EMBRYOGENIC CALLUS CULTURE OF *PERESKIA GRANDIFOLIA* AND *PERESKIA BLEO* AND DETERMINATION OF THEIR CYTOTOXICITY EFFECT

by

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LIST OF ABBREVIATION

MS	Murashige-Skoog
B5	Gamborg B5
BA	6-benzylaminopurine
NAA	1-napthaleneacetic Acid
IBA	3-indole butyric Acid
IAA	3-indole acetic Acid
ABA	Abscisic acid
2,4-D	2,4-dichlorophenoxyacetic acid
Picloram	4,amino-3,5,6-trichloropicolinic Acid
TDZ	Thidiazuron
2-iP	2-isopentenyladenine
PGR	Plant Growth Regulator
SE	Somatic embryo
NaOCl	Sodium Hypochloride
$HgCl_2$	Mercury Chloride
v/v	Volume per Volume
w/v	Weight per Volume
NaOH	Natrium hydroxide
HCl	Hydrochloric acid
ANOVA	Analysis of Varians
EC	Effective Concentration
s.e.	Standard error

ATCC	The American Type Cell Collection
NCI-H23	Lung cancer cell line
Caov-3	Ovarian cancer cell line
T-47D	Breast cancer cell line
$HgCl_2$	Mercuric chloride
NaDCC	Sodium dichloroisocyanurate
MBA	Methylene Blue Assays

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PENGKULTURAN KALUS EMBRIOGENIK *PERESKIA GRANDIFOLIA* DAN *PERESKIA BLEO DAN* PENENTUAN KESAN KESITOTOKSIKANNYA

ABSTRAK

Eksplan daun aseptik tumbuhan *Pereskia grandifolia* dan *Pereskia bleo* dapat diperolehi melalui proses pensterilan permukaan dengan 150 mg/L sodium dikloroisosainurat (NaDCC) selama 15 minit. Hanya kalus embriogenik daripada *P. grandifolia* dapat diinduksi daripada urat tengah daun aseptik selepas dikulturkan dalam medium Gamborg (B5) yang ditambah dengan 6.5 mg/L 2,4-diklorofenoksi asid asetik (2,4-D) selama empat minggu. Kalus embriogenik *P. grandifolia* dapat diperbanyakkan dengan pengsubkulturan setiap empat minggu dengan jisim kalus sebanyak 0.5 g pada setiap 20 ml media pepejal yang sama kandungannya. Sel-sel embriogenik *P. grandifolia* dapat dipropagasi melalui pengkulturan kalus embriogenik yang rapuh dalam media cecair B5 yang ditambahkan 6.5 ml/L 2,4-D. Pengsubkulturan sel embriogenik tersebut dilakukan setiap 12 hari. Eksplan daun *P. bleo* tidak menghasilkan sebarang kalus embriogenik apabila dikulturkan dalam media yang sama formulasi. Media B5 yang mengandungi BA dan NAA (2 – 10 mg/L) merangsangkan pembentukan struktur filamen hijau (GFS) daripada eksplan daun bagi kedua-dua spesis *Pereskia*, *P. grandifolia* dan *P. bleo*. Kajian mikroskopi menunjukkan GFS terdiri daripada rantaian sel-sel yang panjang dan mengandungi granul klorofil dalam setiap sel. Ekstrak heksana pokok induk *P. grandifolia* dan *P. bleo* menunjukkan ciri kesitotoksikan dengan nilai EC_{50} 5.92 μg/ml (76.0 %) dan 15.95 μg/ml (80.7 %) masing-masing terhadap sel kanser paru-paru (NCI-H23). Ekstrak heksana bagi sel-sel embriogenik *in*

vitro juga menunjukkan aktiviti anti-kanser terhadap sel-sel kanser paru-paru tetapi tahap keberkesanannya adalah kurang daripada 50 % dan nilai EC_{50} tidak dapat ditentukan. Ekstrak heksana pokok induk bagi kedua-dua spesis *Pereskia* juga berupaya menghalang pertumbuhan sel-sel kanser ovari (Caov-3) tetapi kesan kesitotoksikannya adalah lebih rendah berbanding dengan kesan terhadap NCI-H23. Ekstrak klorofom dan butanol bagi pokok induk *P. grandifolia* dan *P. bleo* dan kalus embriogenik mempunyai kesan kesitotoksikan yang rendah terhadap kedua-dua sel kanser paru-paru dan ovari. Walaubagaimana pun, GFS tidak menunjukkan kesan kesitotoksikan terhadap kedua-dua jenis sel kanser.

EMBRYOGENIC CALLUS CULTURE OF *PERESKIA GRANDIFOLIA* AND *PERESKIA BLEO* AND DETERMINATION OF THEIR CYTOTOXICITY EFFECT

ABSTRACT

Aseptic leaf explants of *Pereskia grandifolia* and *Pereskia bleo* could be established via surface-sterilization process using 150 mg/L sodium dichloroisocyanurate (NaDCC) for 15 minutes. Only embryogenic callus could be induced from *P. grandifolia* mid-vein leaf explants after culturing on Gamborg (B5) medium supplemented with 6.5 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D) for four weeks. The embryogenic callus of *P. grandifolia* could be multiplied by subculturing every four weeks 0.5 g callus biomass on 20 ml of solid media with the same constituents. Embryogenic cells of *P. grandifolia* could be propagated by culturing the friable embryogenic callus in liquid B5 medium supplemented with 6.5 mg/L 2,4-D. Subculturing of embryogenic cells was carried out every 12 days. Leaf explants of *P. bleo* did not produced any embryogenic callus when they were culture on culture medium with the same formulation. B5 medium containing BA and NAA (2 – 10 mg/L) induced the formation of green filamentous structure (GFS) from the leaf explants of both *Pereskia* species, *P. grandifolia* and *P. bleo*. Microscopy study showed that GFS was made up of chains of long cells and contained chlorophyll granules in each cell. Hexane extracts of *P. grandifolia* and *P. bleo* mother plants showed cytotoxicity effect with EC_{50} values of 5.92 μg/ml (76.0 %) and 15.95 μg/ml (80.7 %) respectively towards lung cancer cells (NCI-H23). Hexane extract of *in vitro* embryogenic cells also showed anti-cancer activities towards lung cancer cells but level of effectiveness was less than 50 % and the EC_{50} value

could not be determined. The hexane extracts of the mother plants of both *Pereskia* species also showed ability to inhibit the growth of ovarian cancer cells (Caov-3) but its cytotoxicity effect was lower as compared to the effect towards NCI-H23 cells. The chloroform and butanol extracts for both the mother plants of *P. grandifolia* and *P. bleo* and the embryogenic calluses possessed low cytotoxicity effects towards both the lung and ovarian cancer cells. However, the GFS did not showed any cytotoxicity effect towards both of the cancer cells.

