TISSUE CULTURE, HISTOLOGICAL AND PIGMENT ANALYSIS OF *Hylocereus polyrhizus* (Weber) Britton AND Rose

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ABSTRACT

An optimum protocol for culturing *Hylocereus polyrhizus* (Weber) Britton and Rose for *in vitro* regeneration and mass propagation was established in the present study. A comparative histological analysis between *in vivo* and *in vitro* grown plants was also successfully investigated. Stem explants cultured on MS medium supplemented with 6-benzylamino purine (BAP), α-Naphthaleneacetic acid (NAA) and Kinetin were placed both vertically and horizontally. MS medium supplemented with low concentration of BAP (0.2 mg/L) gave the highest formation of multiple shoots (8.07 ± 0.721) when stem explants were horizontally cultured. Low concentration of Kinetin (0.2 mg/L) induced the longest shoots in average (1.608 ± 0.128 cm) which was also cultured in horizontal position. Combination of 0.1 mg/L of BAP and 0.1 mg/L of α-Naphthaleneacetic acid (NAA) gave the highest average number of roots (3.031 ± 0.237) in vertical culture position. The combination of 0.5 mg/L of BAP and 0.1 mg/L of NAA resulted in the longest root formation when cultured in horizontal position. In callus induction, the highest percentage (100%) was obtained in MS medium supplemented with combinations of 1.0 mg/L BAP and 0.1 mg/L 2, 4-D; 1.0 mg/L BAP and 1.0 mg/L 2, 4-D and 2.0 mg/L BAP with 1.0 mg/L 2, 4-D. The lowest percentage (27.0 ± 0.082 %) was observed when the stem explants were cultured on MS medium supplemented with 2.0 mg/L 2, 4-D. Green callus was observed on MS medium supplemented with 1.0 mg/L BAP and 0.5 mg/L 2, 4-D; 1.0 mg/L BAP and 1.0 mg/L 2, 4-D, and 2.0 mg/L BAP with 1.0 mg/L 2, 4-D. In the synthetic seed studies, maximum germination (100%) was obtained when the beads were germinated on MS basal medium and emergence of shoots was observed on Day 10. No development of complete plantlets was observed when the hormones were added in the matrix of encapsulated beads. The viability of the beads decreased with the increasing of the storage period. The hardening process of synthetic seed showed negative results with

fungal infection. Complete plants were successfully acclimatized in the combination of red soil, black soil and cocoa peat (1:1:1) with 83.3% survival rate. *Quartz* or silicon oxide (47.33%) was the most abundant element in soil used in acclimatization as examined by X-ray diffractometry (XRD). The multielements such as Si, Al, Fe, Ca, K, Mg, Na, Cu, Zn, Rb, Sr, Zr, Nb and Cl were presence in the soils analyzed by X-ray fluorescence spectrometry (XRF) measurements. For histological analysis, stems and roots derived from *in vivo* and *in vitro* grown plants showed differences in cortical bundle structures, cortex and stomata structures. Histological studies were carried out to compare *in vivo* and *in vitro* plants structures and to monitor any occurrence of somaclonal variation. The established protocol of regeneration system exhibited a high potential in plant production and can help to fulfil the market demand. For pigment analysis, betacyanin pigments extracted from pulp using 80% acetone as solvent at pH 1.0 gave the highest betacyanin content. Betacyanin content decreased when stored under exposure of light compared to dark storage. In addition, increasing concentration of NaCl decreased the absorbance values at faster rates for betacyanin-coated glass slides. The method for detection of betacyanins pigment stability and effectiveness of betacyanins pigments used as natural colorants for coating application was beneficial, recent and novel for environmental friendly and natural plant based products development.

ABSTRAK

Protokol optimum untuk regenerasi *in vitro Hylocereus polyrhizus* (Weber) Britton and Rose telah dikenalpasti dalam kajian ini. Satu analisis perbandingan histologi untuk melihat perbezaan dan persamaan sel-sel antara pokok yang ditanam secara *in vivo* dan *in vitro* telah berjaya dikaji. Eksplan batang *H. polyrhizus* telah dikultur dalam dua posisi; melintang dan menegak. Medium MS yang ditambah dengan kepekatan rendah 6-benzylamino purine (0.2 mg/L BAP) menunjukkan bilangan pucuk berganda tertinggi (8.07 ± 0.721) apabila eksplan batang dikultur dengan kedudukan melintang. Kepekatan rendah Kinetin (0.2 mg/L) telah menghasilkan pucuk yang terpanjang dengan kadar purata (1.608 ± 0.128 cm) juga dengan posisi kultur secara melintang. Kombinasi 0.1 mg/L BAP dan 0.1 mg/L α-Naphthaleneacetic acid (NAA) memberi purata bilangan akar tertinggi (3.031 ± 0.237) dalam posisi kultur secara menegak. Kombinasi 0.5 mg/L BAP dan 0.1 mg/L NAA menghasilkan pembentukan akar terpanjang apabila dikultur secara posisi melintang. Dalam penghasilan kalus, peratus tertinggi telah didapati dalam medium MS yang ditambahkan dengan kombinasi 1.0 mg/L BAP dan 0.1 mg/L 2, 4-D, 1.0 mg/L BAP dan 1.0 2, 4-D dan 2.0 mg/L BAP dan 1.0 mg/L 2, 4-D. Peratus terendah (27.0 ± 0.082 %) telah didapati apabila eksplan batang dikultur di atas medium MS yang ditambahkan dengan 2.0 mg/L 2, 4-D. Kalus berwarna hijau telah terbentuk di atas medium MS yang ditambahkan dengan 1.0 mg/L BAP dan 0.5 mg/L 2, 4-D, 1.0 mg/L BAP dan 1.0 mg/L 2, 4-D, dan 2.0 mg/L BAP dan 1.0 mg/L 2, 4-D. Dalam kajian biji benih sintetik, percambahan maksimum telah didapati apabila biji benih tiruan dicambahkan di atas medium asas MS dan pengeluaran awal pucuk telah dilihat pada hari ke-10. Tiada pembentukan pokok yang lengkap diperhatikan apabila hormon ditambahkan dalam kapsul biji benih tiruan. Daya ketahanan hidup biji benih menurun apabila tempoh jangka penyimpanan dilanjutkan. Proses penanaman biji benih sintetik di luar menunjukkan keputusan negatif akibat

