	465.39 $_{de}$	565.33 $_{cd}$	817.04 $_{c}$
	100µM	2090.07 $_{cd}$	2715.31 $_{bc}$	3388.07 $_{a}$	7.25 $_{e}$	21.75 $_{c}$	38.57 $_{a}$	799.71 $_{c}$	1701.79 $_{a}$	1190.27 $_{bc}$
	200µM	2283.47 $_{cd}$	2968.23 $_{b}$	2501.23 $_{bc}$	16.61 $_{d}$	26.31 $_{c}$	30.55 $_{b}$	928.76 $_{c}$	1080.33 $_{bc}$	1305.57 $_{b}$
Phenylalanine	0 mg/l	416.91 $_{j}$	750.95 $_{i}$	1842.05 $_{h}$	0.97 $_{g}$	2.08 $_{g}$	3.42 $_{f}$	387.81 $_{g}$	405.05 $_{g}$	494.03 $_{f}$
	5 mg/l	2370.33 $_{g}$	2811.69 $_{f}$	3682.79 $_{cd}$	13.43 $_{e}$	29.31 $_{d}$	56.81 $_{c}$	525.96 $_{f}$	1227.29 $_{e}$	1866.99 $_{cd}$
	10 mg/l	3301.12 $_{e}$	3671.15 $_{cd}$	5284.71 $_{a}$	23.15 $_{d}$	49.59 $_{cd}$	93.65 $_{a}$	1098.69 $_{e}$	2247.63 $_{c}$	4190.75 $_{a}$
	20 mg/l	3552.79 $_{de}$	4122.65 $_{c}$	4692.96 $_{b}$	46.24 $_{cd}$	70.24 $_{bc}$	83.56 $_{b}$	1707.54 $_{d}$	2771.94 $_{b}$	3099.75 $_{b}$

* Values for naringenin, quercetin and kaempferol content expressed as µg per 10 g fresh weight samples (FW).
* Values expressed as means of five biological replicated experiments and each biological replicate consisted of three technical replicates.
* Different letters within row and column (lowercase) indicate a significant difference ($p<0.05$) according to Duncan's multiple range test.

Even though the production of naringenin, quercetin and kaempferol in shoot cultures of sweet shoot was found to be dependent on the concentration and contact duration to Phe, further increment in Phe concentration to 20 mg/l led to lower production of each flavonoid (4692.96 µg/10g FW for naringenin, 83.56 µg/10g FW for quercetin and 3099.75 µg/10g FW for kaempferol) in shoot cultures harvested at week three. A statistically significant difference between these two precursor concentrations (10 and 20 mg/l Phe) was noted for each flavonoid ($p<0.05$). These results were in line with the research done by Zia *et al.* (2007) in cultured tissues of *Artemisia absinthium*, whereby the incorporation of 12.5 mg/l Phe increased the production of artemisinin and phenylethanoid glycosides, but decreased as the concentration of Phe increased to 33 mg/l. The possible reason for this adverse outcome could be due to the inconsistency of precursor activity, since many precursors had the tendency to either amplify or interrupt the signaling pathway. There was a possibility of feedback inhibition in the metabolic pathway as mentioned earlier in Chapter 6 (Section 6.3.1.2 and 6.3.2.2). Hence, it is important to optimize the elicitation conditions to maximize the production of each flavonoid compound, since unsuitable precursor concentrations (20 mg/l of Phe) may cause unsuccessful elicitation in shoot cultures of sweet shoot, as evidenced in this study.

In MJ and SA induced shoot cultures, a significant week-to-week rise in flavonoid compound were isolated ($p<0.05$). As the incubation period increased from week 0 to week 3, these MJ and SA induced sweet shoot may undergo biochemical and morphological differentiation process, in order to generate higher numbers of primary and secondary metabolites for plant development and plant defense mechanisms, as previously cited in Chapter 6 (Section 6.3.1.3 and 6.3.1.5). This concept provides a better insight in obtaining higher concentrations of naringenin, quercetin and kaempferol from shoot cultures harvested at week 3.

In order to better elucidate the bioactive flavonoid compounds in Phe-treated culture, Pearson coefficient correlation analysis was conducted between naringenin content, quercetin content, kaempferol content, antioxidant activities (DPPH and FRAP assay) and enzymatic activities (PAL and CHS assay). Based on the results shown in Table 7.4, similarities was observed between shadehouse-grown plants and shoot cultures of sweet

shoot, in which positive relationship was observed in all the parameters mentioned above. There were significant positive correlations between antioxidant activities, naringenin (R_{DPPH}=0.9317 and R_{FRAP}=0.7490, $p<0.05$), quercetin (R_{DPPH}=0.9534 and R_{FRAP}=0.8676, $p<0.05$) and kaempferol (R_{DPPH}=0.9410 and R_{FRAP}=0.8404, $p<0.05$) compounds extracted from shoot cultures fed with Phe. Furthermore, correlations of enzymatic activities of sweet shoot were also significant for naringenin (R_{PAL}=0.9726 and R_{CHS}=0.8659, $p<0.05$), quercetin (R_{PAL}=0.9626 and R_{CHS}=0.9271, $p<0.05$) and kaempferol (R_{PAL}=0.9565 and R_{CHS}=0.9307, $p<0.05$) compounds present in Phe-treated shoot cultures. Excellent correlations also existed between naringenin ($R_{quercetin}$=0.9354 and $R_{kaempferol}$=0.9089, $p<0.05$), quercetin ($R_{kaempferol}$=0.9852, $p<0.05$) and kaempferol in Phe-treated cultures. These results suggested that the addition of Phe may lead to the yield enhancement of naringenin, quercetin and kaempferol, which were responsible for the antioxidant activities in shoot cultures of sweet shoot. Additionally, high PAL and CHS enzymatic activities were also found to parallel with high levels of naringenin, quercetin and kaempferol. This study indicated that the incorporation of Phe may be involved in regulating the metabolic pathway for the production of these flavonoids via enhanced PAL and CHS enzymatic activities, as previously reported in *Arabidopsis* plants by Lillo *et al*. (2008) and Treutter (2010).

Table 7.4. Correlation between measured parameters in *in vitro* shoot cultures of sweet shoot.

	Naringenin	**Quercetin**	**Kaempferol**
DPPH radical scavenging activity	0.9317	0.9534	0.9410
FRAP reducing activity	0.7490	0.8676	0.8404
PAL enzymatic activity	0.9726	0.9626	0.9565
CHS enzymatic activity	0.8659	0.9271	0.9307
Kaempferol	0.9089	0.9852	
Quercetin	0.9354		

7.3.1.3 Light-induced callus cultures of sweet shoot

In this study, untreated light-induced callus cultures harvested at week 0 (baseline) produced an average naringenin content of 138.14 µg/10g FW, an average quercetin content of 1.54 µg/10g FW and an average kaempferol content of 338.79 µg/10g FW. These results were significantly lower than those cultures treated with elicitors and precursor

($p<0.05$) (Table 7.5). The present study showed that untreated light-induced callus culture of sweet shoot has the ability to produce bioactive naringenin, quercetin and kaempferol compounds *in vitro*, as evidenced in light-induced callus cultures of *Haloxylon salicornicum* (Kaur and Bains, 2012), *Terminalia arjuna* (Sharma and Agrawal, 2012) and *Boerhaavia diffusa* (Christian, 2013). As mentioned in Chapter 6, the differentiation process of plant cell definitely played an important role in inducing these bioactive flavonoids from light-induced callus cultures of sweet shoot.

Table 7.5. Comparison of three different flavonoids present in elicited and non-elicited light-induced callus cultures of sweet shoot, after a treatment period of 3 weeks.

Types of elicitors/ precursors	Dosages	Types of flavonoids (µg/10 g FW)								
		Naringenin content			Quercetin content			Kaempferol content		
		week 1	week 2	week 3	week 1	week 2	week 3	week 1	week 2	week 3
Methyl jasmonate	0µM	269.52 $_{h}$	934.71 $_{g}$	2537.75 $_{f}$	1.65 $_{g}$	2.83 $_{g}$	4.04 $_{g}$	441.55 $_{f}$	657.35 $_{e}$	909.69 $_{d}$
	50µM	4310.32 $_{e}$	4832.39 $_{de}$	5099.52 $_{d}$	6.93 $_{f}$	25.21 $_{e}$	39.13 $_{cd}$	903.13 $_{d}$	1082.68 $_{cd}$	1344.17 $_{bc}$
	100µM	4880.29 $_{de}$	5739.53 $_{c}$	6651.12 $_{b}$	23.77 $_{e}$	37.59 $_{cd}$	69.67 $_{b}$	1110.09 $_{cd}$	1203.79 $_{c}$	1483.95 $_{bc}$
	200µM	5531.51 $_{cd}$	5579.99 $_{cd}$	8314.51 $_{a}$	33.57 $_{d}$	60.48 $_{c}$	124.22 $_{a}$	1366.49 $_{bc}$	1750.97 $_{b}$	2927.19 $_{a}$
Salicylic acid	0µM	269.52 $_{h}$	934.71 $_{g}$	2537.75 $_{f}$	1.65 $_{f}$	2.83 $_{f}$	4.04 $_{f}$	441.55 $_{f}$	657.35 $_{e}$	909.69 $_{d}$
	50µM	4020.91 $_{e}$	4722.27 $_{cd}$	4830.49 $_{c}$	5.02 $_{f}$	7.29 $_{ef}$	20.17 $_{d}$	899.06 $_{d}$	1002.46 $_{cd}$	1176.49 $_{c}$
	100µM	4369.16 $_{d}$	5035.78 $_{c}$	6965.52 $_{a}$	9.71 $_{e}$	28.25 $_{cd}$	83.75 $_{a}$	1009.33 $_{cd}$	1084.31 $_{c}$	2150.61 $_{a}$
	200µM	5154.14 $_{c}$	5673.73 $_{b}$	5269.57 $_{bc}$	25.63 $_{cd}$	33.41 $_{c}$	44.65 $_{b}$	1148.79 $_{c}$	1266.80 $_{bc}$	1569.80 $_{b}$
Phenylalanine	0 mg/l	269.52 $_{i}$	934.71 $_{h}$	2537.75 $_{g}$	1.65 $_{g}$	2.83 $_{g}$	4.04 $_{g}$	441.55 $_{j}$	657.35 $_{i}$	909.69 $_{h}$
	5 mg/l	4507.59 $_{f}$	5604.78 $_{e}$	6271.93 $_{d}$	20.03 $_{f}$	42.45 $_{d}$	69.57 $_{bc}$	1529.79 $_{g}$	2841.43 $_{f}$	6057.99 $_{c}$
	10 mg/l	5000.06 $_{ef}$	5815.05 $_{de}$	10658.61 $_{b}$	32.19 $_{e}$	57.13 $_{c}$	99.95 $_{b}$	5500.87 $_{d}$	3713.05 $_{e}$	8095.28 $_{b}$
	20 mg/l	5508.69 $_{e}$	8474.68 $_{c}$	12081.05 $_{a}$	52.74 $_{cd}$	121.04 $_{ab}$	134.36 $_{a}$	2925.83 $_{f}$	5370.51 $_{d}$	11325.13 $_{a}$

* Values for naringenin, quercetin and kaempferol content expressed as µg per 10 g fresh weight samples (FW).
* Values expressed as means of five biological replicated experiments and each biological replicate consisted of three technical replicates.
* Different letters within row and column (lowercase) indicate a significant difference ($p<0.05$) according to Duncan's multiple range test.

The analysis of variance showed significant effects of Phe, MJ and SA treatments on the biosynthesis of naringenin, quercetin and kaempferol compounds in light-induced callus of sweet shoot ($p<0.05$). Flavonoid contents such as naringenin, quercetin and kaempferol were augmented to 87.46, 87.25 and 33.43 fold respectively, when compared to baseline, after the supplementation of 20 mg/l Phe at third week of cultures (Table 7.5; Figure 7.7). These were the highest flavonoid concentrations achieved in all light-induced callus cultures in this present study. Furthermore, it was also higher by 4.76, 33.26 and 12.45 folds in naringenin, quercetin and kaempferol contents respectively, when compared to non-elicited callus cultures harvested at week 3. In other words, Phe at a concentration of 20 mg/l remained the most desirable precursor in enhancing the production of naringenin, quercetin and kaempferol compounds for light-induced callus cultures of sweet shoot. This result concurred with the experiment done in light-induced callus cultures of *Inpluchea lanceolata* (Patni, 2013), whereby the incorporation of Phe (50 mg/l) increased the yield of quercetin by about 1 to 2 folds in comparison to control. Similarly, Phe at the concentration of 75 mg/l, successfully triggered the production of quercetin (1.15 fold) and kaempferol (1.16 fold) in light-induced callus cultures of *Haloxylon recurvum* (Kaur and Bains, 2012).

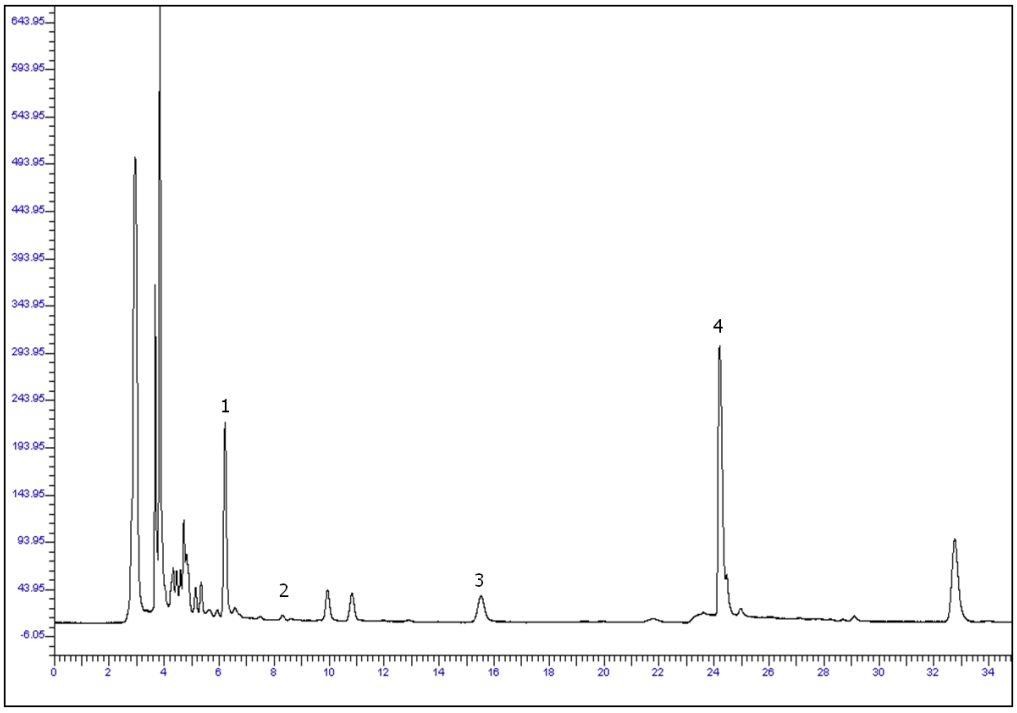


Figure 7.7. Chromatogram of light-induced callus cultures of sweet shoot treated with 20 mg/l of Phe, after three weeks of precursor treatment. (1): Naringenin. (2): Papaverine. (3): Quercetin. (4): Kaempferol.

Elicitors such as MJ and SA were initially used to increase plant resistance to pathogens via the activation of PAL enzyme, which eventually led to the production of phytoalexins such as flavonoids and phenolic acids (Gozzo, 2003; Beckers and Spoel, 2006; Ruiz-Garcia and Gomez-Plaza, 2013). This concept explains the increase in flavonoid production seen in this study when either MJ or SA was added to the light-induced callus cultures. MJ at a dosage of 200 µM significantly stimulated naringenin (8314.51 µg/10g FW), quercetin (124.22 µg/10g FW) and kaempferol (2927.19 µg/10g FW) biosynthesis in light-induced callus cultures of sweet shoot harvested on week 3. These results were significantly higher by 60.19, 80.66 and 8.64 fold in naringenin, quercetin and kaempferol contents respectively, when compared to baseline. A similar observation was obtained in callus cultures of *Emex spinosa*, in which quercetin and kaempferol were increased by 17 fold and 11 fold respectively, after the addition of 100 ml of MJ (El-Mawla and Ibraheim, 2011). Likewise, the addition of SA to light-induced callus cultures also showed significant increment in naringenin (6965.52 µg/10g FW), quercetin (83.75 µg/10g FW) and kaempferol (2150.61 µg/10g FW) concentrations compared to baseline. However, the maximum concentration of these flavonoids varied at different SA concentrations. At

SA concentration of 200 µM, naringenin was produced in the highest concentration, whereas for both quercetin and kaempferol, the highest production was at SA concentration of 100 µM. This observation may be due to the conversion of naringenin into other bioactive flavonoids (anthocyanin, aurones and isoflavone), as previously explained in Figure 7.2 and Section 7.3.1.1. This finding was also supported by Sudha and Ravishankar (2003), where the accumulation of anthocyanin in *Daucus carota* callus cultures treated with SA reduced (from 0.40 to 0.30 mg/100 mg DW) with increasing doses of SA (from 0.01 µM to 200 µM).