CHAPTER ONE INTRODUCTION

Succulent plants and cacti have fascinated human beings since time immemorial. There are many species of cacti with different adaptations and phenotypes. Some grow upright while others creep and cascade. Some have hairy or cylindrical spiny columns, some with flat jointed pads while others are globular in shape. The succulent plants and cacti are grown for their flowers while others for their shape or foliage effect. Their unique characteristic and shapes together with different combinations of colour have attracted much interest to the plant lovers (Lamb, 1995). *Selenicereus macdonaldiae* (*Cerues* of the moon) was an example of fascinating cactus that blooms at night with large beautiful flowers approximately 34 cm in length. This accounted for the popular name 'queen of the night' and it only blooms on mature plant (Grantham and Klaassen, 1999). There are other succulent plants and cacti, instead of producing beautiful flowers, they possess unique morphology or fascinating variety of leaf colour such as *Lithops* spp. and *Echeveria* spp..

The other unique feature of the succulents and cacti was their ability to adapt to the hot and low humidity environment compared to other ornamental plants (Jacobsen, 1960). They can grow in a wide range of temperatures and can even survive only exposing to dew at night. There are groups of cacti like *Lithops formosa* and *Lithops ferox* that grow at altitude more than 4,000 metres (13,000 ft) above sea level in the mountains of Oruro, Bolivia. Cactus like *Rebutia fiebrigii* grows well in shady clefts in the rocks and in bed of mosses, liverworts, club mosses and small ferns (Preston-Mafham, 1994). Although most cacti have their natural habitat in warm semi-desert area, some cacti grow as epiphytes on trees in the tropical forest of America. *Schlumbergera truncate*

and Rhipsalidopsis cacti are epiphytic and prefer indirect sunlight and high humidity environment for active growth (Lamb, 1995).

Besides as an ornamental plant, the succulent plants and cacti have also been used by certain native population as source of food and water. While others used them as raw materials for a range of useful items such as scouring pads to rope, medicine for ailments and poisons for hunting. Leaves of *Mesembrayabthemums* spp. in South Africa and *Fockea damarana* roots in Heroroland have been widely taken by both human and animals as water source. *Euphobias hamata* is fed to the male bull in Africa as energy food so that the animals can work continuously in the field (Jacobsen, 1960; Grantham and Klaassen, 1999).

Some of the succulents and cacti were found to have special medicinal values. The natives of American Church use peyote as medicine for the treatment of angina, arthritis, backache, burns, fever, paralysis, snakebite and headache. Peyote is prepared from the dried tops or the whole plant of *Lophophora williamsii*, a small cactus native to Mexico and southern Texas. *Selenicereus grandiflorus* has been used as traditional herb for anaemia, angina pectoris, bladder inflammation, depression, endocarditis, prostate disease and mycocarditis (Fetrow and Avila, 2000). Four *Opuntia* fruit varieties were found to contain high conjugated flavonoids (quercetin, kaempferol and isorhamnetin), ascorbic acid and carotenoids that can be used as a rich source of natural antioxidants for preparation of health foods (Kuti, 2004).

Pereskia spp. are recently widely used as traditional medicine for cancer treatment. The natives of Brazil crush the leaves of *Pereskia* spp. and apply as poultice for tumour patients (Hartwell, 1982). *P. grandifolia* also

known as 'Rose of Burma' has been used by the Malaysian Chinese as traditional medicine by drinking the leaf extract for the prevention and treatment of uterine cancer and fibroids. The leave extracts of *P. grandifolia* was also found to inhibit the cell growth of NCI-H23 (human non-small lung adenocarcinoma) and Caov-3 (human ovarian carcinoma) cell lines (Ooi *et al.*, 2003). *P. bleo* was found to effectively inhibit the growth of breast carcinoma cell line (T-47D) with EC_{50} of 2.0 µg/mL (Tan *et al.,* 2004). National Cancer Registry of Malaysia recorded a total of 40,000 cancer patients in 2003. The number of cancer patients was reported to increase drastically especially in Malaysia. This is equivalent to one person in every 5 Malaysians will suffer from various type of cancer. This incidence may be one of the factors that make people seek for alternative medicine for treatment. *P. bleo* and *P. grandifolia* are among the numerous plants that are well sought after especially among the Malaysian Chinese for prevention and treatment of cancer.

Pereskia spp. belongs to the family Cactaceae and subfamily Pereskioideae (Watson and Dallwitz, 1992). It was originated from central and South America countries like Mexico, Colombia and Panama. *Pereskia* spp. are unique and different from others in the Cactaceae family because of having semi-woody stems with leaves like other dicotyledonous plants (Lancaster, 1972). They also produce spines at the areoles like other plants in the Cactaceae family such as the *Opuntia* spp. (Britton and Rose, 1963).

The conventional propagation method of *Pereskia* spp. is beset with problems of scanty and delayed rooting of seedlings and vegetative cuttings. This approach can hardly fulfill the increasing demand of the local market due to their medicinal values. *In vitro* culture techniques could then be the

alternative method for the mass production of plant materials for the preparation of herbal products. Tissue culture techniques have long been used as methods for production of raw plant materials for the preparation of traditional medicine. In 1983, MITSUI chemical Industry Ltd (Japan) had successfully produced huge quantity of shikonin from *Lithospermum erythrorhizon* in a 750 L bioreactor using cell culture technique (Endress, 1994). Mass production using *in vitro* culture method was found to be effective in reducing cost and time of production. Therefore, the objective of this study was to establish a protocol for induction of embryogenic calluses from *Pereskia* spp. and evaluated the effectiveness of both *in vivo* and *in vitro* materials toward cytotoxicity activity on the human carcinoma cell lines.