jangkitan kulat. Pokok yang lengkap dari eksperimen regenerasi telah berjaya diaklimatisasikan dengan menggunakan kombinasi tanah merah, tanah hitam dan sabut kelapa (1:1:1) dengan 83.3%. *Quartz* atau Silicon oxide (47.33%) adalah elemen terbanyak dalam tanah yang digunakan untuk aklimatisasi, dianalisis oleh X-ray diffractometry (XRD). Multielemen seperti Si, Al, Fe, Ca, K, Mg, Na, Cu, Zn, Rb, Sr, Zr, Nb dan Cl telah didapati hadir dalam tanah yng telah dianalisis menggunakan X-ray fluorescence spectrometry (XRF). Dalam kajian histologi, sel-sel batang dan akar yang ditanam secara *in vivo* dan *in vitro* telah menunjukkan perbezaan dalam struktur berkas kortikal, kortek dan struktur stomata. Kajian histologi adalah bertujuan untuk meneliti perbezaan di antara pokok *in vivo* dan *in vitro* dalam struktur tumbuhan dan untuk melihat samada wujud variasi somaklon atau tidak apabila kaedah kultur tisu digunakan. Protokol yang telah dikenalpasti dalam kajian ini untuk sistem regenerasi *in vitro* menunjukkan potensi yang tinggi dalam penghasilan tumbuhan untuk memenuhi permintaan pasaran. Dalam kajian analisis pigmen, pigmen betasianin yang diekstrak dari isi buah menggunakan 80% aseton sebagai pelarut pada pH 1.0 menunjukkan kandungan betacyanin yang tertinggi. Kandungan betasianin menurun apabila disimpan dibawah keadaan cahaya berbanding di tempat gelap. Dalam kajian ini juga, apabila kepekatan NaCl meningkat, bacaan kuantiti betasianin menurun lebih cepat untuk slaid kaca yang disalut dengan betasianin. Kaedah pengesanan kestabilan dan keberkesanan pigmen betasianin sebagai pewarna semulajadi untuk kegunaan salutan mempunyai faedah bernilai tinggi untuk pembangunan produk yang mesra alam serta berasaskan tumbuhan semulajadi.

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LIST OF ABBREVIATIONS

%	Percentage
°C	Degree Celcius
µmol	micromole
2, 4-D	2, 4-Dichlorophenoxyacetic acid
BAP	6-benzylamino purine
CAM	Crassulacean acid metabolism
DMRT	Duncan's Multiple Range Test
HCl	Hydrochloric acid
Kn	Kinetin
mg/L	Milligram per litre
MS	Murashige and Skoog
NAA	α-Naphthaleneacetic acid
NaOH	Sodium hydroxide
PGR	Plant Growth Regulator
TBA	tertiary butyl alcohol
UV	Ultraviolet
XRD	X-ray diffractometry
XRF	X-ray fluorescence

CHAPTER 1

INTRODUCTION

Hylocereus polyrhizus (Weber) Britton and Rose, also known as dragon fruit or red-fleshed pitaya is one of the tropical fruits belongs to the family Cactaceae. It is originated from Latin America. Among the main countries cultivating dragon fruit are Vietnam, Colombia, Mexico, Costa Rica, and Nicaragua (Vinas *et al*., 2012). In Malaysia, dragon fruits are also known as "buah naga" or "buah mata naga", which was first introduced in 1999 in Setiawan, Johor and Kuala Pilah regions. It is becoming well-liked in Malaysia due to its attractive colors, sweet, juicy amusing taste and has been considered to be the most beautiful species in cactus family. *Hylocereus* spp. have fleshy stems and may grow from the ground or climb onto trees using aerial roots. Dragon fruit contains high amount of vitamin C and water-soluble fiber (Ruzainah *et al*., 2009). *H. polyrhizus* also contains high amount of betalains, the major bioactive compound and composed of red-violet betacyanin, water soluble pigments that provide colours in flowers and fruits (Wu *et al*., 2006).

This cacti species shows high potential as an ornamental and fruit crop and its demand is high in national and international markets. *Hylocereus* propagation can be done through seed or vegetatively propagated. Vegetative propagation is done by cuttings or stem fraction. Reproduction through seeds is done by extracted the seeds from fruit fleshed and sow the seeds in the potting mix. This fruit contains numerous edible soft black seed covered by mucilage distributed in the pulp of the fruit. However, these conventional methods of propagation are not efficient for producing a large number of this plant within short period. Plant tissue culture can be an alternative method to fulfill the increasing demand of this potent fruit crop. High number of aseptic

plants could be obtained within short period using plant tissue culture protocols. *In vitro* regeneration is considered as reliable and rapid technique for propagating the plants (Thomas & Philip, 2005; Thomas & Jacob, 2004).

Instead of conventional propagation, plant tissue culture is an alternative propagation method which plays a very essential role in plant biotechnology. *In vitro* culture techniques become vital tools in recent years for rapid mass multiplication and germplasm conservation of rare, endangered, and threatened medicinal plants (Anis & Faisal, 2005; Uppendra *et al.*, 2005). Plant tissue culture also is being increasingly applied for clonal propagation of selected tree species to supplement conventional methods which have their limitations, especially when a large number of genetically uniform propagules are required (Mascarenhas *et al.* 1981; Mascarenhas & Muralidharan, 1989). *In vitro* regeneration can increase the availability of disease-free plants and reduces culture space requirement thus lowers the cost of production.

Plant tissue culture is basically a technique of *in vitro* cultivation of pant cells and organs, which divide and regenerate into callus or particular plant organs. Any part of the plant such as root, stem, petiole, leaf or flower called “explants” are used as starting materials. Good and healthy mother plants are preferred because of the physiological state of the plant influence its response to tissue culture process. Contamination from microorganism should be avoided during plant tissue culture. Aseptic environment during culture is important since plant cell division is slower than the growth of microorganism. All the materials such as glassware, equipments, medium and explants must be sterile and freed from microbes to be used in tissue culture work. Laminar flow is mandatory and prerequisite for tissue culture work.

Tissue culture medium is the main requirement for the growth of plant cell. The growth and morphogenesis of plant tissues are influenced by the type and composition of culture media. The choice of tissue culture medium are largely depends on the species to be cultured. The establishment of optimum culture medium formulation is important to determine suitable composition of medium on particular culture system. Murashige and Skoog (1962) or MS medium is the most suitable and commonly used in plant tissue culture until today. Tissue culture medium is consisting of macronutrient, micronutrient, iron, vitamins, sucrose as carbon source, plant growth regulators such as auxin and cytokinin and agar as solidifying agent.