Pearson correlation coefficient analysis, as exhibited in Table 7.6, showed positive correlation between all the parameters in light-induced callus cultures of sweet shoot beyond the significant level of 0.05. This table illustrated that the DPPH and FRAP antioxidant activities of light-induced callus cultures treated with Phe showed strong correlationship with the three flavonoids, ranging from R=0.8686 to R=0.9683. With these positive relationships, the PAL and CHS enzymatic activities were also directly correlated with these three flavonoids (R value between 0.8966 and 0.9832). Interpretation of the relationships between these three flavonoids showed similar positive representation (R=0.9067 to 0.9463). These results strengthen the positive observations shown in shadehouse-grown plants (Section 7.3.1) and *in vitro* shoot cultures (Section 7.3.2) of sweet shoot. The exogenous application of Phe enhanced the PAL and CHS enzymatic activities, thus increasing the yield of naringenin, quercetin and kaempferol that contribute to the increased antioxidant activities seen in light-induced callus cultures of sweet shoot. These observations were in agreement with earlier studies performed in light-induced callus cultures of *Daucus carota* (Heinzmann and Seitz, 1977), *Tribulus alatus* (Jit *et al*., 1985) and *Cassia angustifolia* (Reddy *et al*., 2007), where the production of these flavonoids (quercetin, kaempferol and naringenin) were in association with PAL and CHS enzymatic activities.

Table 7.6. Correlation between measured parameters in light-induced callus cultures of sweet shoot.

	Naringenin	Quercetin	Kaempferol
DPPH radical scavenging activity	0.8962	0.8716	0.8171
FRAP reducing activity	0.8686	0.9683	0.8567
PAL enzymatic activity	0.9832	0.9329	0.9037
CHS enzymatic activity	0.9184	0.8966	0.9494
Kaempferol	0.9263	0.9067	
Quercetin	0.9463		

7.3.1.4 Dark-induced callus cultures of sweet shoot

The effects of MJ, SA and Phe on the production of naringenin, quercetin and kaempferol in dark-induced callus cultures varied according to the elicitor/precursor concentration and contact duration tested in the present study. At week 0, untreated dark-induced callus cultures produced an average naringenin content of 136.11 µg/10g FW, an average quercetin content of 0.68 µg/10g FW and an average kaempferol content of 332.13 µg/10g FW (baseline). The best results were obtained from Phe at a concentration of 10 mg/l on the third week of dark-induced callus cultures (2330.26 µg/10g FW for naringenin, 19.07 µg/10g FW for quercetin and 1092.34 µg/10g FW for kaempferol) (Table 7.7; Figure 7.8). These were 17.12, 28.04 and 3.29 fold higher than the baseline. Data from week 3 of Phe-induced callus cultures at 10 mg/l were also 6.17 (naringenin), 7.86 (quercetin) and 3.01 (kaempferol) folds higher, when compared to non-elicited cultures harvested at third week. The flavonoid levels generally showed marked increase in concentration as the levels of Phe increased from 0 to 10 mg/l in dark-induced callus cultures of sweet shoot. However, at higher levels of Phe (20 mg/l), there was a gradual decline in these flavonoid levels. The differences were also observed in Section 7.3.1.2, whereby the 'overloading' of Phe caused adverse effects in the production of these flavonoids. The similar possible explanation for this observation may be due to the inconsistency of the precursor leading to feedback inhibition in the metabolic pathway as mentioned in the similar chapter.

Table 7.7. Comparison of three different flavonoids present in elicited and non-elicited dark-induced callus cultures of sweet shoot, after a treatment period of 21 days.

Types of elicitors/ precursors	Dosages	Types of flavonoids (µg/10 g FW)								
		Naringenin content			Quercetin content			Kaempferol content		
		week 1	week 2	week 3	week 1	week 2	week 3	week 1	week 2	week 3
Methyl jasmonate	0µM	274.96 $_{d}$	453.03 $_{d}$	839.71 $_{c}$	0.73 $_{d}$	1.41 $_{d}$	2.43 $_{d}$	341.73 $_{e}$	349.61 $_{e}$	362.97 $_{e}$
	50µM	372.31 $_{d}$	794.21 $_{c}$	1235.56 $_{b}$	2.13 $_{d}$	3.36 $_{c}$	5.59 $_{bc}$	386.67 $_{de}$	426.45 $_{d}$	550.63 $_{bc}$
	100µM	684.80 $_{c}$	1245.06 $_{b}$	1690.50 $_{a}$	3.83 $_{c}$	5.91 $_{bc}$	12.68 $_{a}$	464.21 $_{cd}$	483.75 $_{cd}$	706.37 $_{a}$
	200µM	1218.28 $_{b}$	1560.46 $_{ab}$	1295.48 $_{b}$	6.90 $_{b}$	8.66 $_{b}$	10.70 $_{ab}$	542.38 $_{bc}$	607.15 $_{ab}$	447.17 $_{a}$
Salicylic acid	0µM	274.96 $_{e}$	453.03 $_{e}$	839.71 $_{bc}$	0.73 $_{d}$	1.41 $_{d}$	2.43 $_{d}$	341.73 $_{d}$	349.61 $_{d}$	362.97 $_{d}$
	50µM	409.08 $_{e}$	650.10 $_{bcd}$	951.47 $_{ab}$	1.70 $_{d}$	2.17 $_{d}$	3.53 $_{cd}$	368.40 $_{d}$	401.77 $_{cd}$	437.59 $_{c}$
	100µM	536.83 $_{cd}$	864.67 $_{bc}$	1130.21 $_{ab}$	2.23 $_{d}$	4.54 $_{c}$	5.65 $_{b}$	407.01 $_{cd}$	451.82 $_{c}$	488.81 $_{ab}$
	200µM	839.07 $_{bc}$	1045.85 $_{ab}$	1223.98 $_{a}$	4.31 $_{c}$	5.95 $_{b}$	8.16 $_{a}$	477.43 $_{bc}$	496.99 $_{ab}$	532.92 $_{a}$
Phenylalanine	0 mg/l	274.96 $_{e}$	453.03 $_{e}$	839.71 $_{d}$	0.73 $_{e}$	1.41 $_{e}$	2.43 $_{e}$	341.73 $_{e}$	349.61 $_{e}$	362.97 $_{e}$
	5 mg/l	418.83 $_{e}$	953.45 $_{cd}$	1561.54 $_{b}$	3.66 $_{d}$	6.25 $_{c}$	8.30 $_{bc}$	410.67 $_{e}$	610.75 $_{d}$	947.29 $_{ab}$
	10 mg/l	965.39 $_{cd}$	1362.38 $_{bc}$	2330.26 $_{a}$	5.35 $_{c}$	11.24 $_{b}$	19.07 $_{a}$	542.55 $_{de}$	753.63 $_{cd}$	1092.34 $_{a}$
	20 mg/l	1293.89 $_{bc}$	1766.40 $_{b}$	1621.68 $_{b}$	9.02 $_{bc}$	13.73 $_{ab}$	15.55 $_{ab}$	768.39 $_{cd}$	874.47 $_{bc}$	944.48 $_{ab}$

* Values for naringenin, quercetin and kaempferol content expressed as µg per 10 g fresh weight samples (FW).
* Values expressed as means of five biological replicated experiments and each biological replicate consisted of three technical replicates.
* Different letters within row and column (lowercase) indicate a significant difference ($p<0.05$) according to Duncan's multiple range test.

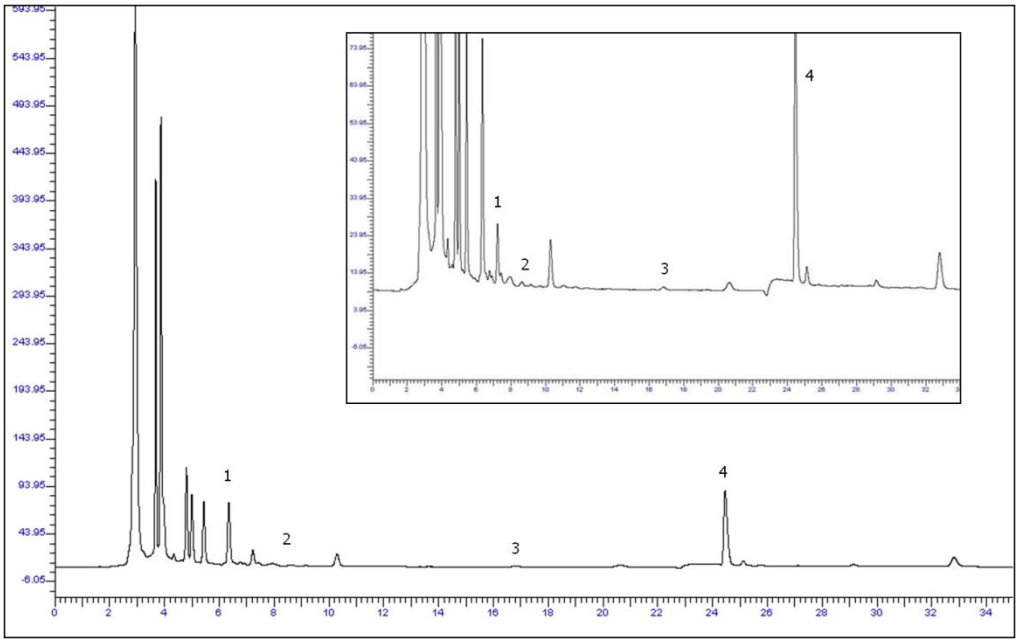


Figure 7.8. Chromatogram of dark-induced callus cultures of sweet shoot treated with 10 mg/l of Phe, after three weeks of precursor treatment. (1): Naringenin. (2): Papaverine. (3): Quercetin. (4): Kaempferol.

In this present study, dark-induced callus cultures showed an overall lower production of flavonoids than light-induced callus cultures of sweet shoot. This observation has been similarly reported in many previous studies in woody plant species (Zhong *et al*., 1993; Merzlyak *et al*., 2005; Ramakrishna and Ravishankar, 2011). Most of these studies hypothesized that the process of photosynthesis is the main reason for the stimulation of secondary metabolite production (Ghasemzadeh and Ghasemzadeh, 2011). According to Chan *et al*. (2010), higher accumulation of anthocyanins (1.13 fold) was observed in irradiated cell suspension cultures of *Melastoma malabathricum* when compared to those cultured primarily in the dark. Besides that, Kim *et al.* (2006) also observed a higher amount of isoflavones (0.56 mg/g FW) being produced in light exposed soybean sprout than those grown under dark conditions (1.38 mg/g fresh weight). Other studies supporting these findings (between light and dark conditions) were in callus cultures of *Zingiber officinale* for gingerol and zingiberene (rise of 1-2 folds) production (Anasori and Asghari, 2008) and *Lotus corniculatus* for tannin production (increased by 9 folds) (Paolocci *et al*., 2005).

The correlation coefficient shown in Table 7.8 were all positive when comparing the enzymatic activity (PAL and CHS assay) and

antioxidant activities (FRAP and DPPH assay) to the levels of naringenin, quercetin and kaempferol produced from dark-induced callus cultures treated with Phe. The coefficients between these flavonoid levels and the antioxidant activities ranged between 0.8387 and 0.9168 ($p<0.05$). Meanwhile, the coefficient range between the enzymatic activities and these flavonoid levels were from 0.9124 to 0.9845 ($p<0.05$). Comparisons between these three flavonoid levels also revealed a direct relationship with positive correlations from 0.9376 to 0.9575 ($p<0.05$). Interestingly, these results were also comparable to Chapter 6 (Section 6.3.2.4), whereby the antioxidant activities of sweet shoot strongly correlated to the total flavonoid content of callus cultures fed with 10 mg/l of Phe. Hence, these results suggested that the antioxidant activities of naringenin, quercetin and kaempferol increased as the enzymatic activities increased in Phe-treated dark-induced callus cultures of sweet shoot.

Table 7.8. Correlation between measured parameters in dark-induced callus cultures of sweet shoot.

	Naringenin	**Quercetin**	**Kaempferol**
DPPH radical scavenging activity	0.9168	0.8974	0.8992
FRAP reducing activity	0.9049	0.8827	0.8387
PAL enzymatic activity	0.9845	0.9621	0.9516
CHS enzymatic activity	0.9440	0.9420	0.9124
Kaempferol	0.9575	0.9376	
Quercetin	0.9476		

7.3.1.5 Somatic embryo cultures of sweet shoot

In this study, the exogenous application of Phe was the most effective in enhancing the yield of naringenin, quercetin and kaempferol in somatic embryos of sweet shoot ($p<0.05$). The overall data in somatic embryos revealed that, higher concentration of Phe and longer duration of precursor contact improved the production of these flavonoids ($p<0.05$). The highest quantitative concentration of these flavonoid compounds were naringenin at 2773.66 µg/10g FW, quercetin at 38.29 µg/10g FW and kaempferol 2139.07 µg/10g FW, in somatic embryos of sweet shoot, after 3 weeks of Phe treatment at a concentration of 20 mg/l ($p<0.05$) (Table 7.9; Figure 7.9). The lowest amount of flavonoid compounds were recorded in untreated somatic embryos harvested at week 0 (baseline), and it amounted to naringenin of 132.13 µg/10g FW, quercetin of 0.83 µg/10g FW and kaempferol of 272.55 µg/10g FW. When compared to

baseline, the highest levels were equivalent to 20.99, 46.23 and 7.85 times more in concentration of naringenin, quercetin and kaempferol, respectively. The maximal value for these flavonoids were also much higher than untreated cultures harvested at week 3 with an increase of 2.18 fold in naringenin, 11.60 fold in quercetin and 5.72 fold in kaempferol. The positive influence of Phe as seen in Chapter 6 (Section 6.3.1.5 and 6.3.2.5) were again observed in the present experiment, in relation to antioxidant activities and total flavonoid content in somatic embryos of sweet shoot. Thus, this study pointed out the possible potential for sweet shoot somatic embryo to synthesize high levels of secondary metabolites with the addition of 20 mg/l Phe into hormone-free MS medium.

Table 7.9. Comparison of three different flavonoids present in elicited and non-elicited somatic embryos of sweet shoot, after 3 weeks of treatments.