The production of somatic embryogenic calluses were fundamentally more efficient than root and shoot induction. The process is usually less elaborate and less diversity of culture medium is required (Endress, 1994). Until today, the induction of somatic embryogenic tissue has not been reported for any of the *Pereskia* spp.. Therefore the objectives of this study are:

a) To establish a surface sterilization protocol for obtaining aseptic cultures of *P. grandifolia* and *P. bleo*

b) To establish an optimum medium for induction of embryogenic tissues/cells

c) To screen the cyctotoxic activities for both the mother plants and the embryogenic tissues of *P. grandifolia* and *P. bleo*

CHAPTER TWO LITERATURE REVIEW

2.1 The Cactaceae

2.1.1 Distribution and habitat

The Cactaceae family can be categorized into two groups, the leafy and the non-leafy cacti. *Pereskia* and *Maihuenia* are two of the genuses that belong to the leafy-cacti while *Opuntia* and *Tephrocactus* genuses belong to the non-leafy cacti. The genus *Pereskia* belongs to the subfamily Pereskioideae (Watson and Dallwitz, 1992). This genus sometimes is named as *Peirescia*, *Peireskia* and *Perescia*. *Pereskia* spp., a type of succulent shrub or tree, grow well in high humidity areas away from direct sunlight. These plants have the characteristic of a woody dicotyledonous plant with abundant leaves and branches. Some of the *Pereskia* spp. are creepers with hooks for attachment (Rendle, 1967). However, the primary characteristic of a cactus like areoles, are covered with long spines is found in this genus.

Pereskia spp. are endemic plants of Brazil, Paraguay, Uruguay, Argentina, North Venezuela, North and West Colombia, certain parts of Florida, and Panama (Grantham and Klaassen, 1999). *Pereskia grandifolia* for example is originated from Brazil especially from Espirito Santo forest, Minas Gerais and Mexico (Faucon, 2004). Meanwhile, *Pereskia bleo* is found in Badilas Colombia area, northwest of USA region and the entire Panama. Besides *P. grandifolia* and *P. bleo*, the genus *Pereskia* is also consisted of many other species such as *P. aculcata, P. nicoyana, P. humboldtii* and *P. bahiensis* (Britton and Rose, 1963).

Maihuenia and *Pereskiopsis* are also leafy cacti. Genus *Maihuenia* belongs to the subfamily Pereskioideae. It grows widely in Agentina and Chile.

Its name was originated from the word 'Maihue' which meant 'women' in the language of Mapuche tribe. This genus only consists of two species, *M. poeppigii* and *M. patagonica.* They can only be located in high humidity environment with an average rainfall of 25 cm per year (Grantham and Klaassen, 1999). *Pereskiopsis* is another genus that is considered as a close relative of *Pereskia* because of the woody stem characteristic. However, this genus was placed under the Opuntioideae subfamily because it produced glochids on the stems and white hard seeds like other cacti in the genus *Opuntia* (Britton and Rose, 1963).

2.1.2 Morphology of *Pereskia bleo* and *Pereskia grandifolia*

The genus *Pereskia* consists of species with woody stems and abundant leaves. However, each species has its specific characteristic with different leaf, flower and fruit morphology. Each species produces spines of different size, number and arrangement. The different morphology and habitat of each *Pereskia* spp. can hence be differentiated from each other.

P. bleo, also known as *P. corrugate*, can be grown successfully in shaded areas with a minimum temperature of 10°C. It is commonly propagated via seeds in spring and grafting in mid summer. Meanwhile, *P. grandifolia* has lower temperature tolerance than *P. bleo.* It can survive well at -3°C and prefers shaded areas rather than direct sunlight. It is usually propagated via seeds and grafting through out the whole year (Faucon, 2004).

In the wild, *P. bleo* can grow up to 7 meters in height with the stem diameter of approximately 10 cm. Old branches of *P. bleo* are usually without spines compare to leafy young shoots with stems that are green-reddish in

colour. It bears red orange flowers **(Fig. 2.1A)**. It produces oblanceolate leaves with acuminate apex and cuneat shaped base. Each leaf is 12.9 ± 0.5 cm in length with a petiole of 1.5 ± 0.2 cm. Each axillary bud is covered with seven spines and each spine is about 1.3 ± 0.1 cm in length (Tang, 2002).

P. grandifolia usually can grow up to 2 – 5 meters high with spiny woody trunk about 10 cm in diameter (Britton and Rose, 1963). It produces spines of approximately 3.2 ± 0.2 cm in length. Even though *P. grandifolia* often found to bear pinkish flowers **(Fig. 2.1B)**, however each branch only produces flowers once it has reached a height of around 60 cm (24 inch). Flowering occurs throughout late summer to early autumn (Grantham and Klaassen, 1999). There are five spines around each axillary bud. Its leaves are oblong with mucronate apex and cuneat shaped base. Each leaf is about 12.3 ± 0.5 cm in length with 0.7 ± 0.03 cm petiole. Both of the *Pereskia* spp. bear fruits of different shape. *P. bleo* bears corn shape fruits with pentagon top **(Fig. 2.2A)** while *P. grandifolia* produces oval shape fruits **(Fig. 2.2B)** (Tang, 2002).

2.1.3 Medicinal value of *Pereskia bleo* and *Pereskia grandifolia*

The extract of *P. bleo* was found to have detoxifying effect and improved the blood circulation. It was used for the treatment of skin rashes and uterine cancer (Foong, 2003). Gupta *et al.* (1996) reported that methanol extract of whole plants of *P. bleo* was found to have cytotoxicity effect with LC_{50} less than 100 ppm for the inhibition of gall tumor. Besides, the methanol extract of *P. bleo* was found to inhibit the growth of human carcinoma breast cancer cell line (T-47D) with EC_{50} of 1.90 ± 0.3 µg/mL and 3.0 µg/mL of the methanol extract was sufficient to inhibit 90% growth of T-47D cell line (Tan *et al.*, 2004).