Micropropagation is *in vitro* propagation of plants vegetatively by tissue culture to produce genetically identical copies of mother plant or explants. Plants which are sexually propagated such as seeds showed high amount of heterogenetic or variation from the mother plants. However, asexual propagation permits genetically similar copies of a cultivar and reserve the same characteristics. Micropropagation can maintain the genotype constitution because it is generally lesser variation than in somatic embryos. Clonal propagation also could reduce growth cycle and rapid multiplication thus selective multiplication can be done.

In plant tissue culture, the ability of plant cells to regenerate into whole plants is known as totipotency concept. The cells obtained from any plant parts when allowed to grow in the specific culture medium containing minerals, nutrients, vitamins and growth regulators can be encouraged cell to divide and grow. The cells in the culture will produce an unorganized proliferative mass of cells which also known as “callus tissue”.

Callus may be able to regenerate back to whole plants when introduced to certain manipulation of medium and culture condition. Manipulation of nutrients and hormones also can produce a cluster of embryos from somatic cells of callus tissue. Somatic embryos will directly transform into the whole plant. This process is known as somatic embryogenesis. Callus culture is also very useful to facilitate the production of secondary metabolites for commercial purposes. Secondary metabolites such as anthocyanin, flavonoids, antimicrobial or anticancer can be directly extracted from the callus tissue instead of the whole plants. Tissue culture method could also help in conserving medicinal plants from extinct in nature.

Somatic embryogenesis is one of the pathways for *in vitro* regeneration. Somatic embryos, shoot tips and axillary buds can be encapsulated and created into synthetic seed. Cryoprotectant material like alginate gel, hydrogel or dimethylsulfoxide (DMSO) can be used as encapsulation matrix. Cryoprotectant materials prevent the explants from mechanical damage during handling and allow germination to occur. Sodium alginate and calcium chloride are widely used to produce seed coat. Synthetic seeds have a great potential for large scale production of plants in low cost and could be propagated similar to true seeds.

The occurrence of somaclonal variation may occur during micropropagation of plants. Somaclonal variation is defined as genetic variation in plants that have been observed among the regenerated plants. Variability could occur spontaneously during *in vitro* culture and can be result of temporary changes or permanent genetic changes. Genetic variability is influence by several factors such as natural selection, mutation, migration and population size. Somaclonal variation can be beneficial for improvement

of crops such as disease resistant traits, improved quality or higher yield of certain species.

In cactus species, plant tissue culture techniques can overcome certain limitations associated with conventional propagation mainly in rare and endangered species. The propagation of native cacti is usually done with seed and rooted offshoots. However, this methods are inadequate for those species that exhibit no to few offshoots, seed dormancy, low germination rates, self-sterility, slow growth, or that require many years to mature. There are at least 24 species of cacti from 16 genera have been micropropagated by rooting of shoots proliferated through axillary branching (Mauseth, 1979; Johnson & Emino, 1979b; Vyskot & Jara, 1984; Starling, 1985; Ault & Blackmon, 1985, 1987; Escobar *et al.*, 1986; Rubluo *et al.*, 1986).

In vitro culture and micropropagation offer useful alternatives for the multiplication and conservation of cactus species. Mass reproduction of plantlets in a short time and in minimal space has been demonstrated in several cacti species. However, each species responds differently to the conditions established for *in vitro* multiplication, rooting and acclimatization, making it necessary to optimize these culture conditions for each cactus species that would be micropropagated (Hubstenberger *et al*., 1992). Several researchers reported micropropagation from different species of *Hylocereus.* Johnson and Emino (1979) reported the potential tissue culture propagation for certain cacti including *H. calcarathus* Britton et Rose. A method for axillary shoot proliferation and embryo formation from seedling explants of yellow pitaya (*Mediocactus coccineus* Britton et Rose) was introduced by Infante (1992).

Mohamad-Yaseen (2002) reported the micropropagation of *Hylocereus undatus* Britton et Rose using thidiazuron (TDZ) and naphthaleneacetic acid (NAA).

Pigment from natural resources such as plants and animals are increasingly in demand as an alternative to synthetic colorants. Natural colorant is renewable and biodegradable and may provide some health benefits such as antioxidant to human. Nevertheless, there are several limitations associated with the use of natural colorant. Natural colorants are usually less stable than synthetic colorants and difficult to achieve color uniformity. Apart from that, it is greatly affected by certain environmental factors such as pH, oxidation, light and temperature. Therefore, enhancement of stability in natural pigment needs to be considered to increase their commercial availability for consumers.

Natural pigment from cactus species such as *Hylocereus polyrhizus* is considered as eco-friendly and no adverse effects for consumers. *H. polyrhizus* contains high amount of red-violet betacyanin pigments. Betacyanins are water soluble polyphenolics pigments which have many benefits such as colorants, antimicrobial properties and antioxidants. However, some environmental factors can cause deprivation of betacyanins pigments and limit the utilization as source of natural colorants.

Figure 1.1: Habit of *Hylocereus polyrhizus* (Weber) Britton and Rose

Figure 1.2: Fruits of *Hylocereus polyrhizus* (Weber) Britton and Rose

1.1 RESEARCH OBJECTIVES

The main aim of this research was to establish an efficient protocol for micropropagation of *Hylocereus polyrhizus* plant via plant tissue culture techniques from various explants. Various hormones with different combinations and concentrations were manipulated to obtain the optimum media for direct regeneration, callus induction and acclimatization of this species. Instead of conventional plant propagation, high yield propagation of *Hylocereus polyrhizus* is vital for commercialization and conservation of this commercial fruit crop.

Other research objectives include observing and comparing morphological differences of *in vivo* and *in vitro* grown plants of *Hylocereus polyrhizus*. This is to observe any occurrences of somaclonal variations of *H. polyrhizus* grown *in vivo* and *in vitro*. In addition, the development of synthetic seed of this species has been done to investigate the ability of the encapsulated seeds to germinate to compare with the normal seeds. The germination of synthetic seed depends on several factors such as the concentrations of sodium alginate and the soaking time to which well-formed beads will be obtained. The pigments analysis from *H. polyrhizus* also has been studied, where its red-violet betacyanin pigments were extracted and the stability of the pigment towards some environmental factors such as pH and light have been observed. Hence, the objectives of the present study are:

1. To establish an efficient protocol for micropropagation of *H. polyrhizus* plant via plant tissue culture techniques from various explants.