Types of elicitors/ precursors	Dosages	Types of flavonoids (µg/10 g FW)								
		Naringenin content			Quercetin content			Kaempferol content		
		week 1	week 2	week 3	week 1	week 2	week 3	week 1	week 2	week 3
Methyl jasmonate	0µM	343.89 $_{f}$	468.98 $_{ef}$	1273.65 $_{de}$	1.01 $_{e}$	1.59 $_{e}$	3.30 $_{d}$	354.19 $_{e}$	365.50 $_{e}$	374.21 $_{e}$
	50µM	394.59 $_{f}$	1495.21 $_{cd}$	2154.15 $_{a}$	3.55 $_{d}$	5.47 $_{cd}$	6.83 $_{c}$	434.88 $_{de}$	520.37 $_{cd}$	1079.29 $_{a}$
	100µM	934.71 $_{e}$	1782.22 $_{bc}$	1777.73 $_{bc}$	4.73 $_{cd}$	11.26 $_{bc}$	16.73 $_{a}$	561.93 $_{cd}$	723.83 $_{bc}$	941.50 $_{ab}$
	200µM	1490.81 $_{cd}$	1999.66 $_{b}$	1499.58 $_{cd}$	9.91 $_{bc}$	12.51 $_{bc}$	13.48 $_{b}$	712.75 $_{bc}$	852.15 $_{ab}$	871.72 $_{ab}$
Salicylic acid	0µM	343.89 $_{e}$	468.98 $_{d}$	1273.65 $_{ab}$	1.01 $_{d}$	1.59 $_{d}$	3.30 $_{cd}$	354.19 $_{d}$	365.50 $_{d}$	374.21 $_{d}$
	50µM	269.52 $_{e}$	1044.39 $_{bc}$	1342.17 $_{ab}$	2.27 $_{d}$	3.68 $_{cd}$	5.21 $_{bc}$	405.49 $_{cd}$	486.68 $_{bc}$	638.90 $_{abc}$
	100µM	794.53 $_{c}$	1235.40 $_{ab}$	1574.77 $_{a}$	3.97 $_{cd}$	6.32 $_{b}$	12.88 $_{a}$	486.55 $_{bc}$	622.63 $_{abc}$	840.51 $_{a}$
	200µM	1034.67 $_{bc}$	1392.71 $_{ab}$	1499.58 $_{a}$	5.37 $_{bc}$	10.63 $_{ab}$	11.60 $_{ab}$	626.51 $_{abc}$	727.73 $_{ab}$	779.77 $_{ab}$
Phenylalanine	0 mg/l	343.89 $_{f}$	468.98 $_{e}$	1273.65 $_{d}$	1.01 $_{f}$	1.59 $_{f}$	3.30 $_{e}$	354.19 $_{e}$	365.50 $_{e}$	374.21 $_{e}$
	5 mg/l	549.55 $_{e}$	1531.09 $_{cd}$	1915.73 $_{bc}$	5.59 $_{de}$	9.00 $_{d}$	18.98 $_{c}$	466.85 $_{de}$	789.35 $_{cde}$	1109.28 $_{c}$
	10 mg/l	1519.70 $_{cd}$	2035.88 $_{bc}$	2215.12 $_{b}$	8.63 $_{d}$	20.45 $_{c}$	33.57 $_{ab}$	692.57 $_{cde}$	1469.65 $_{cd}$	2168.59 $_{bc}$
	20 mg/l	1774.27 $_{bcd}$	2316.89 $_{b}$	2773.66 $_{a}$	18.34 $_{c}$	30.15 $_{b}$	38.29 $_{a}$	1074.41 $_{c}$	1632.81 $_{b}$	2139.07 $_{a}$

* Values for naringenin, quercetin and kaempferol content expressed as µg per 10 g fresh weight samples (FW).
* Values expressed as means of five biological replicated experiments and each biological replicate consisted of three technical replicates.
* Different letters within row and column (lowercase) indicate a significant difference ($p<0.05$) according to Duncan's multiple range test.

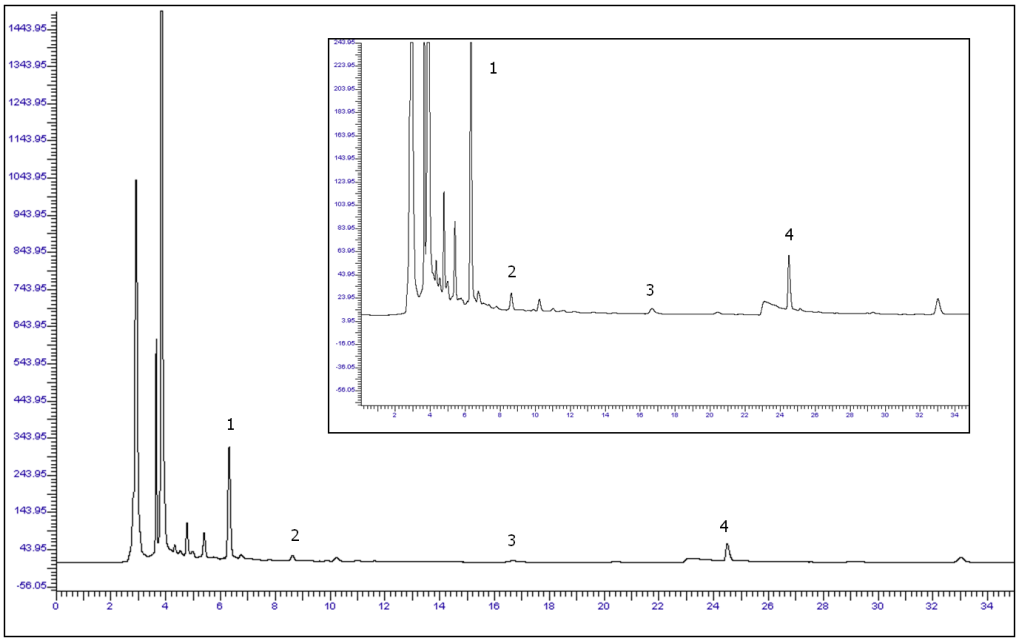


Figure 7.9. Chromatogram of somatic embryos of sweet shoot treated with 20 mg/l of Phe, after three weeks of precursor treatment. (1): Naringenin. (2): Papaverine. (3): Quercetin. (4): Kaempferol.

In this study, Phe was again found to be more effective than MJ and SA, since exogenous incorporation of Phe was able to potentiate the production of flavonoids via the phenylpropanoid pathway leading to increase yield amongst the flavonoids. When compared between the highest values in Phe and SA-treated culture, an increase of 1.28, 2.29 and 1.98 fold was noted in Phe-treated culture for naringenin, quercetin and kaempferol respectively. An increase of 1.76 (naringenin), 2.97 (quercetin) and 2.54 (kaempferol) fold was also detected in Phe-treated culture in comparison to the highest values of MJ-treated cultures. The effectiveness of Phe as a precursor for flavonoid production in somatic embryos of sweet shoot were further illustrated using Pearson correlation coefficient analysis.

The correlation coefficients of antioxidant activities (DPPH and FRAP assay) and enzymatic activities (PAL and CHS assay) with these three flavonoids (naringenin, quercetin and kaempferol) of somatic embryos were shown in Table 7.10. Positive correlations were again seen when comparing the antioxidant activities and enzymatic activities with the flavonoid levels produced from Phe at a concentration of 20 mg/l after three weeks of culture. The coefficients for the antioxidant activities versus these three flavonoid contents ranged from 0.6318 to 0.8240

($p<0.05$). Likewise, the enzymatic activities and flavonoid levels correlated positively between 0.8987 and 0.9599 ($p<0.05$). This further supports the fact that somatic embryos fed with Phe at 20 mg/l had the ability to produce higher amounts of flavonoids in sweet shoot.

Table 7.10. Correlation between measured parameters in somatic embryo cultures of sweet shoot.

	Naringenin	**Quercetin**	**Kaempferol**
DPPH radical scavenging activity	0.7647	0.7816	0.7759
FRAP reducing activity	0.6318	0.8240	0.6839
PAL enzymatic activity	0.9263	0.9098	0.9599
CHS enzymatic activity	0.9519	0.8987	0.9306
Kaempferol	0.8858	0.9334	
Quercetin	0.8689		

7.3.2 Quantification of papaverine in shadehouse-grown plants and light-induced callus cultures of sweet shoot

In order to examine the effects of elicitor/precursor treatment on papaverine production in sweet shoot in this study, shadehouse-grown plant was used as a point of reference for papaverine production, since it closely mimics the natural growth environment of field-grown sweet shoot. Light-induced callus culture of sweet shoot was then chosen as a comparison, since it produced the highest amount of flavonoids (naringenin content of 12081.05 µg/10g FW, quercetin content of 134.36 µg/10g FW and kaempferol content of 11325.13 µg/10g FW) in this study.

The present study showed that papaverine content of shadehouse-grown plants and light-induced callus cultures progressively increased as the contact durations of elicitor/precursor fed cultures increased, as displayed in Table 7.11. The baseline levels of papaverine were 0.06 µg/10g FW for untreated shadehouse-grown plants and 0.03 µg/10g FW for untreated light-induced callus cultures of sweet shoot. The exogenous application of SA and MJ into shadehouse-grown plants revealed a significant rise in papaverine content (34.96 µg/10g FW and 42.28 µg/10g FW respectively), after 3 weeks of elicitor treatment at concentration of 200 µM ($p<0.05$). This corresponded to 2.16 (SA) and 2.61 (MJ) folds higher than non-elicited shadehouse-grown plants harvested at week 3. These results were comparable with the previous report done by Gurkok *et al*. (2014) in opium poppy, whereby the addition of 100 µM MJ significantly induced the production of papaverine from 0.00 to 0.03 mg/g, after 12

hours of MJ elicitation. Concurrently, these authors also pointed out that the number of gene expression related to BIA biosynthetic pathway significantly increased after the addition of 100 μM MJ, with the key enzymes involved in papaverine biosynthetic pathway were (1) norcoclaurine synthase (NCS), (2) (S)-norcoclaurine 6-O-methyltransferase (6OMT), (3) (S)-3'-hydroxy-N-methylcoclaurine 4'-O-methyltransferase (4OMT) and (4) norreticuline 7-O-methyltransferase (N7OMT) (Figure 7.10). Hence, this study suggests that the addition of MJ (200 μM) may activate the enzymatic activities in BIA biosynthetic pathway of shadehouse-grown sweet shoot leading to increased papaverine production.

Table 7.11. Effects of methyl jasmonate, salicylic acid and phenylalanine on the papaverine content of shadehouse-grown plants and light-induced callus cultures of sweet shoot, after 21 days of treatments.

Types of elicitors/ precursors	Dosages	Shadehouse-grown plants			Light-induced callus cultures		
		Papaverine content (µg/10 g FW)			Papaverine content (µg/10 g FW)		
		week 1	week 2	week 3	week 1	week 2	week 3
Methyl jasmonate	0µM	0.70 $_{e}$	1.48 $_{e}$	16.21 $_{bc}$	0.05 $_{d}$	1.47 $_{bc}$	4.19 $_{a}$
	50µM	2.30 $_{e}$	6.63 $_{d}$	23.80 $_{b}$	0.13 $_{d}$	0.95 $_{cd}$	1.62 $_{bc}$
	100µM	4.02 $_{de}$	13.00 $_{c}$	39.63 $_{ab}$	0.33 $_{d}$	2.74 $_{bc}$	2.33 $_{bc}$
	200µM	13.07 $_{c}$	25.66 $_{b}$	42.28 $_{a}$	1.23 $_{bcd}$	3.06 $_{abc}$	3.55 $_{ab}$
Salicylic acid	0µM	0.70 $_{e}$	1.48 $_{e}$	16.21 $_{cd}$	0.05 $_{d}$	1.47 $_{bcd}$	4.19 $_{a}$
	50µM	2.27 $_{e}$	3.78 $_{e}$	19.68 $_{bc}$	0.19 $_{d}$	1.97 $_{bc}$	2.03 $_{bc}$
	100µM	2.87 $_{e}$	10.31 $_{d}$	25.29 $_{b}$	0.39 $_{d}$	2.27 $_{bc}$	3.07 $_{abc}$
	200µM	4.05 $_{e}$	18.90 $_{bc}$	34.96 $_{a}$	0.96 $_{cd}$	2.86 $_{abc}$	3.39 $_{ab}$
Phenylalanine	0 mg/l	0.70 $_{d}$	1.48 $_{d}$	16.21 $_{a}$	0.05 $_{d}$	1.47 $_{bcd}$	4.19 $_{a}$
	5 mg/l	2.39 $_{d}$	2.93 $_{d}$	13.71 $_{ab}$	0.39 $_{d}$	0.83 $_{cd}$	1.53 $_{bcd}$
	10 mg/l	4.87 $_{c}$	3.79 $_{d}$	16.39 $_{a}$	0.33 $_{d}$	1.94 $_{bc}$	2.19 $_{bc}$
	20 mg/l	5.61 $_{c}$	10.28 $_{b}$	15.73 $_{a}$	0.95 $_{cd}$	2.39 $_{bc}$	2.95 $_{b}$

* Values for papaverine content expressed as µg per 10 g fresh weight samples (FW).
* Values expressed as means of five biological replicated experiments and each biological replicate consisted of three technical replicates.
* Different letters within row and column (lowercase) indicate a significant difference ($p<0.05$) according to Duncan's multiple range test.

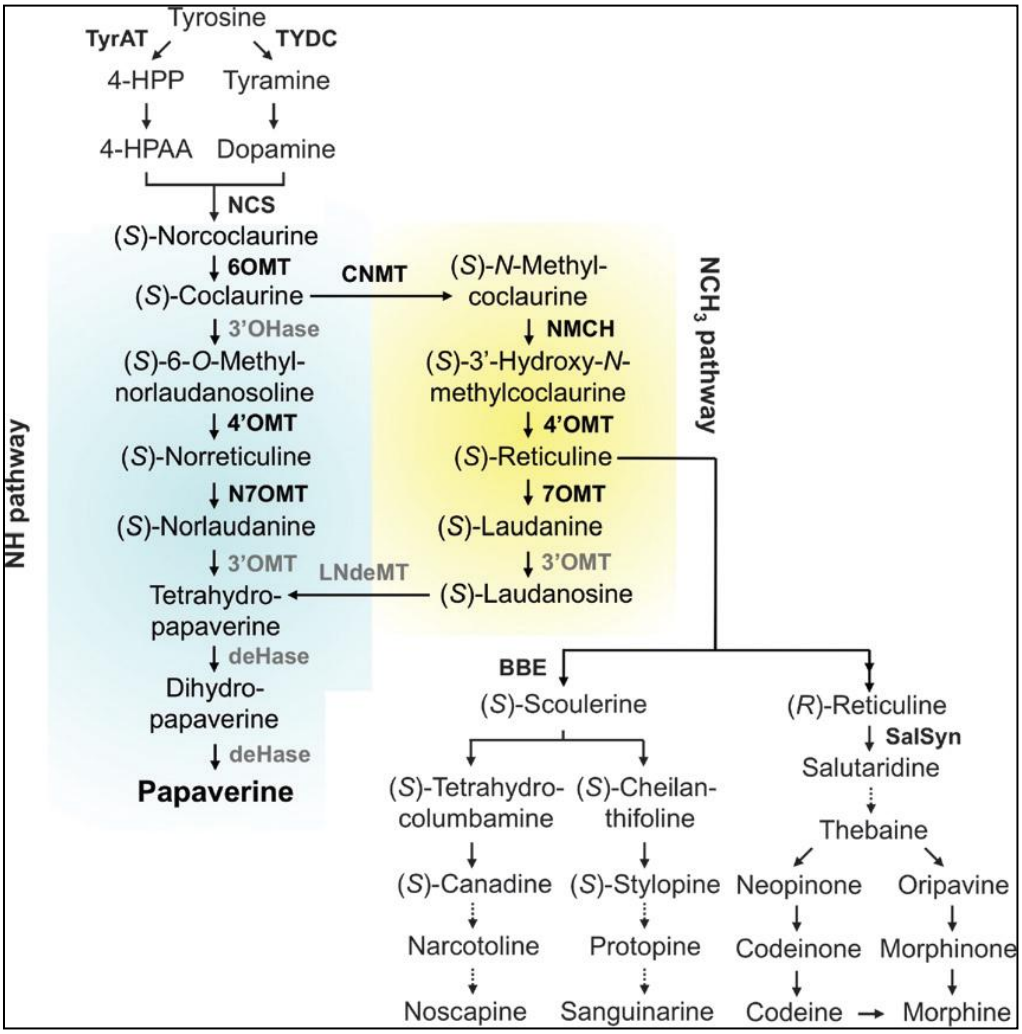


Figure 7.10. The proposed benzylisoquinoline alkaloid (BIA) biosynthetic pathway for the production of papaverine in opium poppy (Desgagne-Penix and Facchini, 2012). Tyrosine may be metabolized into papaverine by a series of enzymatic reactions, namely norcoclaurine synthase (NCS), (*S*)-norcoclaurine 6-*O*-methyltransferase (6OMT), (*S*)-3'-hydroxy-*N*-methylcoclaurine 4'-*O*-methyltransferase (4OMT) and norreticuline 7-*O*-methyltransferase (N7OMT).

However, there was a slight difference in shadehouse-grown plants induced with Phe. The first two weeks of cultures had significant rises in papaverine content, but a non significant elevation of papaverine was seen in plants harvested at week 3. This observation could be attributed to the ineffectiveness of Phe in activating the gene expression of papaverine in BIA biosynthetic pathway (Desgagne-Penix and Facchini, 2012). A previous study done by Desgagne-Penix and Facchini (2012) showed that the suppression of

6OMT, 4OMT and N7OMT enzymes reduced the papaverine content in opium poppy. These results suggested that phenylalanine may be unable to regulate the enzymes involved in the BIA biosynthetic pathway for papaverine production in shadehouse-grown plants of sweet shoot.