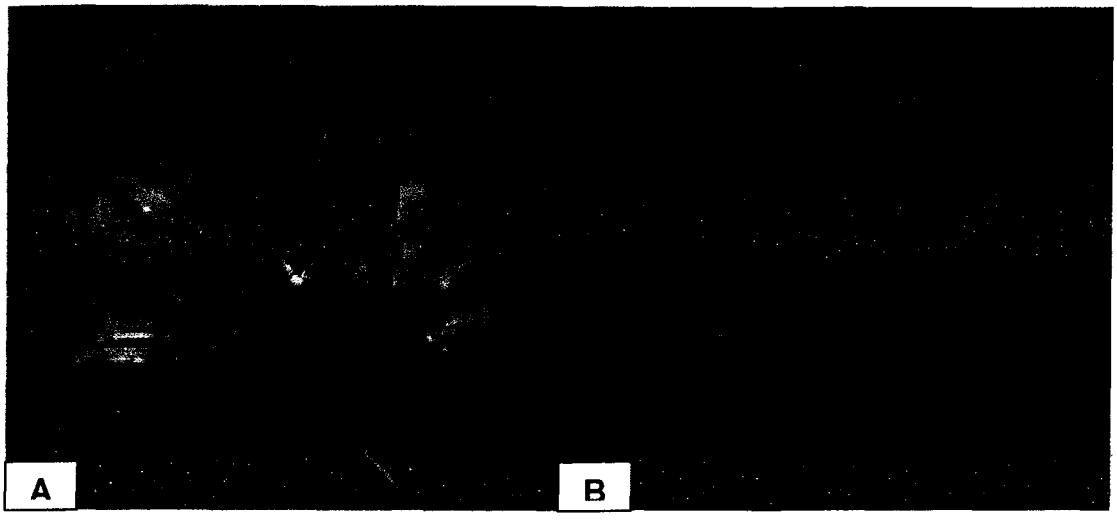

Figure 2.1 Flower of *Pereskia* spp. **A** *Pereskia bleo;* **B** *Pereskia grandifolia*

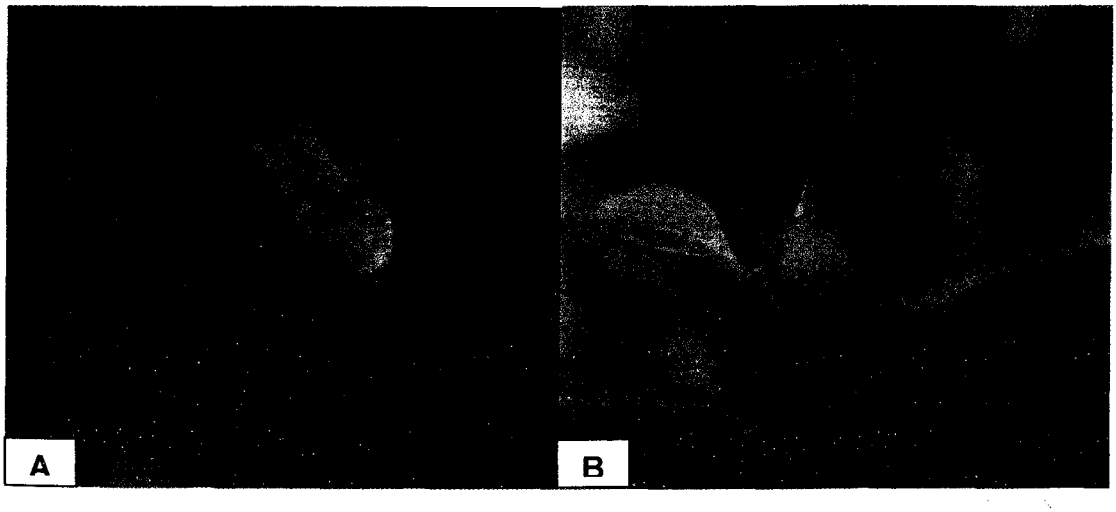

Figure 2.2 Fruit of *Pereskia* spp. **A** *Pereskia bleo;* **B** *Pereskia grandifolia*

The leaf extract of *P. grandifolia* was also found to have anti-tumour effect. The Malaysian Chinese have used the plant extract for the treatment of breast cancer and uterine cancer. The hexane and chloroform extract of *P. grandifolia* leaves showed inhibition on ovarian cancer cell line (Caov-3) and lung cancer cell line (NCI-H23) with EC_{50} less than 30 µg/mL but they were not effective towards breast cancer cell line (T-47D). The butanol extract of *P. grandifolia* was not effective for all the tested cell lines (Ooi *et al.*, 2003).

2.2 *In vitro* culture techniques for production of plant material

2.2.1 Micropropagation

In vitro propagation technique has been used for decades in the mass production of plantlets. For sustainable production of high quality and uniform plant material, micropropagation technique has often been used as an invaluable tool in the big scale production of plantlets. For example, Arinaitwe *et al.* (2000) had successfully micropropagated *Musa* cultivars such as Ndiziwemiti, Kibuzi and Bwara by using modified Murashige and Skoog (MS) medium supplemented with 0.4 mg/L thiamin HCL, 0.5 mg/L nicotinic acid, 0.5 mg/L pyridoxine HCL, 100 mg/L myo-inositol, ascorbic and different type of cytokinins. It was reported that more multiple shoots were produced for the Bwara cultivar when the amount of 6-benzylaminopurine (BA) supplemented into the modified MS medium was increased from 16.8 to 28.8 µM. They also reported that by increasing the kinetin content (16.8 to 24.8 µM) in the modified MS medium could induce more shoots for the Kibuzi cultivar. Ndiziwemiti cultivar produced 10 shoots per shoot apex when cultured on modified MS

medium supplemented with 6.81 µM thidiazuron (TDZ). Their established protocol resulted in successful mass propagation of bananas.