2. To obtain the optimum media for direct regeneration, callus induction and acclimatization of *H. polyrhizus.*

3. To investigate the ability of synthetic seed of *H. polyrhizus* to germinate and regenerated into whole plant.

4. To observe any occurrence of somaclonal variations of *H. polyrhizus* grown *in vivo* and *in vitro* by histological analysis.

5. To determine the effects of pH, light and NaCl concentration on betacyanin contents in *H. polyrhizus* fruit pulp and peel and to observe the stability of the pigment as natural colorants.

CHAPTER 2

LITERATURE REVIEW

2.1 PLANT TISSUE CULTURE AND HISTORY

Plant cell, tissue and organ culture technique has an important contribution for fundamental knowledge of the cell. Plant tissue culture, or aseptic culture of cells, tissues, organ, and their components under specific physical and chemical conditions *in vitro*, is a vital tool in basic, applied and also commercial application. Plant tissue culture is a technique of culturing plant cells, tissues and organs on synthetic media under aseptic environment and controlled conditions of light, temperature, and humidity. Plant tissue culture helps in providing a basic understanding of physical and chemical requirements of cell, tissue, organ culture, their growth and development. The ability to grow plant cells and tissues in culture and to control their development forms the basis of many practical applications in agriculture, horticulture industrial chemistry and is a prerequisite for plant genetic engineering (Evans *et al.* 2003). The success for plant tissue culture is based on the principle called totipotency. Totipotency is the ability of undifferentiated plant tissues to differentiate into functional plants when cultured *in vitro.*

The origin of the *in vitro* work was from a German scientist, Haberlandt, at the beginning of 20th century. He is known as "Father of Tissue Culture. The earliest studies toward plant tissue culture was made by Henri-Lous Duhumel du Monceau in 1756, who during studies on wound healing in plants, observed callus formation. The period in between 1940 to 1960, the development of new techniques and improvement in plant tissue culture were made. Starting in the mid-1960, the availability of these techniques led to the application of tissue culture to five broad areas such as cell behavior

(including cytology, nutrition, metabolism, morphogenesis, embryogenesis, and pathology), plant modification and improvement, pathogen-free plant and germplasm storage, clonal propagation, and product (mainly secondary metabolite) formation. In 1990, the continued expansion in the application of the *in vitro* technologies to an increasing number of plant species. Cell cultures have remained an important tool in the study of basic areas of plant biology and biochemistry.

Tissue culture technique was founded in 1838 by Schleiden and Schwann's when the cell theory was established. However, in 1902, Haberlandt failed on his first experimental approach in isolation of single palisade cells from leaves in Knop's salt solution enriched with sucrose and the cells increased in size but failed to divide. His prediction failed that the plant cultured could grow, divide and develop into embryo and the whole plant.

In 1930s, Vitamin B and natural auxins were discovered necessary for the growth of isolated tissues containing meristems. White (1934) reported that tomato root tips could be repeatedly subcultured to fresh medium of inorganic salts supplemented with yeast extract. In 1937, he replaced yeast extract by vitamin B namely pyridoxine, thiamine and proved their growth promoting effect. First plant growth regulator (PGR) is indolacetic acid (IAA) which was discovered by Fritz Went in 1926.

The first true plant tissue culture was obtained by Gautheret from cambial tissue from *Salix capraea, Robinia pseudoacacia, Populus nigra* and other trees using agar-solidified medium of Knop's solution, glucose and cysteine hydrochloride under aseptic

condition. Gautheret, Nobecourt and White published independently studies on the successful cultivation for prolonged periods of cambial tissues of carrot root (Gautheret, 1939) tobacco (White, 1939) and carrot (Nobecourt, 1939). Nobecourt (1939) found the successful grow of continuous callus cultures of carrot. In 1941, Johannes Van Overbeck used coconut milk in the culture medium to grow the seedlings from heart shaped embryos. In 1954, the principal chapters of this subject were widely opened: Tissue culture, cell culture, improving of nutrients, especially of growth substances, expertness of organ formation and vegetative propagation, attempts to obtain secondary products and application to pathological problems.

In 1955, Skoog and his colleogue reported the addition of adenine and high levels of phosphate allowed non meristematic pith tissues to be cultured and to produce shoots and roots, but only in the presence of vascular tissues. In 1951, Skoog and Tsui demonstrated induction of cell division and bud formation in tobacco by adenine. This led further investigation by Skoog and Miller (1957) who isolated kinetin – a derivative of adenine. Kinetin may showe bud promoting activities called cytokinin. Cytokinin is a cell division promoter in cells of highly mature and differentiated tissue. Skoog and Miller showed high concentration of IAA promoted rooting and high concentration of kinetin induces bud formation or shooting on tobacco pith cultures. Most of the modern tissue culture media derived from the work of Skoog and co-workers during 1950 to 1960. In the year 1962 Skoog and his student Murashige developed a high salt medium. The concentration of some salts was 25 times that of Knop's solution. MS formulation allowed for a further increase in the number of plant species that could be cultured, many of them using only a defined medium consisting of macro- and micro-nutrients, a carbon source, reduced N, B vitamins, and growth regulators. The MS salt formulation is now the most widely used nutrient medium in plant tissue culture.

In 1960s, the next breakthrough of plant tissue culture has been discovered. Kanta *et al.* (1962) demonstrated first role of tissue culture in plant genetic engineering. They developed a technique of first test tube fertilization of excised ovules and pollens grain in order to overcome the incompatibility barriers at sexual level. Guha and Maheshwari (1966) discovered the development of embryos into haploid plants thus initiating androgenesis. The plants can recover from homozygous double haploid cells and express all recessive genes. The involvement of *Agrobacterium tumafaciens* as a plasmid had been discovered by Zaenen *et al.* (1974) and Chilton *et al.* (1977) reported first integration between plasmid and plants.

2.1.1 Micropropagation

Micropropagation is the true-to-type propagation of plants by a variety of tissue, cell and organ culture methods. A small section of tissues and organs are cultured in closed vessel under aseptic conditions on selected culture media. Micropropagation is known as commercially efficient since this technique results in rapid generation of a large number of clonal plants of many plant species. Micropropagation also can produce pathogen free and disease free plants.

There are five major stages of micropropagation for development of plantlets (de Fossard, 1976). Stage 0 is the selection and maintenance of stock plants for culture initiation. At this stage, the preparation of mother plants to provide quality explants for better establishment of aseptic culture. The mother plants should be grown in a glasshouse to minimize the contamination problem and also watered to avoid overhead

irrigation. This stage also involves exposing the stock plants to suitable light, temperature, and growth regulator to enhance the quality of explants.