The light-induced callus cultures of sweet shoot revealed a different observation altogether. Overall, the papaverine content in elicited and non-elicited light-induced callus cultures of sweet shoot were significantly lower than those seen in shadehouse-grown plants at all levels of concentration and contact duration. The present study showed that higher concentration of elicitor/precursor enhanced the production of papaverine. Fortunately, this positive rise dwindled at week 3, whereby the papaverine content in elicited callus cultures were lower than non-elicited callus cultures of sweet shoot, albeit not statistically significant. The non-elicited callus cultures were 1.17 (MJ), 1.23 (SA) and 1.42 (Phe) higher than those elicited at week 3. This is an interesting observation as many studies done previously in an attempt to increase the production of papaverine via wounding (Morimoto *et al*., 2001; Bonilla *et al.*, 2014) were inconclusive. The exact mechanism of papaverine production has not been fully understood and further studies need to be carried out to elucidate this mechanism, especially in shadehouse-grown plants of sweet shoot, as a potentially economical source for production of papaverine for its medicinal properties.

7.4 CONCLUSION

This is the first report in studying the effects of elicitation (methyl jasmonate and salicylic acid) and precursor feeding (phenylalanine) for the production of alkaloid (papaverine) and flavonoid (naringenin, quercetin and kaempferol) compounds in shadehouse-grown plants and cultured tissues of sweet shoot. The present study showed that untreated light-induced callus cultures had the ability to synthesize higher amounts of naringenin (138.14 µg/10g FW), quercetin (1.54 µg/10g FW) and kaempferol (338.79 µg/10g FW), when compared to shadehouse-grown plants and other cultured tissues of sweet shoot. Amongst those plant samples treated with elicitors/precursor, light-induced callus cultures again proved to be the most suitable plant material for the yield enhancement of flavonoids, followed by (1) *in vitro*

shoot cultures, (2) shadehouse-grown plants, (3) somatic embryos and (4) dark-induced callus cultures of sweet shoot. The highest flavonoid values obtained from the treated light-induced callus cultures were between 33.43 and 87.46 fold higher than those untreated light-induced callus cultures harvested at week 0. These results highlight the significance of elicitor/precursor in increasing the production of flavonoids in light-induced callus cultures of sweet shoot.

In the present study, Phe remained the most desirable precursor on the yield enhancement of these flavonoids. The highest levels of naringenin (12081.05 µg/10g FW), quercetin (134.36 µg/10g FW) and kaempferol (11325.13 µg/10g FW) were obtained from light-induced callus cultures treated with 20 mg/l of Phe, after 3 weeks of precursor feeding. These results were significantly higher by 4.76 fold for naringenin, 33.26 fold for quercetin and 12.45 fold for kaempferol, when compared to untreated cultures harvested at week three. Besides that, Phe also exhibited the highest levels of flavonoid production in all the other cultured tissues of sweet shoot, followed by MJ and SA respectively. Hence, the choice of elicitors and precursors played a significant role in the production of flavonoids in shadehouse-grown plants and cultured tissues of sweet shoot.

Additionally, Pearson correlation coefficient analysis was also used to examine the relationship of the tested elicitors/precursor with regards to the flavonoid content, antioxidant activities (DPPH and FRAP assay) and enzymatic activities (PAL and CHS assay) of sweet shoot. This study was able to demonstrate the positive effects of Phe treatment in the production of flavonoid compounds via a series of enhanced enzymatic activities in light-induced callus culture of sweet shoot. The flavonoids obtained in this study were found to display strong antioxidant activities in light-induced callus cultures treated with 20 mg/l of Phe, which was also in agreement with the previous experiment done in Chapter 6. Therefore, this optimized protocol could possibly serve as an alternative method for sustainable production of flavonoids in *in vitro* cultures of sweet shoot.

The current study also showed that the addition of elicitors (MJ and SA) increased the production of papaverine in shadehouse-grown plants of sweet shoot. However, Phe-fed shadehouse-grown sweet shoot did not show significant increment in papaverine production, when compared to untreated plants harvested at week three. Interestingly, lower amount of papaverine were also detected in light-induced callus cultures of sweet shoot, after 3 weeks of elicitation/precursor feeding. Previous studies on opium poppy highlighted that MJ elicitation was able to potentiate the production of papaverine which was similar to the one done in this present study in shadehouse-grown sweet shoot. However, there are no studies that showed poor production of papaverine after elicitation to help explain the negative effects in treated light-induced callus cultures. Hence, further research is needed in order to determine the possible causes for this observation.

CHAPTER 8: GENERAL DISCUSSION AND RECOMMENDATIONS FOR FUTURE RESEARCH

8.1 GENERAL DISCUSSION

An underutilized crop, named *Sauropus androgynus* (sweet shoot) has been cultivated in Southeast Asia for local consumption for many years. However, in the last 15 years, there were only nine studies reported by scientists across the globe, regarding the presence of bioactive compounds such as alkaloid, phenolic and flavonoid compounds in sweet shoot (Miean and Mohamed; Wong *et al*., 2006; Benjapak *et al*., 2008; Maisuthisakul *et al*., 2008; Nahak and Sahu, 2010; Andarwulan *et al*., 2010; Lee *et al*., 2011; Shubha *et al*., 2011; Andarwulan *et al*., 2012). Most of these bioactive compounds extracted from sweet shoot served as an antioxidant in neutralizing the damaging effects of free radicals. However, the present study showed that the shadehouse-grown plants of sweet shoot failed to accumulate significant amounts of antioxidant substances in nature, with only 61.20 µg/10g FW and 193.62 µg/10g FW in total phenolic and flavonoid contents, respectively. Besides that, it also showed very low antioxidant activities in DPPH (54.03%) and FRAP (397.56 µg/10g FW) assay. Therefore, an alternative method using plant tissue culture systems with optimized elicitation conditions is essential for yield enhancement of antioxidant compounds in sweet shoot. This study was undertaken to evaluate the effects of elicitors (methyl jasmonate and salicylic acid) and a precursor (phenylalanine) on antioxidant capacity and on the yield improvement of secondary metabolites in tissue cultures of sweet shoot.

In this study, plant regeneration via (A) adventitious shoot regeneration (Chapter 3), (B) indirect organogenesis (Chapter 4) and (C) indirect somatic embryogenesis (Chapter 5) has been successfully established for sweet shoot using nodal segment, leaf explant and dark-induced callus cultures, respectively. For adventitious shoot multiplication, nodal explants were cultured on semi-solid and liquid MS medium supplemented with different combinations and concentrations of plant growth regulators (Chapter 3). Higher concentrations of BAP enhanced the number of shoots, whereas shoot length decreased as the concentrations of BAP increased from 0.5 to

2.0 mg/l. Semi-solid MS medium containing 2.0 mg/l BAP and 0.5 mg/l IAA was the most effective medium for adventitious shoot proliferation, as the nodal explants produced the highest number of shoots (6.74 shoots per explant) with an average length of 5.74 cm. Besides that, similar medium composition but in liquid culture system was also found to be rather effective in inducing adventitious shoots from nodal explants (7.43 shoots per explant with a mean length of 6.94 cm). The enhancement of shoot proliferation via liquid culture system may be due to better aeration and uptake of nutrients and plant growth regulators compared to semi-solid medium. Despite the promising results mentioned above for liquid culture systems, the generated microshoots showed distinct symptoms of vitrification that shorten their life span. Hence, adventitious shoots cultured on semi-solid MS medium were used as the plant material for the subsequent elicitation experiments.

Secondary metabolites were produced differently between light and dark-induced callus cultures of woody plant species, as shown in previous studies. From these observations, light and dark-induced callus cultures of sweet shoot were then selected as plant materials for the following elicitation experiment. In the present study, aseptic leaf explants of sweet shoot were inoculated on semi-solid MS medium supplemented with different combinations and concentrations of auxin and cytokinin (Chapter 4). Leaf explants of sweet shoot were maintained in tissue culture room temperature of 26±2^{o}C under (A) 16-hour light photoperiod for the proliferation of light-induced callus cultures, or (B) incubated in the dark for dark-induced callus cultures. Results shown in Chapter 4 revealed that MS medium enriched with 2.0 mg/l NAA and 1.0 mg/l kinetin significantly enhanced the callus regeneration capacity (>71.67%), callus fresh weight (>0.45 g) and callus expansion rate (>13.85 cm^2) in both light and dark-induced callus cultures of sweet shoot. A similarity was also seen between light and dark-induced callus cultures, in which the callusing response increased as the auxin levels increased from 0.5 to 2.0 mg/l. The light-induced callus generated in this experiment was transferred into MS medium fortified with 2.0 mg/l BAP and 0.5 mg/l IAA for shoot induction. This medium successfully induced the highest percentage (86.67%) of callus cultures to form shoots, with the highest number (6.23 shoots per callus) and the highest mean length (5.43

cm) of shoots. Thus, the high regeneration capacity of callus cultures served as a feasible and promising method for large-scale production of sweet shoot.

Somatic embryogenesis possesses great potential for clonal propagation of woody plant species, due to its high multiplication rate and potential for scale up in liquid suspension cultures and bioreactor (Merkle and Dean, 2000). The complete ontogeny of somatic embryos in sweet shoot was reported in Chapter 5, in which, the sweet shoot somatic embryos progressed through the globular, heart, torpedo and cotyledonary stages. Since tissues with different levels of competence required a precise balance of plant growth regulators, it is crucial to optimize the concentrations of plant growth regulators for an optimum response at each individual stage of sweet shoot somatic embryos. The three-month-old friable dark-induced callus cultures were cultured on either semi-solid or liquid MS medium fortified with different concentrations of NAA and kinetin. The cultivation of somatic embryos via liquid medium is preferable over semi-solid medium, as it induced the highest packed cell volume of embryogenic cell suspension and produced the highest number of somatic embryos at each individual stage. After three weeks of callus inoculation, high percentage (83.33%) of embryogenic cell achieved its maximum density of 5.2 ml on liquid MS medium supplemented with 2.0 mg/l NAA and 1.0 mg/l kinetin. Liquid MS medium containing 1.0 mg/l NAA and 0.5 mg/l kinetin was used as a histodifferentiation medium for the production of globular embryos (15.60 embryos/g callus), after three weeks of embryo induction. Similar hormone composition was also used to induce the highest yield of heart-shaped and torpedo-shaped embryos after nine weeks of embryo induction, with an average of 14.80 and 13.20 embryos per g callus, respectively. Individual torpedo-shaped embryos generated in this study were then transferred to hormone-free MS medium for the maturation of somatic embryos (90% embryo maturation rate). This study proved that the combination of NAA and kinetin played an important role in initiating the histodifferentiation process of somatic embryos. After two months of shoot induction, cotyledonary somatic embryos (75%) successfully germinated into shoot clumps on semi-solid medium supplemented with 2.0 mg/l BAP and 0.5 mg/l IAA, with a mean of 6.45 shoots per embryo and an average shoot length of 5.69 cm. Subculture of these embryos resulted in the development

of more embryos with no abnormal morphological evidence, as it displayed normal starch grains, nuclei, procambium, cytoplasmic structures and meristematic tissues in all stages of sweet shoot somatic embryos.

Successful acclimatization of rooted plantlets and their subsequent transfer to the field is a crucial step for commercial exploitation of *in vitro* technology (Li and He, 2006). In this study, rooted shoots generated from (A) adventitious shoot regeneration pathway, (B) indirect organogenesis and (C) indirect somatic embryogenesis were transplanted to pots containing a mixture of perlite and compost (1:1) for establishment *ex vitro*. Survival rate of more than 76.67% was achieved after one month of acclimatization in shadehouse. With this improvement in survival rate compared to traditional stem cutting technique (61%), sweet shoot can be exploited for commercial use in large-scale plantations that can be further processed for the production of bioactive metabolites. The protocols described for each regeneration pathway will also form the basis for future research on germplasm conservation and genetic transformation.

Due to the low levels of phenolic content, flavonoid content and antioxidant activity present in non-elicited shadehouse-grown sweet shoot, precursor feeding (Phe) and elicitor (MJ and SA) treatment are necessary to improve the production of these secondary metabolites using plant tissue culture system (Chapter 6). In this study, shadehouse-grown plants, light-induced callus, dark-induced callus and somatic embryos of sweet shoot were treated individually with different concentrations of MJ, SA and Phe at a given treatment period of three weeks. The present study showed that Phe was the preferred choice for antioxidant production in all tested tissue cultures of sweet shoot, followed by MJ and SA. This finding could be due to the role of Phe as an upstream biosynthetic precursor in phenylpropanoid pathway, which was then metabolized by sweet shoot into antioxidant compounds via a series of enzymatic reactions such as phenylalanine ammonia-lyase (PAL) and chalcone synthase (CHS) (Edahiro *et al.*, 2005; Mathur and Goswami, 2012).

Amongst the Phe treatments, hormone-free MS medium containing 20 mg/l of Phe had the most beneficial influence on total phenolic (246.62

µg/10g FW) content, total flavonoid (636.26 µg/10g FW), antioxidant activities (97.35% for DPPH assay and 5941.66 µg/10g FW for FRAP assay) and enzymatic activities (101.18 mmol CA/g FW for PAL assay and 14.49 nkat/mg protein for CHS assay) in light-induced callus cultures of sweet shoot, after three weeks of precursor feeding. These results were significantly higher by 6.99 fold for total phenolic content, 4.64 fold for total flavonoid content, 1.98 fold for DPPH free radical scavenging activity, 17.74 fold for FRAP reducing activity, 8.53 fold for PAL enzymatic activity and 7.96 fold for CHS enzymatic activity, when compared to untreated light-induced callus cultures of sweet shoot (baseline). Additionally, Pearson correlation coefficient analysis was also used to examine the relationship of Phe treatment with regards to the total phenolic content, total flavonoid content, antioxidant activities (DPPH and FRAP assay) and enzymatic activities (PAL and CHS assay) in light-induced callus cultures of sweet shoot. Positive correlations were seen in all the parameters mentioned above, suggesting that higher phenolic and flavonoid contents of sweet shoot led to the multiplication of antioxidant activities via enhanced enzymatic activities (PAL and CHS enzyme) in light-induced callus cultures treated with Phe. The results obtained in this study indicated that Phe can be used as a substrate for the initial step of phenylpropanoid metabolism for the production of secondary metabolites that are responsible for the antioxidant activity in sweet shoot.

In this study, selected flavonoid compounds particularly naringenin, quercetin and kaempferol were chosen to be extracted from sweet shoot, due to its promising medicinal properties (Chapter 7). Similarly, shadehouse-grown plant and tissue cultures of sweet shoot were singly supplemented with different concentrations of MJ, SA or Phe for a period of three weeks (week 0, 1, 2 and 3). The present study revealed that higher concentrations of Phe and longer duration of precursor treatment significantly improved the production of these flavonoids. Phe treatment once again proved to be the most effective in enhancing the yield of naringenin, quercetin and kaempferol in light-induced callus cultures of sweet shoot. The highest concentration of these flavonoid compounds were naringenin at 12081.05 µg/10g FW, quercetin at 134.36 µg/10g FW and kaempferol 11325.13 µg/10g FW, in

light-induced callus cultures of sweet shoot, after 3 weeks of Phe treatment at a concentration of 20 mg/l. These results were significantly higher by 87.46 fold for naringerin, 87.25 fold for quercetin and 33.43 fold for kaempferol content, when compared to untreated light-induced callus cultures of sweet shoot harvested at week 0 (baseline). Pearson correlation coefficient analysis was additionally done to examine the relationship of Phe treatment with regards to the naringenin, quercetin and kaempferol content, antioxidant activities (DPPH and FRAP assay) and enzymatic activities (PAL and CHS assay) in light-induced callus cultures of sweet shoot treated with Phe. Again, there were positive correlations seen in all the parameters mentioned above, suggesting that the higher flavonoid contents of sweet shoot were due to enhancement in enzymatic activities (PAL and CHS enzyme) leading to improved antioxidant capabilities in light-induced callus cultures treated with Phe. With elevated antioxidant activity as shown here, sweet shoot cultivation can be used as a source of medicinal compounds by pharmaceutical companies. These optimized protocols for mass production of bioactive secondary metabolites may be exploited for this purpose.

In conclusion, this is the first study in sweet shoot that manages to explore tissue culture with the addition of plant growth regulators as an alternative method for large-scale production as it has shown to improve growth and proliferation of sweet shoot compared to the conventional methods available currently. This is also the first study in reporting the effectiveness of elicitors and precursors for yield improvement of bioactive antioxidants in sweet shoot. With these optimized protocols, sweet shoot not only can be harvested at larger quantities, it can also be used for the production of antioxidants for medicinal purposes. This study provides a benchmark for future experiments to further explore the varied possibilities for this underutilized, yet promising crop.