Micropropagation method can also be used to obtain pure breed ornamental plants. Sigh and Syamal (2001) have successfully micropropagated true-to-type hybrids and induced multiple shoots for tea roses (*Rosa hybrida*), Sonia and Raktagandha. They were established from the nodal segments of Sonia and Raktagandha on MS medium supplemented with 16.56 µM BA and 2.69 µM 1-naphthaleneacetic acid (NAA). The results showed that cultivar Sonia produced highest shoots (17 shoots per explant) by dipping the explants in 100 µM TDZ followed by cultivar Raktagandha (15 shoots per explant) by dipping the explants in 50 µM TDZ. Jones *et al.* (1996) successfully micropropagated a group of non-mutated, uniform in height and trunk girth birch tree *(Betula pendula*) from the nodal segments via somatic embryogenesis technique. The conventional plant breeding and propagation methods are difficult to perform on this woody plant once they are matured (6 years in field). Micropropagation technique was also applied for the production of uniform true-to-type papaya seedlings. Chan and Teo (2002) had successfully produced uniform true to type high quality *Carica papaya* cv Eksotika clone using axillary buds obtained from good quality mature mother plants.

Micropropagation technique was also widely used to study plant regeneration behaviour, production of metabolite or even the effect of different culture ingredients on callus induction. Nhut *et al.* (2001) studied the plant regeneration behavior of *Lilium longiflorum* using the flower stalk, receptacle, folded inner petal, outer petal sheath, ovary, stigma, stamens and anthers of

closed flower buds as explants. It was showed that the receptacle tissues (5 mm) had the highest shoots regeneration with approximately 17 shoots produced from each explant when cultured on MS medium supplemented with 5.4 µM NAA and 2.2 µM BA while the other explants did not survived. They also reported that the best receptacle size was 3 mm which could produce 41 surviving shoots when cultured on to the same medium. Tang *et al.* (2006) had also used micropropagation method to study the influence of different basal medium supplemented with 2,4-dichlorophenoxyacetic acid (2,4-D) (15 µM), NAA (12 µM) and 2-isopentenyladenine (2iP) (6 µM) on induction of embryogenic callus of slash pine (*Pinus elliottii*) from mature seeds for reforestation project and timber plantation.

Micropropagation method was also applicable in preserving some endangered and valuable medicinal plant from extinction. As an example, the roots of *Curculigo orchioides* (Hypoxidaceae) are widely used as tonic at south-east India for strength, vigour and vitality due to the presence of flavanone glycoside-I, three steroids, six triterpenoids, three saponins and other metabolites. Most of the species are collected from the forest without proper replanting and has endangered this plant. Therefore, Suri *et al.* (1999) had developed a rapid multiplication method for *Curculigo orchioides* (Hypoxidaceae) through direct organogenesis and shoot multiplication using leaf explants to provide alternative source for consumer to extract the alkaloid compounds without harvesting it from the forest. Figueiredo *et al.* (2001) also applied the micropropagation method for *Rollinia mucosa,* a medicinal herbs using epicotyl and hypocotyl as explants to induce multiple shoots that could be used as alternative sources for phytochemical extraction purposes.

Various types of cacti have been propagated via *in vitro* culture technique. Guisti *et al.* (2002) had successfully micropropagated three endangered cactus species, *Escobaria minima* (Baird), *Mammillaria pectinifera* (Ruempler) F.A.C. Weber and *Pelecyphora aselliformis* Ehrenbery using shoot tip as the explants. The shoot explants were cultured on MS medium supplemented with NAA, TDZ, BA and kinetin. Their results showed that the best shoot tip proliferation medium for *E. minima* and *M. pectinifera* was MS medium supplemented with 0.05 µM NAA and 22.2 µM BA. Meanwhile *Pelecyphora aselliformis* had the highest shoot proliferation on MS medium supplemented with 0.05 µM NAA and 23.23 µM kinetin. Tissue culture techniques had been used as propagation tool especially for the plant species that are difficult to obtain seeds due to incompatibility, low seed germination rate and slow growing seedlings. Martínez-Vázquez and Rubluo (1989) used *in vitro* technique to mass propagate the near extinct *Mammillaria san-angelensis* Sánchez-Mejorada. The near extinction was due to over harvesting to fulfil the market demand. Mass regeneration of *M. san-angelensis* was most successful when the lateral shoots of the seedlings were maintained on MS basal medium supplemented with 0.1 mg/L BA; MS + 0.1 mg/L BA + 0.01 mg/L NAA or MS + 1.0 mg/L BA. *In vitro* micro grafting had been carried out on selected species of prickly pear cactus (*Opuntia* spp.). The cultures were maintained on MS medium supplemented with 2.5 mg/L BA and 5 mg/L N^6-[Δ^2-isopentanyl] adenine (Estrada-Luna *et al.,* 2001).

2.2.2 Somatic embryogenesis

Somatic embryogenesis technique has been widely recognized as an alternative method of micropropagation. Somatic embryogenesis is a process in which a bipolar structure, resembling zygotic embryo, develops from a non-zygotic cell without vascular connection with the original tissue (Vajrabhaya, 1988). It has become an important tool for the study and analysis of molecular and biochemical processes. It has also been used to establish pathogen-free planting stock, genetically improved crops, and preservation of germplasm. Santacruz-Ruvalcaha *et al.* (2004) reported that somatic embryos were successfully induced from the callus and cell suspension cultures of some cacti and succulent plants like *Aztekium ritteri*, *Opuntia ficus-indica*, *Turbinicarpus pseudomacrochele*, *Agave Victoria-reginae* and *Agave tequilana*. In general, the induction and development of *in vitro* somatic embryos were carried out in three stages: (I) extablishment of an aseptic explant (II) induction of somatic embryos and (III) maturation of embryos culture.