Stage I is the preparation and establishment of explants on suitable medium. The nature of explants to be used for *in vitro* propagation is governed by the method of shoot multiplication. For enhanced axillary branching, only the explants which carry a pre-formed vegetative bud are suitable. Suitable explants to be chosen are vital for micropropagation. A special precautions and sterilization need to be taken to minimize the loss of cultures due to microbial contamination. Disinfectant agents such as ethyl alcohol, bromine water, mercuric chloride, silver nitrate, sodium hypochlorite and calcium hypochlorite can be used. MS medium are frequently modified for the growth of particular explants. Stage II involves the multiplication of shoots or somatic embryo formation using defined cultured medium. In this stage, rapid multiplication of regenerative system is carried out to obtain mass number of shoots. Cytokinin is commonly used in the culture medium to increase the production of numerous shoots. A few cycles are repeated until a desired number of shoots are developed to carry out for rooting. Stage III is formation of roots for regenerated shoots or germination of somatic embryos *in vitro*. Multiple shoots are separated and transferred to a rooting medium containing auxin. At this stage, the elongation of shoots and roots before being transferred to soil are important to improve the rate of survival on natural environment. Stage IV involves transferring the plantlets to sterilized soil for hardening under greenhouse. Several factors such as humidity, light, soil mixture and nutrients are need to be maintained for enabling them to survive and grow into complete plantlets.

Micropropagation had been done to regenerate mass production of many species such as *Spilanthes acmella* (Saritha *et al.* 2002), *Cardiospermum halicacabum* (Babber *et al.* 2001), *Psidium guajava* L. (Ali *et al.* 2008), *Amorpha fructicosa* L. (Gao *et al.* 2003), *Sophora flavescens* (Zhao *et al.* 2003) and *Ixia* (Ruffoni *et al.* 2005). For Cactaceae, Clayton *et al*. (1990) reported axillary shoot proliferation of 11 rare members of Cactaceae such as *Escobaria missouriensis, Mammillaria wrightii, Sclerocactus spinosior,Pediocactus despainii, P. paradinei, P. winkleri, Toumeya papyracantha, E. robbinsorum, S. mesae-verdae, P. bradyi, P. knowltonii.*

2.1.2 Callus Induction

In plant regeneration, *in vitro* cultures allow plants to be regenerated from unorganized tissues called callus. Callus is an unorganized proliferative mass of cells produced from isolated plant cells, tissues or organs. Small excised portion of explants are grown on artificial nutrient medium in glass vials under controlled experimental conditions. Plant regeneration from callus is possible by *de novo* organogenesis or somatic embryogenesis. Manipulation of plant growth regulators promotes the induction of callus from explants. In addition, the identification and screening of useful cultivars for callus induction and plant regeneration *in vitro* are prerequisites in genetic improvement programs (Abe & Futsuhara, 1986). The origin, physiological state, age of explants and the degree of differentiation of tissue have been identified as the main factors influencing regeneration through somatic embryogenesis. According to Morrish *et al.* (1987), undifferentiated cells of immature organs and meristematic tissues are more suitable for plant regeneration compared with mature organs.

Shah *et al.* (2003) reported that callus formation is influenced by hormones such as cytokinin and auxin present in the medium. Prem *et al.* (2005) obtained green and friable morphogenic callus from embryo or cotyledon of *Cyamopsis tetragonoloba* explants when using combination of cytokinin and auxin in the culture medium. The combination of auxin and cytokinin promotes cellular differentiation and also organogenesis. Organogenesis is a process involving redifferentiation of meristematic cells present in callus into shoot buds. The stimulation of shoot bud differentiation in plants depends on many factors which differ for different plant species.

2.1.3 Synthetic Seed

Synthetic seed is encapsulated explants such as shoot tips, axillary buds and somatic embryos in cryoprotectant material like hydrogel, alginate gel, enthylene glycol and dimethylsulfoxide (DMSO). The idea of synthetic seeds was first suggested by Murashige in 1977 and it is restricted to encapsulation of somatic embryos in protective jelly. The coating protects the explants from mechanical damage during handling and allows germination and conversion to occur without inducing undesirable variations (Harikrishna & Ong, 2002). Synthetic seeds have a great potential for large scale production of plants at low cost as alternative to true seeds.

Synthetic seed production is a potential technique for plant multiplication and preservation, especially for propagation of non-seed producing plants, transgenic plants and other plants that need to keep superior traits by means of asexual propagation (Saiprasad, 2001). Karnada (1985) presented a general concept of plant artificial seed, in which all plants with germination ability can be used for synthetic seed production. However, the effect is varied depends on plant species, coating materials, maintained solution and its concentration and condition (Nhut *et al*. 2005).

There are various advantages of synthetic seeds. One of the advantages is germplasm conservation of elite and endangered or extinct plant species. Synthetic seeds also are easy to handle during storage, transportation and planting. The alginate encapsulation technique and cryogenic procedures are reliable methods for long term storage of plant genetic resources without the risk of genetic instability using minimum space and with low labour and maintenance costs. Ghosh and Sen (1994) reported that synthetic seeds have a potential for long term storage without losing viability.

2.1.4 Acclimatization

After micropropagation, complete *in vitro* plantlets are ready to be transferred to the natural environment. Acclimatization can be defined as the process of an individual organism adjusts to a gradual change in temperature, humidity, photoperiod or pH in order to maintain performance across a range of environmental conditions. During acclimatization, plants may undergo physiological and morphological changes in response to such alterations in the environment. Preece and Sutter (1991) reported that water deficits are one of the main causes of plant mortality after transfer from culture to greenhouse. Water deficits in plants will increase whenever transpiration exceeds water uptake. In tissue-cultured plants, high transpiration rates have been attributed to poor stomatal function (Brainerd & Fuchigami, 1982), reduced leaf epicuticular wax (Sutter & Langhans, 1982) and high stomatal density (Desjardins *et al*., 1988).

During *in vitro* regeneration, plantlets were grown under special conditions in air tight vessel in order to prevent microbial contamination. The closed vessel also decrease air turbulence and limits the inflow of CO_2 and outflow of gaseous plant products from

the vessel. However, in the greenhouse or field, irradiance is much higher and air humidity much lower than in the vessel. Many plantlets die during process of acclimatization. Therefore, *ex vitro* transplantation plantlets commonly need some weeks of acclimatization with gradual lowering in air humidity.