8.2 RECOMMENDATIONS FOR FUTURE RESEARCH

The present study demonstrated the validity of using elicitor and precursor in enhancing the yield of secondary metabolites and improving the antioxidant activity in tissue cultures of sweet shoot. The optimized methods for the establishment of plant tissue cultures and the yield enhancement of

antioxidant compounds via precursor and elicitor treatment could be potentially used in other underutilized crops, especially for those of economic interest. The recommendations for future research include:

1. Ensuring sustainable production of high-quality plantlets and secondary metabolites via liquid suspension culture and bioreactor.
2. Enhance the production of secondary metabolites using different types of elicitors and precursors.
3. Study of metabolic pathway in relation to phenolic and flavonoid development using molecular and genetic approaches.
4. Advanced spectroscopic studies for structural elucidation of secondary metabolites and to identify novel pharmaceutical compounds of sweet shoot.

Recommendation 1: Ensuring sustainable production of high-quality plantlets and secondary metabolites via liquid suspension culture and bioreactor. Plant tissue culture has been proven to produce high levels of flavonoid compounds with subsequent increased antioxidant activity, as shown in Chapter 6 and 7. To maximize this potential, there are a few plant regeneration methods that can be adopted for mass multiplication of sweet shoot plantlets and secondary metabolites, such as stirred tank bioreactor (Ducos *et al*., 1999), liquid suspension culture (Hellwig *et al*., 2004) and RITA temporary immersion bioreactor (Etienne *et al*., 2006). The present study showed that liquid culture system has the ability to allow mass propagation of sweet shoot, as shown in Chapter 3. However, vitrification symptoms were observed in sweet shoot plantlets generated in liquid MS medium which shorten its life span. Therefore, strategies need to be formulated to rectify this issue and to maximize the yield of sweet shoot and antioxidant compounds using liquid culture medium.

Besides that, several scientists also reported that higher multiplication rate and better quality of plantlets can be obtained using temporary immersion bioreactor in non-woody and woody plant species, such as coffee, banana, sugarcane, *Morinda citrifolia* and *Saussurea involucrata* (Jia *et al*., 2005; Roels *et al*., 2005; Jang *et al*., 2013). The application of temporary immersion bioreactor was shown to eliminate the hyperhydricity in plantlets,

since the liquid medium comes in contact with the plantlets intermittently (Hui *et al*., 2012). Hence, protocols for scaling up the biomass and secondary metabolites using bioreactors need to be optimized for sweet shoot, especially the types of bioreactors (batch, fed batch and two-stage batch culture) and culture conditions (oxygen supply and carbon dioxide exchange, cell density and agitation speed).

Recommendation 2: Enhance the production of secondary metabolites using different types of elicitors and precursors. Current study revealed the beneficial effects of MJ, SA and Phe in enhancing the production of antioxidant compounds in sweet shoot (Chapter 6 and 7). Some success in stimulating phenolic and flavonoid production has also been achieved by other types of elicitors and precursors, such as chitosan, tyrosine, silver nitrate, fungal homogenates and ultraviolet radiations (Reymond and Farmer, 1998; Ruiz-Garcia and Gomez-Plaza, 2013; Bharati and Bansal, 2014). It was reported that the production of antioxidant compounds responded differently to the types of elicitor and precursor used in cultured tissues of woody plant species (Ramakrishna and Ravishankar, 2011). Therefore, different types of elicitors and precursors can also be used in enhancing the production of antioxidant compounds in tissue cultures of sweet shoot.

Recently, the synergistic effects of both elicitor and precursor were also reported for the production of phenolic and flavonoid compounds in plant tissue cultures of woody plant species, such as *Curcuma longa* (Cousins *et al*., 2010), *Vitis vinifera* (Qu *et al*., 2011) and *Exacum affine* (Skrzypczak-Pietraszek *et al*., 2014). Hence, the combined effects of Phe and methyl jasmonate can also be examined in the future studies, in order to obtain a better productivity of antioxidant compounds in tissue cultures of sweet shoot.

Recommendation 3: Study of metabolic pathway in relation to phenolic and flavonoid development using molecular and genetic approaches. Current study revealed that the production of antioxidant compounds were positively correlated to the enzymatic activities of light-induced callus cultures treated with Phe (Chapter 6 and 7). This finding

suggested that PAL and CHS enzymes may regulate the phenylpropanoid pathway by converting Phe into naringenin, quercetin and kaempferol. However, it was also documented that other enzymes such as 4-coumaroyl-coenyzme A ligase (4CL) and UDP-glucose:flavonoid 3-O-glycosyltransferase (UFGT) played a significant role in synthesizing the antioxidant compounds in *Solanum tuberosum* (Hu *et al*., 2011) and *Ginkgo biloba* (Cheng *et al*., 2012). Therefore, molecular and genetic techniques are needed for further quantification of these enzymatic genes (*PAL* and *CHS*) in relation to phenolic acid and flavonoid development in sweet shoot. Isolation of the full length cDNA and genomic DNA sequence of the relevant genes can facilitate the expression analysis of *PAL* and *CHS* genes in light-induced callus cultures of sweet shoot treated with Phe, as was done with *Ginkgo biloba* (Xu *et al*., 2008). In other words, molecular techniques such as RT-PCR and northern blot can be used to study the functional analysis of sweet shoot enzymatic genes in response to stress created by elicitors and precursors.

Recommendation 4: Advanced spectroscopic studies for structural elucidation of secondary metabolites and to identify novel pharmaceutical compounds of sweet shoot. The HPLC chromatogram showed a large number of unknown peaks detected in light-induced callus cultures treated with elicitors and precursor. This showed that tissue culture of sweet shoot has the potential to synthesize a relatively huge number of secondary metabolites, which could be responsible for the medicinal properties such as antioxidant, antimicrobial and anticancer activities of sweet shoot. Therefore, a high-throughput screening technique such as liquid chromatography-mass spectrometry (LC-MS), gas chromatography (GC) and capillary electrochromatography (CEC) can be used in future studies, in order to thoroughly identify the unknown secondary metabolites present in tissue cultures of sweet shoot (Rijke *et al*., 2006; Stalikas, 2007). Furthermore, nuclear magnetic resonance (NMR) can also be used for complete structure elucidation of flavonoid extracted in this study and to identify novel pharmaceutical compounds present in sweet shoot (Petersen, 2007).

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APPENDICES

Table 1. Constituents of MS basal media for the preparation of six stock solutions.

Constituents (analytical grade)	gL^{-1}
SOLUTION 1	
Ammonium nitrate (NH_4NO_3)	165
Potassium nitrate (KNO_3)	190
Magnesium sulphate ($MgSO_4.7H_20$)	37
SOLUTION 2	
Potassium hydrogen phosphate (KH_2PO_4)	17
SOLUTION 3	
Calcium chloride ($CaCl_2.6H20$)	44
SOLUTION 4	
Iron sodium EDTA (FeNaEDTA)	3.67
SOLUTION 5	
Cobalt chloride ($CoCl_2.6H20$)	0.0025
Copper sulphate ($CuCo_4.H20$)	0.0025
Sodium molybdate ($NaMoO_4.4H20$)	0.025
Boric acid (H_3BO_3)	0.62
Potassium iodide (KI)	0.083
Magnesium sulphate ($MgSO_4.4H_20$)	2.23
Zinc sulphate ($ZnSO_4.7H_20$)	0.86
SOLUTION 6	
Inositol	10
Nicotinic acid	0.05
Thiamine HCl	0.01
Pyridoxine HCl	0.05
Glycine	0.2

Table 2. Effect of cutting origin on the rooting of sweet shoot cuttings.

Cutting origin	Number of cuttings	Percentage of rooting	Root number per rooted cutting	Longest root length (cm)	Total shoot number per rooted cutting	Longest shoot length (cm)
Shoot tip	60	41.33 ± 5.00 $_{A}$	6.41 ± 0.48 $_{C}$	16.59 ± 0.52 $_{C}$	8.45 ± 0.47 $_{A}$	24.21 ± 0.54 $_{A}$
Internodal	60	61.00 ± 2.89 $_{A}$	8.58 ± 0.35 $_{B}$	27.20 ± 0.71 $_{B}$	5.10 ± 0.31 $_{B}$	9.89 ± 0.25 $_{C}$
Nodal	60	56.67 ± 2.89 $_{A}$	12.29 ± 0.56 $_{A}$	38.88 ± 0.63 $_{A}$	3.54 ± 0.45 $_{C}$	14.54 ± 0.37 $_{B}$

* Values expressed as means with standard deviations.

* Each mean was based on 3 replicates, each of which consists of 20 cultures.

* The same letter in the same column (uppercase) indicates treatment means are not significantly different at 5% level (Duncan's multiple range test).

Table 3. Effects of various concentrations and combinations of NAA and kinetin on shoot formation from juvenile leaf and stem explants of sweet shoot after 2 months of culture.

Treatment code	Growth regulators (mg/l)		Types of explants											
			Leaf explants				Internodal explants				Nodal explants			
	KIN	NAA	% of responded explant	Number of shoot per explant	Longest shoot length (cm)	Number of root	% of responded explant	Number of shoot per explant	Longest shoot length (cm)	Number of root	% of responded explant	Number of shoot per explant	Longest shoot length (cm)	Number of root
T0	0.0	0.0	0.00 $_{e}$	0.00 $_{h}$	0.00 $_{g}$	0.00 $_{a}$	0.00 $_{d}$	0.00 $_{h}$	0.00 $_{h}$	0.00 $_{b}$	0.00 $_{c}$	0.00 $_{g}$	0.00 $_{d}$	0.00 $_{d}$
T1	0.0	0.5	80.00 $_{c}$	2.29 $_{ef}$	1.99 $_{e}$	0.00 $_{a}$	63.33 $_{abc}$	2.70 $_{de}$	1.01 $_{ed}$	0.00 $_{b}$	70.00 $_{ab}$	3.15 $_{e}$	3.78 $_{b}$	0.00 $_{d}$
T2	0.0	1.0	83.33 $_{bcd}$	2.00 $_{fg}$	2.22 $_{d}$	0.33 $_{a}$	56.67 $_{abc}$	2.22 $_{ef}$	0.58 $_{fg}$	1.50 $_{ab}$	73.33 $_{ab}$	3.06 $_{ef}$	4.52 $_{ab}$	1.25 $_{ab}$
T3	0.0	2.0	70.00 $_{d}$	1.68 $_{g}$	1.50 $_{f}$	0.33 $_{a}$	50.00 $_{c}$	1.82 $_{g}$	0.60 $_{g}$	2.00 $_{a}$	60.00 $_{b}$	2.34 $_{f}$	2.67 $_{c}$	1.92 $_{a}$
T4	0.5	0.0	80.00 $_{c}$	3.17 $_{cd}$	2.00 $_{e}$	0.00 $_{a}$	65.00 $_{abc}$	2.33 $_{def}$	1.50 $_{d}$	0.00 $_{b}$	70.00 $_{ab}$	3.85 $_{d}$	3.92 $_{b}$	0.00 $_{d}$
T5	0.5	0.5	86.67 $_{bc}$	2.76 $_{de}$	2.60 $_{bc}$	0.33 $_{a}$	66.67 $_{abc}$	3.25 $_{bc}$	1.99 $_{c}$	1.18 $_{ab}$	75.00 $_{ab}$	4.25 $_{cd}$	4.67 $_{a}$	1.17 $_{ab}$
T6	0.5	1.0	88.33 $_{abc}$	2.49 $_{ef}$	2.74 $_{ab}$	0.33 $_{a}$	58.33 $_{abc}$	2.75 $_{cd}$	1.17 $_{de}$	1.33 $_{ab}$	76.67 $_{ab}$	3.64 $_{b}$	4.75 $_{a}$	1.00 $_{ab}$
T7	0.5	2.0	73.33 $_{d}$	1.72 $_{fg}$	1.89 $_{ef}$	0.33 $_{a}$	53.33 $_{bc}$	2.12 $_{fg}$	1.01 $_{efg}$	1.29 $_{ab}$	66.67 $_{ab}$	2.91 $_{ef}$	3.43 $_{b}$	1.25 $_{ab}$
T8	1.0	0.0	85.00 $_{bc}$	4.19 $_{b}$	2.66 $_{bc}$	0.00 $_{a}$	68.33 $_{abc}$	2.80 $_{cd}$	2.33 $_{bc}$	0.00 $_{b}$	75.00 $_{ab}$	4.39 $_{bc}$	4.69 $_{a}$	0.00 $_{d}$
T9	1.0	0.5	90.00 $_{a}$	4.12 $_{abc}$	2.80 $_{a}$	0.33 $_{a}$	70.00 $_{ab}$	3.36 $_{b}$	2.45 $_{b}$	0.51 $_{b}$	83.33 $_{a}$	4.37 $_{b}$	4.72 $_{a}$	0.67 $_{b}$
T10	1.0	1.0	88.33 $_{abc}$	3.80 $_{cd}$	2.88 $_{ab}$	0.33 $_{a}$	65.00 $_{abc}$	3.44 $_{b}$	2.22 $_{bc}$	0.67 $_{b}$	81.67 $_{a}$	4.39 $_{bc}$	4.43 $_{ab}$	0.67 $_{b}$
T11	1.0	2.0	76.67 $_{cd}$	2.61 $_{ef}$	2.29 $_{cd}$	0.33 $_{a}$	58.33 $_{abc}$	2.50 $_{def}$	1.35 $_{de}$	0.67 $_{b}$	70.00 $_{ab}$	3.09 $_{ef}$	3.75 $_{b}$	0.42 $_{c}$
T12	2.0	0.0	83.33 $_{bcd}$	4.53 $_{a}$	2.21 $_{d}$	0.00 $_{a}$	66.67 $_{abc}$	2.67 $_{de}$	2.00 $_{c}$	0.00 $_{b}$	73.33 $_{ab}$	5.04 $_{a}$	5.55 $_{a}$	0.00 $_{d}$
T13	2.0	0.5	88.33 $_{abc}$	4.48 $_{ab}$	2.98 $_{a}$	0.33 $_{a}$	75.00 $_{a}$	4.10 $_{a}$	3.04 $_{a}$	0.42 $_{b}$	80.00 $_{a}$	5.00 $_{a}$	4.67 $_{a}$	0.42 $_{bc}$
T14	2.0	1.0	88.33 $_{abc}$	3.47 $_{d}$	2.77 $_{ab}$	0.33 $_{a}$	63.33 $_{abc}$	3.29 $_{b}$	2.02 $_{c}$	0.35 $_{b}$	80.00 $_{a}$	4.29 $_{c}$	4.26 $_{ab}$	0.33 $_{c}$
T15	2.0	2.0	73.33 $_{d}$	2.56 $_{efg}$	2.15 $_{de}$	0.33 $_{a}$	58.33 $_{abc}$	2.27 $_{ef}$	1.28 $_{de}$	0.25 $_{b}$	68.33 $_{ab}$	2.99 $_{e}$	3.72 $_{b}$	0.17 $_{cd}$

* Values expressed as means of 12 replicated experiments, each replicate consisted of 5 cultures.

* Different letters within column (lowercase) indicate a significant difference ($p<0.05$) according to Duncan's multiple range test.

Table 4. Effect of plant growth regulator treatment (2,4-D and kinetin) on the number of shoots, shoot length and number of root on leaf and stem explants *in vitro* of sweet shoot, after 60 days of culture.