2.2.2.1 Establishment of aseptic explant

Contaminations by fungi and bacteria in *in vitro* cultures often affect the growth of plant tissues. Viable contaminants on the surface of explants will proliferate rapidly once in contact with nutrient medium. This is because both plants tissue and microorganisms have similar basic requirements. However, the growth rate of the fungi and bacteria are faster compared to the plant tissue. Therefore, they will deplete the nutrient and excrete metabolic compounds into the culture medium which will affect the growth of the plant tissue (Street, 1977; Endress, 1994). Therefore, sterilization agents are used

to decontaminate the surface of an explant. Chemical agents often used for this purpose include antibiotics and fungicides, alcohols, mercuric chloride ($HgCl_2$), oxidize biocides such as halogen compounds (chlorine, bromine, and iodine) and hydrogen peroxide. The effectiveness of these sterilization chemicals can be further enhanced if a small amount of wetting agents (0.05%) like Teepol is incorporated into the sterilizing solution. The additions of the wetting agents enable the sterilizing agents to come in close contact with the microbes and destroy them (Street, 1977). However, the usage of chemical agents for surface-sterilization should be moderate. The concentration of the sterilizing agents and the duration of treatment should be adjusted to minimize tissue damage and increase the recovery of aseptic tissues. The amount of the sterilization agents used depends on the plant species, type of explants, phytotoxicity, type of contaminants and cost (Niedz and Bausher, 2002). Consequently, different sterilization process can be used such as single stage or double stage surface-sterilization with suitable sterilizing agents.

In single stage surface-sterilization, explants were washed with moderate concentration of sterilizing agents. Various type of sterilizing agents and concentration with different sterilization duration were tested on explants to determine the best protocol. For an example, sodium or calcium hypochlorite was one of the most common sterilization agents used in plant tissue culture. Bhojwani and Razdan (1985) suggested 0.5 – 5% of sodium hypochlorite or 9 – 10% of calcium hypochlorite treatment on explants for approximately 5 – 30 minutes was sufficient to decontaminate most plant tissue. Higher concentration of sterilization agents is toxic to the plant tissues. Chen *et al.* (2001) used single stage surface-sterilization method with 0.5% sodium

hypochlorite for 5 minutes to obtain aseptic leaf explants of *Adenophora triphylla*. Single stage surface-sterilization method with 1% sodium hypochlorite for 10 minutes was found to be effective for obtaining aseptic stem tissues of *A. triphylla*. However for seeds, higher concentration of sterilization agents and longer duration had to be used in a single stage surface-sterilization because the seeds were protected by the testa. Catapan *et al.* (2002) used commercial bleach (2.5% sodium hypochlorite) for 30 minutes to surface-sterilize seeds of *Phyllanthus urinaria*.

Besides sodium hypochlorite, $HgCl_2$ was also found to be effective in single stage surface-sterilization to decontaminate the explants. Pierik (1987) suggested that the concentration of $HgCl_2$ to be used should be in the range of 0.01 – 0.05% (w/v) for 2 – 12 minutes. The explants required very thorough rinsing to prevent any $HgCl_2$ residues attached to the surface. Amin and Jaiswal (1993) reported that aseptic apical buds of *Artocrpus heterophyllus* (jackfruits) could be obtained by using 0.1% $HgCl_2$ for 5 minutes. Liu and Bao (2003) also used one stage surface-sterilization of 0.1% $HgCl_2$ for 10 minutes to decontaminate *Platanus acerifolia* seeds.

Sodium dichloroisocyanurate (NaDCC) is another type of sterilizing agent. It is an organic form of chlorine with pH 7 (Niedz and Bausher, 2002). It was found to be effective for surface-sterilization of plant tissues because it was less toxic compared to sodium hypochlorite or $HgCl_2$. Furthermore, phytotoxicity of NaDCC was relatively low and allowed shoots to be cultured without rinsing. According to Heiling *et al.* (2001), NaDCC was as effective as sodium hypochlorite to eliminate bacterial contaminants such as *Streptococcus mutans*, *Streptococcus salivarius*, *Enterococcus faecalis* and *Streptococcus*

sobrinu. Parkinson *et al.* (1996) reported that single stage sterilization with NaDCC was effective in eliminating a wide range of bacteria from *Spathyphyllum petite* shoots and oak somatic embryo cultures. The explants became aseptic and survived although they were exposed to NaDCC for a long duration.

However, some explants are very sensitive to the sterilizing agents and they are very difficult to decontaminate using low concentration of the sterilizing agents. Therefore, double stage surface-sterilization method was often recommended for eliminating microorganism gradually by repeated sterilization twice with a moderate concentration of sterilization agents within a short duration. For obtaining *Eurycoma longifolia* Jack aseptic leaf explants, Luthfi *et al.* (2003) used double stage surface-sterilization method with 13% Clorox® solution for 15 minutes in the first stage followed by 5% Clorox® solution for 5 minutes in the second stage. Double stage surface-sterilization could be carried out with combination of two different sterilizing agents such as mercury chloride and sodium hypochlorite. Combination of different sterilizing agents enabled the establishment of aseptic explants that could not be decontaminated using single agent. For example, Ang and Chan, (2003) had successfully obtained aseptic axillary buds of *Spilanthus acmella* L. by surface-sterilizing the nodal segment explants with 0.08% (w/v) $HgCl_2$ for 5 minutes followed by 15% (w/v) Clorox® for 15 minutes. Meanwhile, Lee and Chan (2004) used Hegel solution (0.02 g $HgCl_2$ in 100 mL water) for 5 minutes followed by 20% Clorox® for 20 minutes to surface-sterilize the nodal segments of *Orthosiphon stamineus* Benth. Occasionally $HgCl_2$ was combined with other detergents that were less toxic to prevent tissue damage. Chan and Chan

(2002) had used double stage surface-sterilization method with 100 mg/L $HgCl_2$ for 10 minutes followed by 300 mg/L NaDCC for 24 hours to obtain aseptic and viable shoots of *Cyperus aromaticus* that commonly contaminated with fungi and bacteria.