2.1.5 Histological Analysis

Histology analysis is the branch of biology concerned with the composition and structure of plant tissues in relation to their specialized functions. It is use to determine the organization of tissues at all structural levels, from cells and intercellular substances to organs. In histological studies, variety techniques are used such as using various fixatives, stains, the use of microtome for preparing thin sections, light microscopy, electron microscopy and X-ray diffraction. The development of bipolar structures of somatic embryos on semi-solid cultures has been demonstrated histologically in *Musa* spp. (Escalant & Teisson, 1989; Novak *et al.*, 1989).

There are five main stages in the preparation of histological slides. The first step is fixation and it is the most important step in the entire process. The aim of fixation is to preserve the tissue in the state that most reflect the living cell. The explants are preserving in the fixative agents such as buffered aldehyde and formalin/acid/alcohol mixtures. The next step is dehydration step. Dehydration removes fixative and water from the tissue and replaces them with dehydrating fluid. Another step is clearing process where the dehydrating fluid is replace with another fluid that is totally miscible with both the dehydrating fluid and the embedding medium. Clearing agents such as toluene, xylene, chloroform and benzene are commonly used in this process. After tissues have been dehydrated, the tissues will be embedded and sectioned. Generally, wax, polyethylene glycol (PEG), or resins (e.g. LR white) are used as embedding

material for histology. Paraffin wax is probably the most commonly used embedding material, being easy to section. Sectioning an embedded tissue sample is the next step necessary to produce sufficiently thin slices of sample that the detail of the microstructure of the cells/tissue can be clearly observed using microscopy techniques. Last step for preparing histology slides is staining. Staining is applied to give both contrast to the plant tissue and highlight the features of interest. Dyes are used to enhance and improve the visibility of the specimen and allowing quick and easy identification of specific cell types and cellular components.

2.1.6 Pigment Analysis

Pigment is a substance that produces a colour characteristic in tissues. Natural pigments can be found in plants, animals and minerals. Betalain, anthocyanins, carotenoids, chlorophylls and others flavonoids are good source of natural colorants and harmless for consumer use. *H.polyrhizus* contains high amount of betalains, the major bioactive compound and composed of red-violet betacyanin, water soluble pigments that provide colours in flowers and fruits (Wu *et al*., 2006).

Betalains are water soluble polyphenolic pigments and divided into two structural groups; the yellow betaxanthins and red-purple betacyanins. Polyphenolic compounds in fruits have many functions such as colorant, antimicrobial properties, and antioxidants values. Stevenson and Hurst (2007) reported that polyphenolic compounds can reduce the risk of cardiovascular disease and cancer. Le Bellec *et al.* (2006) found that *H. polyrhizus* are free from betaxanthins and only betacyanin can be found. The stability of betalains is greatly influenced by heat, oxygen, light, pH and moisture (Woo *et al.*, 2011). According to Huang and von Elbe (1987), discolouration of betacyanin is

relatively higher in storage of 25°C compared to 4°C storage. Betacyanins structures are characterized by cyclo-dopa with addition of acylation pattern on C_5 and C_6 (Figure 2.1). Betacyanins are classified into four kinds: betanin, amaranthine, gomphrenin and bougainvillea (Stintzing & Carle, 2004; Cai *et al.*, 2005). Betalamic acid and an acyclic amine group in betacyanins could act as excellent electron donors and enables them to scavenge free radicals. Several studies on betacyanin pigment stability have been investigated from various other plant species such as *Beta vulgaris* (de Azeredo *et al.*, 2009), *Basella alba* (Devi *et al.*, 2012), *Opuntia stricta* (Castellar *et al.*, 2012) and *Chenopodium formasanum* (Tsai *et al*., 2010).

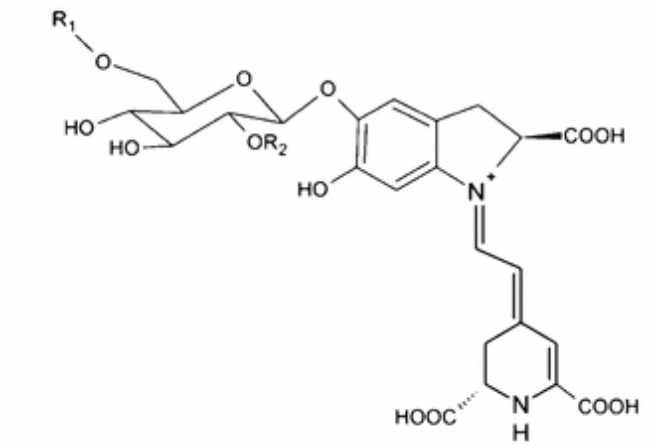

Figure 2.1: Structure of betacyanin, R_1=H; R_2=H (Wong & Siow, 2014)

2.2 CLASSIFICATION OF *Hylocereus* spp.

Cactaceae are species that mostly have spiny succulent photosynthetic stem. The cactus family is endemic from southern Canada to southern South America. Cacti are most common in semiarid habitats with low rainfall. There are about 300 species occur in dessert region. Cacti species have the ability for adaption in dry climate. It usually uses its spine to retain water. Cactaceae have 30-200 genera and 1000-2000 species characterized by the presence of betalain and p-plastids. Leaves of the cacti are associated with highly modified axillary buds or shoots called areoles.

Hylocereus polyrhizus belongs to the family of Cactaceae. It is also known as pitaya, strawberry pear, dragon fruit and night blooming cereus. Edible cacti species are classified based on the nature of the stem, color of the skin and color of fruit pulp. Various species of dragon fruit are identified by its different color skin and pulp (Table 2.1). Edible vine cacti species belong to two different genera *Hylocereus* and *Selenicereus,* while columnar cacti belong to three genera, *Cereus, Pachycereus* and *Stenocereus* (Crane & Balerdi, 2004).

Table 2.1: Various species of dragon fruit with different color skin and pulp.

Species	Color	
	Skin	**Pulp**
Hylocereus undatus	Red	White
Hylocereus triangularis	Yellow	White
Hylocereus costaricenes	Red	Red
Hylocereus polyrhizus	Red	Red
Hylocereus ocamponis	Red	Red
Selenicereus megalanthus	Yellow	White
Cereus triangularis	Yellow	White
Acanthocereus pitajaya	Yellow	White
Cereus ocamponis	Red	Red

The nomenclature of this species is as shown below:

Domain : Eukaryota

Kingdom : Plantae

Phylum : Spermatophyta

Subphylum : Angiospermae

Class : Dicotyledonae

Order : Caryophyllales

Family : Cactaceae

Genus : *Hylocereus*

Species : *Hylocereus polyrhizus*

Hylocereus spp. are perennial, terrestrial, epiphytic or climbing vine. They have triangular stems with corneous margins and 1-3 small spines and form aerial roots to

adhere or climb. The stem may reach about 20 ft height. The flower is hermaphroditic and some species are self incompatible. Flower is edible and white in color with 14 inch long and 9 inch wide. Temperature and light intensity may affect the blooming of the flower. The flower starts to open in the evening and completed by night. The flower will close after pollination and if not pollinated at night, they remain open until the next morning.