Treatment code	Growth regulators (mg/l)		Types of explants											
			Leaf explants				Internodal explants				Nodal explants			
	KIN	2,4-D	% of responded explant	Number of shoot per explant	Longest shoot length (cm)	Number of root	% of responded explant	Number of shoot per explant	Longest shoot length (cm)	Number of root	% of responded explant	Number of shoot per explant	Longest shoot length (cm)	Number of root
T0	0.0	0.0	0.00 $_{e}$	0.00 $_{e}$	0.00 $_{h}$	0.00 $_{b}$	0.00 $_{d}$	0.00 $_{h}$	0.00 $_{h}$	0.00 $_{b}$	0.00 $_{d}$	0.00 $_{h}$	0.00 $_{h}$	0.00 $_{d}$
T1	0.0	0.5	73.33 $_{c}$	2.02 $_{cd}$	1.66 $_{de}$	0.00 $_{b}$	56.67 $_{abc}$	1.64 $_{ef}$	0.70 $_{ef}$	0.00 $_{b}$	63.33 $_{abc}$	2.76 $_{e}$	2.44 $_{b}$	0.00 $_{d}$
T2	0.0	1.0	81.67 $_{b}$	1.55 $_{d}$	2.10 $_{cd}$	0.50 $_{a}$	58.33 $_{abc}$	1.24 $_{fg}$	0.61 $_{fg}$	2.00 $_{a}$	68.33 $_{abc}$	2.14 $_{f}$	2.11 $_{bcd}$	2.25 $_{a}$
T3	0.0	2.0	63.33 $_{d}$	1.22 $_{d}$	1.18 $_{g}$	0.50 $_{a}$	43.33 $_{c}$	0.97 $_{g}$	0.51 $_{g}$	2.00 $_{a}$	51.67 $_{c}$	1.65 $_{g}$	1.23 $_{g}$	2.42 $_{a}$
T4	0.5	0.0	68.33 $_{cd}$	2.65 $_{c}$	1.41 $_{ef}$	0.00 $_{b}$	51.67 $_{abc}$	2.17 $_{d}$	1.20 $_{d}$	0.00 $_{b}$	60.00 $_{abc}$	3.22 $_{e}$	2.08 $_{cde}$	0.00 $_{d}$
T5	0.5	0.5	78.33 $_{bc}$	3.11 $_{bc}$	2.15 $_{c}$	0.50 $_{a}$	58.33 $_{abc}$	2.30 $_{de}$	1.34 $_{c}$	1.50 $_{a}$	71.67 $_{ab}$	3.82 $_{bc}$	2.53 $_{b}$	1.50 $_{ab}$
T6	0.5	1.0	83.33 $_{ab}$	2.37 $_{c}$	2.64 $_{a}$	0.50 $_{a}$	61.67 $_{abc}$	1.97 $_{e}$	0.14 $_{de}$	1.33 $_{ab}$	73.33 $_{ab}$	2.79 $_{de}$	2.24 $_{bc}$	1.67 $_{ab}$
T7	0.5	2.0	66.67 $_{d}$	1.86 $_{d}$	1.30 $_{ef}$	0.50 $_{a}$	46.67 $_{bc}$	1.53 $_{f}$	0.92 $_{ef}$	1.33 $_{ab}$	55.00 $_{bc}$	2.17 $_{f}$	1.70 $_{fg}$	1.75 $_{ab}$
T8	1.0	0.0	73.33 $_{c}$	3.89 $_{ab}$	1.86 $_{d}$	0.00 $_{b}$	56.67 $_{abc}$	3.54 $_{bc}$	1.90 $_{bc}$	0.00 $_{b}$	66.67 $_{abc}$	4.19 $_{ab}$	2.40 $_{bcd}$	0.00 $_{d}$
T9	1.0	0.5	83.33 $_{ab}$	3.85 $_{ab}$	2.59 $_{ab}$	0.50 $_{a}$	60.00 $_{ab}$	3.59 $_{ab}$	2.00 $_{b}$	0.67 $_{b}$	76.67 $_{a}$	4.27 $_{ab}$	2.88 $_{a}$	0.75 $_{bc}$
T10	1.0	1.0	85.00 $_{a}$	3.63 $_{b}$	2.74 $_{a}$	0.50 $_{a}$	65.00 $_{abc}$	3.43 $_{e}$	1.88 $_{bc}$	0.67 $_{ab}$	75.00 $_{a}$	3.83 $_{bc}$	2.44 $_{b}$	0.92 $_{b}$
T11	1.0	2.0	68.33 $_{cd}$	2.28 $_{cd}$	1.40 $_{ef}$	0.50 $_{a}$	51.67 $_{abc}$	1.84 $_{e}$	1.07 $_{de}$	0.75 $_{ab}$	58.33 $_{abc}$	2.64 $_{e}$	2.00 $_{def}$	1.25 $_{ab}$
T12	2.0	0.0	73.33 $_{c}$	4.31 $_{a}$	1.78 $_{d}$	0.00 $_{b}$	53.33 $_{abc}$	3.99 $_{a}$	1.52 $_{c}$	0.00 $_{b}$	66.67 $_{abc}$	4.80 $_{a}$	2.21 $_{bcd}$	0.00 $_{d}$
T13	2.0	0.5	81.67 $_{b}$	3.98 $_{a}$	2.31 $_{bc}$	0.50 $_{a}$	65.00 $_{a}$	3.67 $_{a}$	2.60 $_{a}$	0.42 $_{b}$	75.00 $_{a}$	4.48 $_{a}$	3.04 $_{a}$	0.50 $_{c}$
T14	2.0	1.0	83.33 $_{ab}$	3.49 $_{bc}$	2.48 $_{b}$	0.50 $_{a}$	63.33 $_{abc}$	3.51 $_{c}$	1.91 $_{c}$	0.50 $_{b}$	73.33 $_{ab}$	3.48 $_{cd}$	2.20 $_{bc}$	0.67 $_{c}$
T15	2.0	2.0	66.67 $_{d}$	2.03 $_{cd}$	1.33 $_{ef}$	0.50 $_{a}$	50.00 $_{abc}$	1.77 $_{ef}$	0.97 $_{de}$	0.25 $_{b}$	58.33 $_{abc}$	2.31 $_{ef}$	1.87 $_{ef}$	0.75 $_{bc}$

* Values expressed as means of 12 replicated experiments, each replicate consisted of 5 cultures.

* Different letters within column (lowercase) indicate a significant difference ($p<0.05$) according to Duncan's multiple range test.

Table 5. Effect of different concentrations of NAA and kinetin on shoot regeneration in liquid medium using nodal explants of sweet shoot, after 60 days of culture.

Treatment code	Growth regulators (mg/l)		% of responded explant	Number of shoot per explant	Longest shoot length (cm)	% of root formation	Number of root
	KIN	NAA					
T0	0.0	0.0	0.00 $_{d}$	0.00 $_{d}$	0.00 $_{d}$	0.00 $_{e}$	0.00 $_{e}$
T1	0.0	0.5	80.00 $_{bc}$	4.25 $_{c}$	2.75 $_{c}$	20.00 $_{bc}$	7.25 $_{b}$
T2	0.0	1.0	78.33 $_{c}$	4.22 $_{c}$	2.92 $_{c}$	28.33 $_{a}$	8.08 $_{a}$
T3	0.5	0.0	75.00 $_{c}$	4.55 $_{c}$	2.90 $_{c}$	0.00 $_{e}$	0.00 $_{e}$
T4	0.5	0.5	83.33 $_{b}$	5.02 $_{bc}$	3.78 $_{bc}$	15.00 $_{bc}$	5.33 $_{cd}$
T5	0.5	1.0	83.33 $_{b}$	5.10 $_{bc}$	3.90 $_{b}$	23.33 $_{ab}$	6.08 $_{c}$
T6	1.0	0.0	83.33 $_{b}$	5.09 $_{bc}$	3.53 $_{bc}$	0.00 $_{e}$	0.00 $_{e}$
T7	1.0	0.5	90.00 $_{a}$	5.52 $_{ab}$	4.44 $_{ab}$	13.33 $_{c}$	4.13 $_{d}$
T8	1.0	1.0	86.67 $_{ab}$	4.53 $_{c}$	4.44 $_{ab}$	15.00 $_{bc}$	5.92 $_{c}$
T9	2.0	0.0	81.67 $_{bc}$	6.26 $_{a}$	3.36 $_{c}$	0.00 $_{e}$	0.00 $_{e}$
T10	2.0	0.5	90.00 $_{a}$	6.29 $_{a}$	4.75 $_{a}$	8.33 $_{d}$	2.75 $_{d}$
T11	2.0	1.0	85.00 $_{ab}$	4.26 $_{c}$	4.32 $_{ab}$	11.67 $_{cd}$	3.50 $_{d}$

* Values expressed as means of 12 replicated experiments, each replicate consisted of 5 cultures.

* Different letters within column (lowercase) indicate a significant difference ($p<0.05$) according to Duncan's multiple range test.

Table 6. Effect of plant growth regulator treatment, IAA and BAP on the incidence (%) of callus formation, callus fresh weight and callus expansion of the leaf and stem explants incubated under the light condition, after 60 days of culture.

Treatment code	Growth regulators (mg/l)		Types of explants											
			Leaf explants				Internodal explants				Nodal explants			
	IAA	BAP	% of callus formation	Estimated callus fresh weight (g)	Callus expansion factor (cm^2)	% of Browning	% of callus formation	Estimated callus fresh weight (g)	Callus expansion factor (cm^2)	% of Browning	% of callus formation	Estimated callus fresh weight (g)	Callus expansion factor (cm^2)	% of Browning
T0	0.0	0.0	0.00 $_{d}$	0.00 $_{e}$	0.00 $_{g}$	100.00 $_{a}$	0.00 $_{c}$	0.00 $_{d}$	0.00 $_{c}$	0.00 $_{a}$	0.00 $_{c}$	0.00 $_{d}$	0.00 $_{d}$	0.00 $_{a}$
T1	0.0	0.5	0.00 $_{d}$	0.00 $_{e}$	0.00 $_{g}$	100.00 $_{a}$	0.00 $_{c}$	0.00 $_{d}$	0.00 $_{c}$	0.00 $_{a}$	0.00 $_{c}$	0.00 $_{d}$	0.00 $_{d}$	0.00 $_{a}$
T2	0.0	1.0	0.00 $_{d}$	0.00 $_{e}$	0.00 $_{g}$	100.00 $_{a}$	0.00 $_{c}$	0.00 $_{d}$	0.00 $_{c}$	0.00 $_{a}$	0.00 $_{c}$	0.00 $_{d}$	0.00 $_{d}$	0.00 $_{a}$
T3	0.0	2.0	0.00 $_{d}$	0.00 $_{e}$	0.00 $_{g}$	100.00 $_{a}$	0.00 $_{c}$	0.00 $_{d}$	0.00 $_{c}$	0.00 $_{a}$	0.00 $_{c}$	0.00 $_{d}$	0.00 $_{d}$	0.00 $_{a}$
T4	0.5	0.0	0.00 $_{d}$	0.00 $_{e}$	0.00 $_{g}$	0.00 $_{f}$	0.00 $_{c}$	0.00 $_{d}$	0.00 $_{c}$	0.00 $_{a}$	0.00 $_{c}$	0.00 $_{d}$	0.00 $_{d}$	0.00 $_{a}$
T5	0.5	0.5	38.33 $_{c}$	0.38 $_{d}$	2.25 $_{f}$	46.67 $_{b}$	6.67 $_{bc}$	0.16 $_{cd}$	1.00 $_{bc}$	0.00 $_{a}$	3.33 $_{b}$	0.09 $_{c}$	0.50 $_{c}$	0.00 $_{a}$
T6	0.5	1.0	46.67 $_{bc}$	0.80 $_{c}$	4.35 $_{d}$	41.67 $_{bc}$	8.33 $_{b}$	0.22 $_{c}$	1.25 $_{b}$	0.00 $_{a}$	5.00 $_{ab}$	0.11 $_{c}$	0.75 $_{c}$	0.00 $_{a}$
T7	0.5	2.0	46.67 $_{bc}$	0.75 $_{c}$	4.01 $_{de}$	31.67 $_{c}$	8.33 $_{b}$	0.18 $_{cd}$	1.17 $_{bc}$	0.00 $_{a}$	6.67 $_{ab}$	0.15 $_{c}$	0.92 $_{c}$	0.00 $_{a}$
T8	1.0	0.0	0.00 $_{d}$	0.00 $_{e}$	0.00 $_{g}$	0.00 $_{f}$	6.67 $_{bc}$	0.17 $_{cd}$	1.17 $_{bc}$	0.00 $_{a}$	5.00 $_{ab}$	0.12 $_{c}$	0.83 $_{c}$	0.00 $_{a}$
T9	1.0	0.5	45.00 $_{bc}$	0.68 $_{c}$	3.88 $_{e}$	43.33 $_{bc}$	11.67 $_{ab}$	0.47 $_{abc}$	2.25 $_{ab}$	0.00 $_{a}$	8.33 $_{ab}$	0.31 $_{b}$	1.50 $_{b}$	0.00 $_{a}$
T10	1.0	1.0	53.33 $_{ab}$	1.75 $_{ab}$	7.01 $_{b}$	31.67 $_{c}$	15.00 $_{ab}$	0.65 $_{ab}$	3.00 $_{ab}$	0.00 $_{a}$	11.67 $_{ab}$	0.49 $_{ab}$	2.33 $_{ab}$	0.00 $_{a}$
T11	1.0	2.0	50.00 $_{ab}$	1.59 $_{b}$	6.43 $_{c}$	21.67 $_{de}$	13.33 $_{ab}$	0.48 $_{abc}$	2.25 $_{ab}$	0.00 $_{a}$	11.67 $_{ab}$	0.42 $_{ab}$	2.08 $_{ab}$	0.00 $_{a}$
T12	2.0	0.0	0.00 $_{d}$	0.00 $_{e}$	0.00 $_{g}$	0.00 $_{f}$	6.67 $_{bc}$	0.28 $_{bc}$	1.42 $_{b}$	0.00 $_{a}$	6.67 $_{ab}$	0.27 $_{b}$	1.33 $_{b}$	0.00 $_{a}$
T13	2.0	0.5	48.33 $_{b}$	0.77 $_{c}$	4.00 $_{de}$	23.33 $_{d}$	13.33 $_{ab}$	0.50 $_{abc}$	2.58 $_{ab}$	0.00 $_{a}$	11.67 $_{ab}$	0.43 $_{ab}$	2.25 $_{a}$	0.00 $_{a}$
T14	2.0	1.0	58.33 $_{a}$	2.19 $_{a}$	8.54 $_{a}$	18.33 $_{de}$	20.00 $_{a}$	0.85 $_{a}$	4.08 $_{a}$	0.00 $_{a}$	15.00 $_{a}$	0.62 $_{a}$	3.00 $_{a}$	0.00 $_{a}$
T15	2.0	2.0	56.67 $_{a}$	1.98 $_{a}$	7.96 $_{a}$	13.33 $_{e}$	13.33 $_{ab}$	0.57 $_{abc}$	2.75 $_{ab}$	0.00 $_{a}$	13.33 $_{ab}$	0.50 $_{ab}$	2.42 $_{a}$	0.00 $_{a}$

* Values expressed as means of 12 replicated experiments, each replicate consisted of 5 cultures.

* Different letters within column (lowercase) indicate a significant difference ($p<0.05$) according to Duncan's multiple range test.

Table 7. Effect of kinetin and 2,4-D on the percentage of callus formation, callus fresh weight and callus expansion factor and percentage of browning of the leaf and stem explants incubated under the light condition, 60 days after culture initiation.