Even though these sterilization agents were capable of decontaminating surfaces of explants but they were not able to remove systemic contaminants. Systemic contaminants are usually the rod bacteria (*Bacillus licheniformis* and *Bacillus subtilis*). They grow slowly in the explants and can only be detected when they come in contact with the nutrient medium (Pierik, 1987). This kind of contaminants can be eliminated by using shoot tip and meristem culture because such explants have low systemic contamination. However, meristem culture needs a skilful hand to perform. There is an alternative method to eliminate the systemic contaminants with addition of antibiotic in the nutrient medium. The systemic bacteria will be eliminated when the explants come in contact with the antibiotic supplemented nutrient medium. There are a few antibiotics that are commonly used such as Penicillin, Streptomycin, Bacitracin, and Sparsomycin. However, plant tissues are usually sensitive to antibiotics and show variable responses depending on the genotype. Antibiotic may damage the plastids or mitocondrias within the plant cells. Cells or tissues that were exposed to antibiotics for long period can result in the development of resistance within these organelles or result in tissue dormancy (George and Sherrington, 1984).

2.2.2.2 Induction of somatic embryos

Induction of somatic embryos (SE) started with the formation of pro-embryogenic masses followed by SE formation, maturation, desiccation and plant regeneration. SE might arise directly from explants or indirectly through callus. Embryos derived directly from a cell or tissue without going through callus phase to form pre-embryos determined cells (PEDC). The PEDC required certain amount of plant growth regulators (PGR) or favorable conditions to stimulate cell division to form a complete embryo. Only certain cells gave rise to SE such as the embryogenic predetermined cells and the hypocotyl epidermis cells of young seedlings. SE of *Eurycoma longifolia* were developed directly from immature cotyledonary explants after 14 days of culture on MS medium supplemented with NAA. *E. longifolia* callus was induced from leaf explants after 20 days cultured on MS supplemented with 2,4-D and the callus formed SE after 35 days of culture (Aziz *et al.*, 2000).

Meanwhile indirect SE could be formed from undetermined cells and non-embryogenic callus (Arnold *et al.*, 2002). Indirect induction of SE from callus was affected by certain stress factors. Cells from which the embryos developed were called induced embryogenically determined cells (IEDC) and formed embryos when they were induced further (William and Maheswaran, 1986). Indirect SE induction was observed from the floral tissue of *Feijoa sellowiana* Berg. (Stefanello *et al.*, 2005). They reported that SE were induced when *Feijoa sellowiana* calluses were transfered to LPm medium supplemented with different level of picloram, kinetin and/or 2,4-D. The highest embryogenic calluses induction was obtained on LPm medium supplemented with 10 µM picloram and 1 µM kinetin.

Auxin such as 2,4-D, NAA, 3-indol acetic acid (IAA), picloram and TDZ were commonly used to induce SE. According to Bhojwani and Razdan (1985), 2,4-D was the most common PGR for the induction of SE. It was effective for promoting proliferation of embryogenic cultures. It was less metabolized by the cells compared to other auxins (Arnold *et al.*, 2002). Liu *et al.* (2001) used MS medium supplemented with 9.05 µM 2,4-D to induce embryogenic calluses and established embryogenic suspension cultures from the shoot apices of sweet potatos (*Ipomoea batatas* L.). Combination of different PGR was found to be effective for the induction of SE. For example, Choun *et al.* (2004) obtained optimum embryogenic callus proliferation for *Bambusa edulis* on MS medium supplemented with 0.046 µM TDZ plus 13.6 µM 2,4-D and 3% (w/v) sucrose. This combination of PGR was found to promote germination of the bamboo SE. Meanwhile medium supplemented with either TDZ (10 µM), NAA (5.3 µM), BAP (0.9 µM) or 0.9 µM kinetin was found to stimulate proliferation of secondary SE of *Malus* x *domestica* Burkh (Daigny *et al.*, 1996).

Cytokinin was also found to be as effective as auxin in the induction of SE. It could induce SE with or without auxin. Sagare *et al.* (2000) successfully induced SE using primary callus cultured on MS medium supplemented with 0.5 – 4.0 mg/L BA, kinetin or zeatin without any addition of auxin. However, Kumria *et al.* (2003) had found that induction of SE for *Gossypium hirsutum* required combination of both cytokinin and auxin in the culture medium. In order to produce SE of *G. hirsutum,* the hypocotyl or cotyledonary leaf sections of *G. hirsutum* were cultured on MS medium supplemented with 0.1 mg/L 2,4-D plus 0.5 mg/L kinetin and 3% maltose for eight weeks before transferred

to PGR free MS medium for another four weeks. Gupta *et al.* (1997) also found that combination of BA and NAA was suitable for the induction SE of *Psophocarpus tetragonolobus* (L.) DC. Their experimental results showed that culture medium either supplemented with only auxin or cytokinin could not induce SE of *P. tetragonolobus.* They could be induced successfully when the leaf segments were cultured on MS medium supplemented with combination of BA (1.0 – 2.0 mg/L) and NAA (0.1 – 0.5 mg/L). Though high concentration of cytokinin was favorable in inducing more SE of *P. tetragonolobus,* an addition of low amount of auxin was necessary.

Beside PGR, other components in the nutrient medium also played an important role in the induction of SE. Tomaz *et al.* (2001) had studied the effect of different carbohydrate source (galactose, glucose, lactose, maltose, sucrose and glycerol) in nutrient medium on SE induction of *Citrus* spp. Their results showed that formation of SE of *Citrus* spp. responsed differently with different carbohydrate sources in the medium. The cultures of Valencia, Caipira sweet oranges and Cleopatra mandarin line 1 induced high amount of embryos on Murashige and Tucker medium that contained galactose, lactose and maltose compared to other carbohydrates. The numbers of SE derived from Caipira and Cleopatra line 1 calluses decreased when the concentration of maltose increased. SE of Valencia was best obtained by using culture medium containing maltose. The number of Valencia SE increased with increasing amount of maltose and lactose compared to galactose.