Hylocereus fruit is a fleshy berry, oblong, red skin and red pulp. Fruits develop from both ovary (pulp) and the receptacle that surrounds the ovary (peel). After anthesis, the peel color will change from green to red after 25 days. After 25-41 days of anthesis, the dry weight of fruit pulp increases significantly whilst peel dry weight and the water content in the peel decreases. Wybraniec *et al*. (2007) reported that the red colour of pitaya fruit is accredited by betacyanins, water soluble pigments.

Pitaya is propagated by seed and stem cutting. Seeds are small, numerous and black within the pulp. Seeds from selected fruits of mother plants were washed with water and germinating them on wet blotting papers or sand clay mixture. After germination, the seedlings can be potted for 4-5 weeks and ready for field planting by 9-10 months. Although the method is very simple, the quality of the offspring cannot be guaranteed due to cross pollination. Seedling also grow slowly and time consuming to reach bearing age longer than propagated by cutting. Asexual propagation or stem cutting are most preferred. The stems were slanted cut and treated with fungicide before planting. Mature cuttings are preferred because it is resistant to insect and snail damages. Rooting hormone is usually use to encourage quick formation of roots. The cut end of the stems was dipped in the hormone before inserting into soil. However,

according to Cavalcante *et al.* (2007) roots from the cuttings are very sensitive to water salinity and cause growth problem. Thus, cutting needs to be shaded and minimal water is required together with fertilizer before the roots develops. When the root development has occurred, the plants can be sun-hardened and planted into a mounded area. Dragon fruit also can be propagated by grafting. However, grafting is inconvenient method of propagation. Grafting method is beneficial when using selected rootstocks and scions.

2.2.1 MEDICINAL VALUES OF *Hylocereus* spp.

Hylocereus spp. contains many health benefits substances for human. Dragon fruits, like many other tropical fruits and vegetables, are believed to be rich in antioxidants known as betalains. Wu *et al.* (2006) indicated that the flesh and peel were both rich in polyphenols and were good sources of antioxidants. The presence of high level of vitamin C, minerals and pytoalbumin is regarded as relevant in fighting free radicals and possess anti-oxidant properties. The dragon fruit helps the digestive process, prevent colon cancer and diabetes, neutralize toxic substances such as heavy metal, reduce cholesterol levels and high blood pressure and consumed regularly the dragon fruit can help against asthma and cough. It is also rich with potassium, protein, fiber, sodium and calcium which goods for health than other fruits (Ruzainah *et al.*, 2009).

Aqueous extracts of the leaves, rind, fruit pulp and flowers of *Hylocereus undatus* have been studied for their wound healing properties (Perez *et al.,* 2005). Topical applications of *Hylocereus undatus* were shown to produce increases in hydroxyproline, tensile strength, total proteins, DNA collagen content and better

epithelization. Dragon fruit offer cosmetic scientists so much as a naturally active ingredient. Not only does this fascinating natural ingredient have proven anti aging properties but the story of the dragon fruit also offers a rich and exotic narrative for the cosmetic copywriter.

Dragon fruit also acts as an antibacterial agent to inhibit the growth of microorganisms. This is proved by Nurmahani *et al.*(2012), whereby the chloroform extract of red flesh pitaya peel inhibits the growth of *Bacillus cereus, Listeria monocytogenes* and *Campylobacter jejuni.* The dragon fruit juice also works as a wonderful sun shield because it rich in Vitamin B3 soothes and moisturizes dry sunburned skin.

2.2.2 OTHER PROPERTIES OF DRAGON FRUIT

Dragon fruit is a nutritious fruit with variety of uses for human consumption. A nutritional content of dragon fruit is stated below in Table 2.2. The fruit pulp of dragon fruit is commonly eaten raw as a fresh fruit. The fruit pulp contains various nutritional values. It is widely used in fruit salads and the flavour of the fruit pulp similar to the Kiwi fruit. Dragon fruit can be processed into a range of industrial products such as juice, ice cream, jam, syrup, yogurt, jelly, candy and pastries. Dragon fruit also can be used as food colouring agent. The flowers buds of dragon fruit are used to make soups, salads and tea.

Dragon fruit is an ideal fruit crop and could be an asset in plantation sector. It is a fast return perennial fruit crop with production in the second year after planting and full production within five years. It is also a good candidate plant for the development of sustainable agroforestry systems. Dragon fruit bears within six to nine months and yields could be obtained from the second year onwards. The average yield is about 10-12000 lg/ha at the end of the third year. The proper management of the vines and fruit thinning improves the size of the fruits and yield. The flower and fruits can be thinned to improve and maintain fruit size and quality when exported.

Table 2.2: Nutritional contents of stem and flesh of dragon fruit

Nutritional contents	Flesh	Stem
Moisture	82.5-83 g	96.0-98.0 g
Protein	0.159-0.229 g	0.120-0.270 g
Fat	0.21-0.61 g	0.09-0.23 g
Crude Fiber	0.7-0.9 g	0.02-0.05 g
Ascorbic acid	8-9 mg	63.71-132.95 mg L^{-1}
Ash	-	0.03-0.09 g
Water activity (A_w)	-	0.545-0.865 A_w
Glucose	-	0.263-0.552 g L^{-1}

Source: Ruzainah *et al.* (2009)

CHAPTER 3

IN VITRO REGENERATION OF *Hylocereus polyrhizus*

3.1 EXPERIMENTAL AIMS

Tissue culture is a technique that involves exposing plant tissues to a specific procedure of nutrients, hormones, and lights under sterile, *in vitro* conditions to produce many new plants, each a clone of the original mother plant, over a very short period of time. Composition of culture medium is one of the important factors in promoting the growth in plant tissue culture. The basic nutrient requirements of cultured plant cells are very similar to those of intact plants. The Murashige and Skoog (MS) medium is the medium widely used for plant regeneration. MS medium consist of macronutrients, micronutrients, irons and vitamins which are vital for the growth of plants. The supplementation of plant growth regulators also enhanced the micropropagation of plant under aseptic conditions. Plant growth regulators act by controlling or modifying plant growth processes, such as formation of leaves and flowers, elongation of stems, development and ripening of fruit. The aim of this chapter is to observe the effect of different media and hormones and subsequently identify the optimum medium for efficient micropropagation of *Hylocereus polyrhizus*.