Treatment code	Growth regulators (mg/l)		Types of explants											
			Leaf explants				Internodal explants				Nodal explants			
	2,4-D	KIN	% of callus formation	Estimated callus fresh weight (g)	Callus expansion factor (cm^2)	% of Browning	% of callus formation	Estimated callus fresh weight (g)	Callus expansion factor (cm^2)	% of Browning	% of callus formation	Estimated callus fresh weight (g)	Callus expansion factor (cm^2)	% of Browning
T0	0.0	0.0	0.00 $_{e}$	0.00 $_{f}$	0.00 $_{f}$	100.00 $_{a}$	0.00 $_{c}$	0.00 $_{c}$	0.00 $_{d}$	0.00 $_{a}$	0.00 $_{d}$	0.00 $_{d}$	0.00 $_{e}$	0.00 $_{a}$
T1	0.0	0.5	0.00 $_{e}$	0.00 $_{f}$	0.00 $_{f}$	100.00 $_{a}$	0.00 $_{c}$	0.00 $_{c}$	0.00 $_{d}$	0.00 $_{a}$	0.00 $_{d}$	0.00 $_{d}$	0.00 $_{e}$	0.00 $_{a}$
T2	0.0	1.0	0.00 $_{e}$	0.00 $_{f}$	0.00 $_{f}$	100.00 $_{a}$	0.00 $_{c}$	0.00 $_{c}$	0.00 $_{d}$	0.00 $_{a}$	0.00 $_{d}$	0.00 $_{d}$	0.00 $_{e}$	0.00 $_{a}$
T3	0.0	2.0	0.00 $_{e}$	0.00 $_{f}$	0.00 $_{f}$	100.00 $_{a}$	0.00 $_{c}$	0.00 $_{c}$	0.00 $_{d}$	0.00 $_{a}$	0.00 $_{d}$	0.00 $_{d}$	0.00 $_{e}$	0.00 $_{a}$
T4	0.5	0.0	0.00 $_{e}$	0.00 $_{f}$	0.00 $_{f}$	0.00 $_{g}$	0.00 $_{c}$	0.00 $_{c}$	0.00 $_{d}$	0.00 $_{a}$	0.00 $_{d}$	0.00 $_{d}$	0.00 $_{e}$	0.00 $_{a}$
T5	0.5	0.5	43.33 $_{d}$	0.75 $_{e}$	4.05 $_{e}$	31.67 $_{b}$	10.00 $_{bc}$	0.25 $_{bc}$	1.25 $_{c}$	0.00 $_{a}$	6.67 $_{c}$	0.16 $_{c}$	0.75 $_{d}$	0.00 $_{a}$
T6	0.5	1.0	53.33 $_{bc}$	1.78 $_{c}$	7.09 $_{d}$	31.67 $_{b}$	13.33 $_{b}$	0.38 $_{bc}$	2.08 $_{b}$	0.00 $_{a}$	10.00 $_{b}$	0.28 $_{bc}$	1.50 $_{bc}$	0.00 $_{a}$
T7	0.5	2.0	48.33 $_{cd}$	1.34 $_{d}$	6.01 $_{d}$	26.67 $_{bc}$	13.33 $_{b}$	0.32 $_{bc}$	2.00 $_{bc}$	0.00 $_{a}$	10.00 $_{b}$	0.22 $_{c}$	1.42 $_{cd}$	0.00 $_{a}$
T8	1.0	0.0	46.67 $_{d}$	1.19 $_{de}$	4.88 $_{e}$	26.67 $_{bc}$	11.67 $_{bc}$	0.27 $_{bc}$	1.75 $_{c}$	0.00 $_{a}$	8.33 $_{bc}$	0.20 $_{c}$	1.25 $_{cd}$	0.00 $_{a}$
T9	1.0	0.5	51.67 $_{c}$	3.19 $_{b}$	10.20 $_{c}$	21.67 $_{c}$	13.33 $_{b}$	0.52 $_{b}$	2.58 $_{b}$	0.00 $_{a}$	10.00 $_{b}$	0.39 $_{b}$	2.00 $_{b}$	0.00 $_{a}$
T10	1.0	1.0	60.00 $_{ab}$	3.69 $_{ab}$	13.09 $_{ab}$	18.33 $_{de}$	18.33 $_{ab}$	0.68 $_{b}$	3.58 $_{ab}$	0.00 $_{a}$	15.00 $_{ab}$	0.55 $_{ab}$	2.83 $_{ab}$	0.00 $_{a}$
T11	1.0	2.0	58.33 $_{b}$	3.21 $_{b}$	10.90 $_{c}$	18.33 $_{de}$	13.33 $_{b}$	0.48 $_{b}$	2.33 $_{b}$	0.00 $_{a}$	15.00 $_{ab}$	0.53 $_{ab}$	2.58 $_{ab}$	0.00 $_{a}$
T12	2.0	0.0	51.67 $_{c}$	1.70 $_{cd}$	6.51 $_{a}$	18.33 $_{de}$	11.67 $_{bc}$	0.36 $_{bc}$	2.08 $_{bc}$	0.00 $_{a}$	10.00 $_{b}$	0.30 $_{b}$	1.58 $_{bc}$	0.00 $_{a}$
T13	2.0	0.5	58.33 $_{b}$	3.31 $_{b}$	11.00 $_{bc}$	13.33 $_{e}$	16.67 $_{b}$	0.64 $_{b}$	3.33 $_{ab}$	0.00 $_{a}$	15.00 $_{ab}$	0.58 $_{a}$	2.92 $_{ab}$	0.00 $_{a}$
T14	2.0	1.0	65.00 $_{a}$	4.15 $_{a}$	16.28 $_{a}$	13.33 $_{e}$	23.33 $_{a}$	0.93 $_{a}$	4.17 $_{a}$	0.00 $_{a}$	18.33 $_{a}$	0.73 $_{a}$	3.67 $_{ab}$	0.00 $_{a}$
T15	2.0	2.0	60.00 $_{ab}$	3.74 $_{ab}$	14.59 $_{ab}$	6.67 $_{f}$	13.33 $_{b}$	0.59 $_{b}$	2.67 $_{b}$	0.00 $_{a}$	13.33 $_{ab}$	0.52 $_{ab}$	2.67 $_{ab}$	0.00 $_{a}$

* Values expressed as means of 12 replicated experiments, each replicate consisted of 5 cultures.

* Different letters within column (lowercase) indicate a significant difference ($p<0.05$) according to Duncan's multiple range test.

Table 8. Effect of plant growth regulator treatment, IAA and BAP on the incidence (%) of callus formation, callus fresh weight and callus expansion factor of the leaf and stem explants incubated in the dark, after 2 months of culture.

Treatment code	Growth regulators (mg/l)		Types of explants											
			Leaf explants				Internodal explants				Nodal explants			
	IAA	BAP	% of callus formation	Estimated callus fresh weight (g)	Callus expansion factor (cm^2)	% of Browning	% of callus formation	Estimated callus fresh weight (g)	Callus expansion factor (cm^2)	% of Browning	% of callus formation	Estimated callus fresh weight (g)	Callus expansion factor (cm^2)	% of Browning
T0	0.0	0.0	0.00 $_{e}$	0.00 $_{h}$	0.00 $_{f}$	100.00 $_{a}$	0.00 $_{d}$	0.00 $_{c}$	0.00 $_{d}$	0.00 $_{a}$	0.00 $_{a}$	0.00 $_{a}$	0.00 $_{a}$	0.00 $_{a}$
T1	0.0	0.5	0.00 $_{e}$	0.00 $_{h}$	0.00 $_{f}$	100.00 $_{a}$	0.00 $_{d}$	0.00 $_{c}$	0.00 $_{d}$	0.00 $_{a}$	0.00 $_{a}$	0.00 $_{a}$	0.00 $_{a}$	0.00 $_{a}$
T2	0.0	1.0	0.00 $_{e}$	0.00 $_{h}$	0.00 $_{f}$	100.00 $_{a}$	0.00 $_{d}$	0.00 $_{c}$	0.00 $_{d}$	0.00 $_{a}$	0.00 $_{a}$	0.00 $_{a}$	0.00 $_{a}$	0.00 $_{a}$
T3	0.0	2.0	0.00 $_{e}$	0.00 $_{h}$	0.00 $_{f}$	100.00 $_{a}$	0.00 $_{d}$	0.00 $_{c}$	0.00 $_{d}$	0.00 $_{a}$	0.00 $_{a}$	0.00 $_{a}$	0.00 $_{a}$	0.00 $_{a}$
T4	0.5	0.0	38.33 $_{d}$	0.22 $_{gh}$	1.75 $_{f}$	55.00 $_{b}$	0.00 $_{d}$	0.00 $_{c}$	0.00 $_{d}$	0.00 $_{a}$	0.00 $_{a}$	0.00 $_{a}$	0.00 $_{a}$	0.00 $_{a}$
T5	0.5	0.5	46.67 $_{bcd}$	0.43 $_{fg}$	3.25 $_{e}$	43.33 $_{bc}$	5.00 $_{c}$	0.12 $_{bc}$	0.67 $_{c}$	0.00 $_{a}$	0.00 $_{a}$	0.00 $_{a}$	0.00 $_{a}$	0.00 $_{a}$
T6	0.5	1.0	53.33 $_{abcd}$	0.94 $_{cd}$	4.19 $_{de}$	31.67 $_{cd}$	8.33 $_{abc}$	0.20 $_{bc}$	1.08 $_{bc}$	0.00 $_{a}$	0.00 $_{a}$	0.00 $_{a}$	0.00 $_{a}$	0.00 $_{a}$
T7	0.5	2.0	53.33 $_{abcd}$	0.67 $_{def}$	3.58 $_{e}$	25.00 $_{d}$	6.67 $_{abc}$	0.18 $_{bc}$	1.00 $_{bc}$	0.00 $_{a}$	0.00 $_{a}$	0.00 $_{a}$	0.00 $_{a}$	0.00 $_{a}$
T8	1.0	0.0	45.00 $_{cd}$	0.60 $_{ef}$	3.94 $_{e}$	43.33 $_{bc}$	0.00 $_{d}$	0.00 $_{c}$	0.00 $_{d}$	0.00 $_{a}$	0.00 $_{a}$	0.00 $_{a}$	0.00 $_{a}$	0.00 $_{a}$
T9	1.0	0.5	58.33 $_{abc}$	1.07 $_{c}$	4.67 $_{cd}$	25.00 $_{d}$	8.33 $_{abc}$	0.23 $_{bc}$	1.42 $_{bc}$	0.00 $_{a}$	0.00 $_{a}$	0.00 $_{a}$	0.00 $_{a}$	0.00 $_{a}$
T10	1.0	1.0	63.33 $_{ab}$	1.56 $_{b}$	5.98 $_{b}$	21.67 $_{d}$	8.33 $_{abc}$	0.26 $_{bc}$	1.58 $_{abc}$	0.00 $_{a}$	0.00 $_{a}$	0.00 $_{a}$	0.00 $_{a}$	0.00 $_{a}$
T11	1.0	2.0	61.67 $_{abc}$	1.43 $_{b}$	5.48 $_{bc}$	20.00 $_{d}$	6.67 $_{abc}$	0.18 $_{bc}$	1.08 $_{bc}$	0.00 $_{a}$	0.00 $_{a}$	0.00 $_{a}$	0.00 $_{a}$	0.00 $_{a}$
T12	2.0	0.0	56.67 $_{abc}$	0.71 $_{de}$	3.94 $_{de}$	41.67 $_{bc}$	0.00 $_{d}$	0.00 $_{c}$	0.00 $_{d}$	0.00 $_{a}$	0.00 $_{a}$	0.00 $_{a}$	0.00 $_{a}$	0.00 $_{a}$
T13	2.0	0.5	65.00 $_{a}$	1.44 $_{b}$	5.66 $_{b}$	23.33 $_{d}$	10.00 $_{ab}$	0.31 $_{b}$	1.83 $_{ab}$	0.00 $_{a}$	0.00 $_{a}$	0.00 $_{a}$	0.00 $_{a}$	0.00 $_{a}$
T14	2.0	1.0	68.33 $_{a}$	1.88 $_{a}$	6.96 $_{a}$	18.33 $_{d}$	13.33 $_{a}$	0.53 $_{a}$	2.58 $_{a}$	0.00 $_{a}$	0.00 $_{a}$	0.00 $_{a}$	0.00 $_{a}$	0.00 $_{a}$
T15	2.0	2.0	68.33 $_{a}$	1.57 $_{b}$	5.93 $_{ab}$	16.67 $_{d}$	11.67 $_{ab}$	0.40 $_{ab}$	2.17 $_{a}$	0.00 $_{a}$	0.00 $_{a}$	0.00 $_{a}$	0.00 $_{a}$	0.00 $_{a}$

* Values expressed as means of 12 replicated experiments, each replicate consisted of 5 cultures.

* Different letters within column (lowercase) indicate a significant difference ($p<0.05$) according to Duncan's multiple range test.

Table 9. Effect of kinetin and 2,4-D on the percentage of callus formation, callus fresh weight and callus expansion factor and percentage of browning of the leaf and stem explants incubated in the dark, 8 weeks after culture initiation.

Treatment code	Growth regulators (mg/l)		Types of explants											
			Leaf explants				Internodal explants				Nodal explants			
	2,4-D	KIN	% of callus formation	Estimated callus fresh weight (g)	Callus expansion factor (cm^2)	% of Browning	% of callus formation	Estimated callus fresh weight (g)	Callus expansion factor (cm^2)	% of Browning	% of callus formation	Estimated callus fresh weight (g)	Callus expansion factor (cm^2)	% of Browning
T0	0.0	0.0	0.00 $_{d}$	0.00 $_{h}$	0.00 $_{i}$	100.00 $_{a}$	0.00 $_{c}$	0.00 $_{c}$	0.00 $_{d}$	0.00 $_{a}$	0.00 $_{a}$	0.00 $_{a}$	0.00 $_{a}$	0.00 $_{a}$
T1	0.0	0.5	0.00 $_{d}$	0.00 $_{h}$	0.00 $_{i}$	100.00 $_{a}$	0.00 $_{c}$	0.00 $_{c}$	0.00 $_{d}$	0.00 $_{a}$	0.00 $_{a}$	0.00 $_{a}$	0.00 $_{a}$	0.00 $_{a}$
T2	0.0	1.0	0.00 $_{d}$	0.00 $_{h}$	0.00 $_{i}$	100.00 $_{a}$	0.00 $_{c}$	0.00 $_{c}$	0.00 $_{d}$	0.00 $_{a}$	0.00 $_{a}$	0.00 $_{a}$	0.00 $_{a}$	0.00 $_{a}$
T3	0.0	2.0	0.00 $_{d}$	0.00 $_{h}$	0.00 $_{i}$	100.00 $_{a}$	0.00 $_{c}$	0.00 $_{c}$	0.00 $_{d}$	0.00 $_{a}$	0.00 $_{a}$	0.00 $_{a}$	0.00 $_{a}$	0.00 $_{a}$
T4	0.5	0.0	51.67 $_{c}$	0.33 $_{gh}$	2.21 $_{hi}$	33.33 $_{b}$	0.00 $_{c}$	0.00 $_{c}$	0.00 $_{d}$	0.00 $_{a}$	0.00 $_{a}$	0.00 $_{a}$	0.00 $_{a}$	0.00 $_{a}$
T5	0.5	0.5	60.00 $_{bc}$	0.64 $_{fg}$	3.41 $_{gh}$	26.67 $_{bc}$	6.67 $_{bc}$	0.15 $_{c}$	0.75 $_{d}$	0.00 $_{a}$	0.00 $_{a}$	0.00 $_{a}$	0.00 $_{a}$	0.00 $_{a}$
T6	0.5	1.0	65.00 $_{abc}$	1.43 $_{e}$	5.35 $_{ef}$	20.00 $_{bcd}$	10.00 $_{ab}$	0.32 $_{b}$	1.83 $_{c}$	0.00 $_{a}$	0.00 $_{a}$	0.00 $_{a}$	0.00 $_{a}$	0.00 $_{a}$
T7	0.5	2.0	65.00 $_{abc}$	1.24 $_{e}$	4.78 $_{fg}$	15.00 $_{cd}$	8.33 $_{abc}$	0.21 $_{c}$	1.33 $_{c}$	0.00 $_{a}$	0.00 $_{a}$	0.00 $_{a}$	0.00 $_{a}$	0.00 $_{a}$
T8	1.0	0.0	65.00 $_{abc}$	1.06 $_{ef}$	4.52 $_{fg}$	26.67 $_{bc}$	8.33 $_{abc}$	0.26 $_{c}$	1.50 $_{c}$	0.00 $_{a}$	0.00 $_{a}$	0.00 $_{a}$	0.00 $_{a}$	0.00 $_{a}$
T9	1.0	0.5	70.00 $_{ab}$	2.96 $_{c}$	8.53 $_{c}$	20.00 $_{bcd}$	8.33 $_{abc}$	0.30 $_{b}$	1.58 $_{c}$	0.00 $_{a}$	0.00 $_{a}$	0.00 $_{a}$	0.00 $_{a}$	0.00 $_{a}$
T10	1.0	1.0	73.33 $_{ab}$	3.28 $_{b}$	10.80 $_{b}$	13.33 $_{cd}$	11.67 $_{ab}$	0.58 $_{b}$	2.58 $_{bc}$	0.00 $_{a}$	0.00 $_{a}$	0.00 $_{a}$	0.00 $_{a}$	0.00 $_{a}$
T11	1.0	2.0	66.67 $_{abc}$	2.19 $_{d}$	6.39 $_{de}$	13.33 $_{cd}$	8.50 $_{abc}$	0.39 $_{b}$	1.83 $_{c}$	0.00 $_{a}$	0.00 $_{a}$	0.00 $_{a}$	0.00 $_{a}$	0.00 $_{a}$
T12	2.0	0.0	68.33 $_{abc}$	1.29 $_{e}$	5.12 $_{ef}$	26.67 $_{bc}$	11.67 $_{ab}$	0.48 $_{b}$	2.33 $_{bc}$	0.00 $_{a}$	0.00 $_{a}$	0.00 $_{a}$	0.00 $_{a}$	0.00 $_{a}$
T13	2.0	0.5	75.00 $_{ab}$	3.39 $_{ab}$	11.63 $_{b}$	11.67 $_{d}$	15.00 $_{ab}$	1.25 $_{a}$	4.42 $_{b}$	0.00 $_{a}$	0.00 $_{a}$	0.00 $_{a}$	0.00 $_{a}$	0.00 $_{a}$
T14	2.0	1.0	78.33 $_{a}$	3.62 $_{a}$	12.88 $_{a}$	8.33 $_{d}$	18.33 $_{a}$	1.75 $_{a}$	6.58 $_{a}$	0.00 $_{a}$	0.00 $_{a}$	0.00 $_{a}$	0.00 $_{a}$	0.00 $_{a}$
T15	2.0	2.0	71.67 $_{ab}$	2.36 $_{d}$	6.51 $_{d}$	6.67 $_{d}$	10.00 $_{ab}$	0.42 $_{b}$	1.92 $_{c}$	0.00 $_{a}$	0.00 $_{a}$	0.00 $_{a}$	0.00 $_{a}$	0.00 $_{a}$

* Values expressed as means of 12 replicated experiments, each replicate consisted of 5 cultures.