Beside the carbon source, other nutrient supplements such as vitamins or certain amino acids had been reported to affect SE formation. Al-khayri (2001) reported the optimum induction of date palm (*Phoenix dactylifera* L.)

embryos was observed on MS medium containing 0.5 mg/L thiamine and 2 mg/L biotin. Embryos elongation was highest on medium containing 0.5 – 2 mg/L thiamine combined with 1 mg/L biotin. It was found that the effect of thiamine on embryo growth was biotin dependent. Embryos number gradually increased as biotin concentration reached 2 mg/L in medium supplemented with 0.1, 0.5 or 2 mg/L thiamine. Shetty and McKersie (1993) reported that the addition of 10 – 25 mM proline into the induction medium containing 2,4-D induced embryogenesis of alfalfa. The addition of thioproline, a proline analog, in the culture medium at concentrations of 0.1 – 0.5 mM also stimulated embryogenesis. Thioproline at higher concentrations (0.5 – 1.0 mM) was found to decrease the size of embryogenic callus. However, at lower concentration it reduced the embryo numbers but increased the size of SE. Combination of proline (10 – 25 mM) and thioproline (0.4 mM) with addition of 50 mM potassium in the induction medium enhanced the size of embryogenic callus and significantly enhanced subsequent embryo formation.

The elevation of certain elements in the culture medium was reported to affect SE formation as well. As an example, the increase of calcium ion in the MS medium supplemented with 0.5 mg/L 2,4-D increased the growth of carrot somatic embryogenic suspension cell culture especially at the early stage of induction. The SE that were pre-cultured on lower concentration of PGR at high pH medium increased rapid uptake calcium ions as the cells began to grow (Takeda *et al.*, 2002). Beside calcium, potassium was reported to affect the size of embryogenic callus of alfalfa (*Medicago sativa* L.). Addition of potassium in the medium also promoted embryo formation when its level was increased from 25 mM to 75–100 mM in the Schenk and Hilderbrandt (SH) medium

supplemented with 1 mg/L 2,4-D. However, the addition of 125 mM potassium had reduced embryogenic callus size and reduced embryo number (Pasternak *et al.,* 2002). Therefore, the type and concentration of elements in the culture medium had to be optimized to achieve desirable result.

Different form of nitrogen sources in the culture medium had also proven to affect the *in vitro* growth of somatic embryos. Bhojwani and Razdan (1985) suggested that, in general, lower concentration of NH_4Cl as nitrogen source together with KNO_3 could result in the formation of SE. However, the frequency of embryo formation with the presence of only KNO_3 or NH_4Cl was not high. This was because NH_4Cl combined with KNO_3 resulted the pH value of the medium to re-adjust to 5.4 which was suitable for SE formation. However, NH_4Cl alone would cause the pH to drop below 4 and this could inhibit embryo induction. Das *et al.* (2001) had tested on the effect different inorganic nitrogen source in optimisation of SE production of *Santalum album* L. Results showed that 10 mM nitrate and 5 mM ammonium in McCown basal medium induced the formation of maximum embryos. The amount of ammonium at this concentration was sufficient for proper growth and maturation of embryos. However, higher concentration or completely absence of ammonium would not support growth and differentiation to SE. Hence culture medium containing both forms of nitrogen source was needed for induction of *S. album* L. SE.

2.2.2.3 Maturation of embryos

During the maturation stage, the somatic embryos will undergo various morphological and biochemical changes. Embryos need some degree of

drying before they can germinate (Arnold *et al.*, 2001). Malabadi *et al.* (2004) had successfully promoted maturation of *Pinus kesiya* (Royle ex. Gord) SE by partial desiccation and using high concentration of Gellan gum. The embryogenic calluses were desiccated by keeping them in the petri dish contained two pieces of sterile Whatman filter paper and kept aseptically at 25 ± 2°C in the dark for different period of time (12, 24, 48, 72 and 96 hours). The embryos were cultured on modified MS medium with high myo-inositol, 60 g/L maltose, 37.84 µM abscisic acid (ABA) and 5 g/L Gellan gum (III). Results showed that maturation of embryos increased from 4.2% to 21.5% after 12 hours of desiccation treatment. After 24 hours of desiccation, the maturation rate of embryo increased to 67.3%. Those SE that did not undergo desiccation process had low maturation rate (approximately 5%). Therefore, a certain degree of drying was necessary for embryos maturation process.

Elevation of sucrose level in the culture medium could induce desiccation effect for maturation purpose. Increase of sucrose concentration affected the osmotic and metabolic rate and induced desiccation tolerance on embryos. Since both metabolic and osmotic effects were associated with accumulation of storage reserves, ratio of dry weight and fresh weight (DW/FW) would be expected to increase with the elevated sucrose concentration in the medium. Sucrose concentration at 6.0% or 7.5% in Lloyd and McCown medium, with 1 g/L casein hydrolysate produced highest dry weight and fresh weight (DW/FW) ratio value in maturation and germination of American Chestnut SE. The high DW/FW ratio indicated that the embryos had depleted the storage of food within it and ready to regenerate. Higher

sucrose level of more than 7.5% did not induce maturation of embryos. At low sucrose concentration, embryos remained translucent (Robichaud *et al.,* 2004).

Maturation of SE could also be induced by using different type of PGR. Aderkas *et al.* (2001) found that somatic embryos maturation of larch was effective on semi-solid MS medium supplemented with 60 μM ABA, 1 μM 3-indole butyric acid (IBA) and 0.2 M sucrose. It was found that ABA was steadily uptake by the embryos during maturation period and this resulted in the maturation of the conifer SE. This was because ABA was able to enhance SE quality by increasing desiccation tolerance. It also increased the germination rate of the embryos through elevation of sucrose level.

Even though ABA was effective in promoting maturation on SE but it could only be effective for certain plants. Some embryos might require combination of ABA with osmotic regulator such as polyethylene glycol (PEG) or elevated sugar levels to promote maturation of SE. Langhansová *et al.* (2004) found that SE treated with ABA and polyethylene glycol (PEG 4000) resulted in maturation of *Panax* ginseng torpedo-stage embryos. *Panax* ginseng SE without treatment could successfully germinated to form less shoots (31%) as compared with SE that were treated with ABA and PEG (70%). Treatment with ABA and PEG resulted 75% of the embryos formed young plantlets.

2.3 Cell cytotoxicity studies

2.3.1 Animal cell culture

Animal tissue culture has become a generic term and encompasses organ culture, where a small fragment of tissue or whole embryogenic organ is taken as explant to retain tissue and cell culture. This tissue can be propagated