* Different letters within column (lowercase) indicate a significant difference ($p<0.05$) according to Duncan's multiple range test.

Table 10. Total phenolic and total flavonoid contents in different crude extracts of sweet shoot based on the results obtained from Folin-Ciocalteu phenol assay and aluminium chloride colourimetric assay, respectively.

Types of crude extracts	Total phenolic content (mg GAE/100 g DW)		Total flavonoid content (mg RE/100 g DW)	
	Leaf	Stem	Leaf	Stem
Diethyl ether	7.91 ± 0.32 $_{e}$	5.34 ± 0.35 $_{c}$	56.34 ± 0.48 $_{b}$	32.15 ± 0.38 $_{b}$
Chloroform	12.84 ± 0.62 $_{d}$	9.58 ± 0.47 $_{b}$	35.78 ± 0.34 $_{c}$	18.56 ± 0.43 $_{c}$
Ethyl acetate	15.82 ± 0.56 $_{c}$	10.85 ± 0.59 $_{b}$	30.41 ± 0.37 $_{d}$	15.43 ± 0.28 $_{d}$
Ethanol	26.46 ± 0.31 $_{b}$	20.33 ± 0.67 $_{a}$	36.56 ± 0.45 $_{c}$	20.67 ± 0.32 $_{c}$
Methanol	38.12 ± 0.27 $_{a}$	18.08 ± 0.52 $_{a}$	62.72 ± 0.41 $_{a}$	41.81 ± 0.49 $_{a}$

* Values expressed as means of 3 replicated experiments.

* Different letters within column (lowercase) indicate a significant difference ($p<0.05$) according to Duncan's multiple range test.

Table 11. Growth yield of shadehouse-grown plants and *in vitro* shoot cultures of sweet shoot treated with methyl jasmonate, salicylic acid and phenylalanine, after 3 weeks of treatment.

Types of elicitors/ precursors	Dosages	Weight of plant material (g)					
		Shadehouse-grown plants			*In vitro* shoot cultures		
		week 1	week 2	week 3	week 1	week 2	week 3
Methyl jasmonate	0µM	16.49 $_{b}$	17.20 $_{b}$	18.67 $_{ab}$	16.75 $_{b}$	17.40 $_{b}$	19.56 $_{a}$
	50µM	17.08 $_{b}$	17.61 $_{b}$	18.99 $_{ab}$	17.41 $_{b}$	17.95 $_{b}$	19.67 $_{a}$
	100µM	17.40 $_{b}$	19.12 $_{a}$	19.53 $_{a}$	17.88 $_{b}$	18.26 $_{a}$	20.35 $_{a}$
	200µM	18.55 $_{ab}$	19.59 $_{a}$	19.55 $_{a}$	18.77 $_{a}$	19.68 $_{a}$	20.01 $_{a}$
Salicylic acid	0µM	16.32 $_{b}$	16.79 $_{b}$	17.13 $_{b}$	15.94 $_{b}$	16.79 $_{b}$	17.38 $_{b}$
	50µM	16.88 $_{b}$	16.95 $_{b}$	17.90 $_{a}$	16.54 $_{b}$	17.05 $_{b}$	18.61 $_{a}$
	100µM	17.28 $_{ab}$	17.87 $_{a}$	18.39 $_{a}$	17.09 $_{b}$	17.92 $_{b}$	19.13 $_{a}$
	200µM	17.56 $_{ab}$	18.34 $_{a}$	18.61 $_{a}$	17.77 $_{b}$	18.37 $_{a}$	19.45 $_{a}$
Phenylalanine	0 mg/l	17.23 $_{b}$	18.48 $_{b}$	19.27 $_{ab}$	17.30 $_{b}$	18.34 $_{b}$	19.79 $_{a}$
	5 mg/l	17.95 $_{b}$	18.63 $_{b}$	20.60 $_{ab}$	18.55 $_{b}$	19.22 $_{a}$	19.84 $_{a}$
	10 mg/l	18.67 $_{b}$	19.02 $_{ab}$	21.39 $_{a}$	19.06 $_{a}$	19.65 $_{a}$	20.15 $_{a}$
	20 mg/l	19.11 $_{ab}$	20.57 $_{ab}$	21.45 $_{a}$	19.83 $_{a}$	20.12 $_{a}$	20.42 $_{a}$

* Values expressed as means of five biological replicated experiments and each biological replicate consisted of three technical replicates.

* Different letters within row and column (lowercase) indicate a significant difference ($p<0.05$) according to Duncan's multiple range test.

Table 12. Comparison of total phenolic and total flavonoid contents in *in vitro* shoot cultures of sweet shoot cultured on MS medium supplemented with 2.0 mg/l BAP, 0.5 mg/l IAA and various concentration of methyl jasmonate, salicylic acid and phenylalanine, after 3 weeks of elicitor treatment.

Types of elicitors/precursors	Concentration	Total Phenolic Content (µg GAE/10 g FW)			Total Flavonoid Content (µg RE/10 g FW)		
		week 1	week 2	week 3	week 1	week 2	week 3
methyl jasmonate	0µM	35.72 $_{d}$	37.61 $_{d}$	49.03 $_{bc}$	116.33 $_{c}$	129.38 $_{c}$	156.13 $_{bc}$
	50µM	40.44 $_{cd}$	50.52 $_{bc}$	73.59 $_{a}$	130.87 $_{c}$	178.93 $_{ab}$	196.40 $_{a}$
	100µM	43.13 $_{cd}$	56.23 $_{b}$	62.33 $_{ab}$	154.20 $_{bc}$	182.27 $_{ab}$	169.55 $_{ab}$
	200µM	46.97 $_{bcd}$	60.41 $_{ab}$	56.93 $_{b}$	163.46 $_{ab}$	166.82 $_{ab}$	141.60 $_{bc}$
Salicylic acid	0µM	28.13 $_{c}$	33.69 $_{bc}$	42.16 $_{b}$	88.99 $_{c}$	109.33 $_{bc}$	129.49 $_{a}$
	50µM	33.59 $_{bc}$	34.21 $_{bc}$	40.41 $_{b}$	96.90 $_{c}$	115.71 $_{ab}$	123.07 $_{a}$
	100µM	35.51 $_{bc}$	37.82 $_{bc}$	42.19 $_{b}$	111.56 $_{bc}$	129.40 $_{a}$	131.71 $_{a}$
	200µM	38.93 $_{bc}$	40.26 $_{b}$	49.33 $_{a}$	117.04 $_{ab}$	120.28 $_{ab}$	116.74 $_{ab}$
Phenylalanine	0 mg/l	33.67 $_{g}$	40.27 $_{fg}$	49.06 $_{e}$	129.47 $_{f}$	142.71 $_{f}$	173.23 $_{e}$
	5 mg/l	40.27 $_{fg}$	52.35 $_{de}$	60.40 $_{c}$	143.55 $_{f}$	168.99 $_{e}$	238.89 $_{bc}$
	10 mg/l	43.83 $_{ef}$	56.83 $_{cd}$	90.45 $_{a}$	169.59 $_{e}$	190.27 $_{cde}$	330.90 $_{a}$
	20 mg/l	53.47 $_{de}$	67.00 $_{b}$	73.54 $_{b}$	213.03 $_{cd}$	201.30 $_{cd}$	273.71 $_{b}$

* Values for total phenolic content expressed as µg gallic acid equivalent (GAE) per 10 g fresh weight samples (FW).
* Values for total flavonoid content expressed as µg rutin equivalent (RE) per 10 g fresh weight samples (FW).
* Values expressed as means of five biological replicated experiments and each biological replicate consisted of three technical replicates.
* Different letters within row and column (lowercase) indicate a significant difference ($p<0.05$) according to Duncan's multiple range test.

Table 13. Phytochemical screening of shadehouse-grown plants of sweet shoot treated with different concentration of phenylalanine at a given period of 3 weeks.

Phytochemical tests	Elicitation treatment											
	Week1				Week 2				Week 3			
	0 mg/l	5 mg/l	10 mg/l	20 mg/l	0 mg/l	5 mg/l	10 mg/l	20 mg/l	0 mg/l	5 mg/l	10 mg/l	20 mg/l
Wagner's test	-	+	+	+	-	+	+	+	-	+	+	+
Hager's test	-	+	+	+	-	+	+	+	-	+	+	+
Dragendorff's test	-	+	+	+	-	+	+	+	-	+	+	+
Determination of phenolics												
Ferric chloride test	+	+	+	+	+	+	+	+	+	+	+	+
Gelatin test	+	+	+	+	+	+	+	+	+	+	+	+
Lead acetate test	+	+	+	+	+	+	+	+	+	+	+	+
Determination of flavonoids												
Sodium hydroxide test	+	+	+	+	+	+	+	+	+	+	+	+
Shinoda's test	+	+	+	+	+	+	+	+	+	+	+	+
Sulphuric acid test	+	+	+	+	+	+	+	+	+	+	+	+
Determination of glycosides												
Keller-Kiliani test	+	+	+	+	+	+	+	+	+	+	+	+
Determination of saponins												
Frothing test	+	+	+	+	+	+	+	+	+	+	+	+
Determination of sterol and steroids												
Salkowski test	+	+	+	+	+	+	+	+	+	+	+	+

Table 14. Phytochemical screening of *in vitro* shoot cultures of sweet shoot treated with different concentration of phenylalanine at a given period of 3 weeks.

Phytochemical tests	**Elicitation treatment**											
	Week1				**Week 2**				**Week 3**			
	0 mg/l	**5 mg/l**	**10 mg/l**	**20 mg/l**	**0 mg/l**	**5 mg/l**	**10 mg/l**	**20 mg/l**	**0 mg/l**	**5 mg/l**	**10 mg/l**	**20 mg/l**
Wagner's test	-	+	+	+	-	+	+	+	-	+	+	+
Hager's test	-	+	+	+	-	+	+	+	-	+	+	+
Dragendorff's test	-	+	+	+	-	+	+	+	-	+	+	+
Determination of phenolics												
Ferric chloride test	-	+	+	+	+	+	+	+	+	+	+	+
Gelatin test	-	+	+	+	+	+	+	+	+	+	+	+
Lead acetate test	-	+	+	+	+	+	+	+	+	+	+	+
Determination of flavonoids												
Sodium hydroxide test	-	+	+	+	+	+	+	+	+	+	+	+
Shinoda's test	-	+	+	+	+	+	+	+	+	+	+	+
Sulphuric acid test	-	+	+	+	+	+	+	+	+	+	+	+
Determination of glycosides												
Keller-Kiliani test	-	+	+	+	-	+	+	+	+	+	+	+
Determination of saponins												
Frothing test	-	+	+	+	-	+	+	+	+	+	+	+
Determination of sterol and steroids												
Salkowski test	-	+	+	+	-	+	+	+	+	+	+	+

Table 15. Phytochemical screening of somatic embryos of sweet shoot treated with different concentration of phenylalanine at a given period of 3 weeks.

Phytochemical tests	Phenylalanine treatment											
	Week1				Week 2				Week 3			
	0 mg/l	5 mg/l	10 mg/l	20 mg/l	0 mg/l	5 mg/l	10 mg/l	20 mg/l	0 mg/l	5 mg/l	10 mg/l	20 mg/l
Wagner's test	-	+	+	+	-	+	+	+	-	+	+	+
Hager's test	-	+	+	+	-	+	+	+	-	+	+	+
Dragendorff's test	-	+	+	+	-	+	+	+	-	+	+	+
Determination of phenolics												
Ferric chloride test	-	+	+	+	+	+	+	+	+	+	+	+
Gelatin test	-	+	+	+	+	+	+	+	+	+	+	+
Lead acetate test	-	+	+	+	+	+	+	+	+	+	+	+
Determination of flavonoids												
Sodium hydroxide test	-	+	+	+	+	+	+	+	+	+	+	+
Shinoda's test	-	+	+	+	+	+	+	+	+	+	+	+
Sulphuric acid test	-	+	+	+	+	+	+	+	+	+	+	+
Determination of glycosides												
Keller-Kiliani test	-	+	+	+	-	+	+	+	+	+	+	+
Determination of saponins												
Frothing test	-	+	+	+	-	+	+	+	+	+	+	+
Determination of sterol and steroids												
Salkowski test	-	+	+	+	-	+	+	+	+	+	+	+

Table 16. Comparison of PAL and CHS enzymatic activities in shadehouse-grown plants and tissue cultures of sweet shoot treated with methyl jasmonate, salicylic acid and phenylalanine, after 3 weeks of elicitor treatment.

Types of explants	Penylalanine concentration	PAL activity (mmol CA/g FW)			CHS activity (nkat/mg protein)		
		week 1	week 2	week 3	week 1	week 2	week 3
Shadehouse-grown plants	**0 mg/l**	22.20 $_{d}$	32.86 $_{d}$	40.72 $_{bcd}$	2.43 $_{c}$	3.67 $_{c}$	6.70 $_{abc}$
	5 mg/l	29.16 $_{d}$	37.84 $_{cd}$	48.10 $_{bc}$	3.72 $_{c}$	4.59 $_{c}$	6.28 $_{bc}$
	10 mg/l	40.88 $_{bcd}$	48.14 $_{bc}$	56.76 $_{ab}$	4.26 $_{c}$	6.32 $_{bc}$	8.56 $_{ab}$
	20 mg/l	51.02 $_{bc}$	60.70 $_{ab}$	71.10 $_{a}$	5.87 $_{bc}$	7.48 $_{ab}$	10.13 $_{a}$
***In vitro* shoot cultures**	**0 mg/l**	16.98 $_{f}$	27.86 $_{e}$	30.48 $_{e}$	2.14 $_{d}$	3.45 $_{d}$	4.53 $_{cd}$
	5 mg/l	32.28 $_{e}$	44.14 $_{d}$	51.92 $_{cd}$	2.84 $_{d}$	3.32 $_{d}$	7.30 $_{bc}$
	10 mg/l	49.14 $_{cd}$	60.30 $_{bc}$	80.10 $_{a}$	4.52 $_{cd}$	5.40 $_{cd}$	12.08 $_{a}$
	20 mg/l	56.46 $_{bcd}$	66.44 $_{bc}$	72.82 $_{b}$	6.99 $_{bcd}$	7.31 $_{bc}$	8.29 $_{b}$
Dark-induced callus cultures	**0 mg/l**	10.78 $_{d}$	14.22 $_{d}$	18.14 $_{cd}$	1.42 $_{c}$	2.04 $_{c}$	3.48 $_{c}$
	5 mg/l	12.28 $_{d}$	18.04 $_{cd}$	26.90 $_{bc}$	2.16 $_{c}$	3.22 $_{c}$	4.47 $_{b}$
	10 mg/l	17.26 $_{cd}$	25.86 $_{bc}$	39.14 $_{a}$	3.79 $_{c}$	4.61 $_{b}$	6.29 $_{a}$
	20 mg/l	24.60 $_{bc}$	29.04 $_{bc}$	31.76 $_{b}$	4.51 $_{b}$	5.21 $_{ab}$	6.54 $_{a}$
Somatic embryo cultures	**0 mg/l**	12.80 $_{d}$	17.96 $_{d}$	20.68 $_{d}$	1.87 $_{c}$	2.59 $_{c}$	4.12 $_{bc}$
	5 mg/l	16.14 $_{d}$	25.22 $_{cd}$	31.02 $_{cd}$	2.34 $_{c}$	3.77 $_{c}$	4.30 $_{bc}$
	10 mg/l	27.92 $_{cd}$	37.94 $_{bc}$	40.94 $_{bc}$	3.58 $_{c}$	5.82 $_{bc}$	6.11 $_{ab}$
	20 mg/l	40.96 $_{bc}$	46.66 $_{b}$	57.16 $_{a}$	4.99 $_{bc}$	6.34 $_{ab}$	8.24 $_{a}$

* Values for PAL assay expressed as mmol of cinnamic acid (CA) equivalent per g fresh weight samples (FW).
* Values for CHS assay expressed as nanokatal (nkat) per mg protein.
* Values expressed as means of five biological replicated experiments and each biological replicate consisted of three technical replicates.
* Different letters within row and column (lowercase) indicate a significant difference ($p<0.05$) according to Duncan's multiple range test.