3.2 MATERIALS AND METHODS

3.2.1 Preparation of explants

Seeds were removed from *Hylocereus polyrhizus* fruit fleshed and washed thoroughly under running tap water for 30 minutes followed by soaked in 70% ethanol for 2 minutes. The seeds were then soaked in 1% sodium hypochlorite and 6 % Tween 20 for 10 minutes. The seeds were washed by using sterile distilled water in the laminar flow chamber to maintain sterile condition. Seeds were dried for about 15 minutes before being cultured on the selected media.

3.2.2 Culture media compositions

Composition of culture medium is the most important factor promoting the growth and morphogenesis of plant tissue culture. The Murashige and Skoog (MS) salts compositions are the most widely used mainly in plant regeneration procedures. For seed germination in order to obtain normal growth, only the basal MS medium without any plant growth regulators was used.

MS media contains macronutrients, micronutrients, vitamins and irons stock. For macronutrient stock, 4.4 g/L of $CaCl_2.2H_2O$, 16.5 g/L of NH_4NO_3, 19.0 g/L of KNO_3, 17.0g/L of KH_2PO_4 and 3.7 g/L of $MgSO_4.7H_2O$ were diluted in 1L of distilled water in Scott bottle. For micronutrient, 0.0415 g of KI, 0.0013g of $CoCl_2.6H_2O$, 0.31g of H_3BO_3, 0.0125g of $Na_2MoO_4.2H_2O$, 0.845g of $MnSO_4.4H_2O$, 0.0013g of $CuSO_4.5H_2O$ and 0.43g of $ZnSO_4.7H_2O$ were mixed in 500ml of distilled water. By using 100 ml conical flasks, the FeEDTA and vitamins stock solution was obtained. For irons, 0.27785 g of $FeSO_4.7H_2O$ and 0.3725 of $Na_2EDTA.2H_2O$ were diluted in 100ml

of distilled water. For vitamins, 0.02g of glycine, 0.005g nicotinic acid and 0.005g pyridoxine were added in 100ml of distilled water. The conical flasks were wrapped with aluminium foil to prevent direct exposure of the light. All the stock solution was kept in the fridge 6 °C for further use.

For preparation of MS basal medium, 100 ml macronutrient, 10 ml micronutrient, 10 ml FeEDTA and 10 ml vitamins were added in conical flask and stirred. Next, 30 g of sucrose was added and the pH of the medium was adjusted to 5.8 using 1.0 N Hydrochloric acid (HCl) or 1.0 N Sodium hydroxide (NaOH). Technical agar and gelrite were used as solidifying agent and autoclaved at 121°C (15 psi) for 20 minutes. After autoclaved, MS medium were dispensed into 30 ml sterile container. The medium was allowed to cool prior to use. The combinations and concentrations of hormones which were used for plant regeneration study as shown below:

Table 3.1: List of combinations and concentrations of hormones for plant regeneration of *Hylocereus polyrhizus*

No.	Combinations and concentration of hormones
1.	MS basal (no hormone)
2.	MS + 0.1 mg/l BAP
3.	MS + 0.2 mg/l BAP
4.	MS + 0.3 mg/l BAP
5.	MS + 0.4 mg/l BAP
6.	MS + 0.5 mg/l BAP
7.	MS + 0.1 mg/l Kinetin
8.	MS + 0.2 mg/l Kinetin
9.	MS + 0.3 mg/l Kinetin
10.	MS + 0.4 mg/l Kinetin
11.	MS + 0.5 mg/l Kinetin
12.	MS + 0.1 mg/l BAP + 0.1mg/l NAA
13.	MS + 0.2 mg/l BAP + 0.1mg/l NAA
14.	MS + 0.3 mg/l BAP + 0.1mg/l NAA
15.	MS + 0.4 mg/l BAP + 0.1mg/l NAA
16.	MS + 0.5 mg/l BAP + 0.1mg/l NAA

3.2.3 Culture conditions

All the apparatus involved in tissue culture procedure such as scalpel and forceps must be in aseptic conditions to prevent any contamination. The culture apparatus were autoclaved for 20 minutes. UV light was switched on for at least 30 minutes and wiped with 70% ethanol before use to ensure sterility inside the laminar flow chamber. All the aseptic cultures were maintained in the culture room (16 hours light and 8 hours dark photoperiod and the temperature was maintained at 25 ± 1°C). The plantlets obtained were subcultured on the same medium at 2 months interval to maintain supply of fresh nutrient.

3.2.4 *In vitro* plant regeneration

The aseptic seedlings germinated from the seeds were used as explants sources in this study. The young stems (4 week-old) of *H. polyrhizus* were excised about 3-5mm and cultured on the MS medium supplemented with various concentrations of auxin and cytokinin such as 6-benzylamino purine (BAP), α-Naphthaleneacetic acid (NAA) and Kinetin (Kn). MS medium without any hormones (MS0) were used as control. The explants were also placed vertically and horizontally in the culture media. The cultures were maintained at a temperature of 25 ± 2°C with 16 hours light and 8 hours dark with 25 µmol m^{-2} s^{-1} of light intensity.

3.2.5 Data analysis

Data obtained were analysed using Duncan's Multiple Range Test (DMRT). The mean with the different letters in the same column differ significantly at p= 0.05.

3.3 RESULTS

3.3.1 *In vitro* Regeneration of *Hylocereus polyrhizus*

Plant regeneration or micropropagation is the technique whereby shoots and roots are being developed directly from parts of mother plants or explants. The addition of plant growth regulators in the media triggers the success of regeneration process. Plant growth regulators or hormones such as auxin and cytokinin influence the ability of the plant cells to segregate and differentiate to produce new organs.

In this chapter, young and healthy stems of *H. polyrhizus* were obtained from the 4-week-old aseptic seedlings cultured on MS basal media. The stems were cultured on two different positions which were horizontal and vertical. The development of the new shoots was observed after 1 week being cultured on MS medium supplemented with various concentrations and combinations of hormones. The highest average number of shoots was counted as 8.07 ± 0.721 on MS medium supplemented with 0.2 mg/L of BAP with explants being placed horizontally in the medium, while, 0.2 mg/L of Kn induced the longest shoots (1.608 ± 0.128 cm) when cultured in horizontal position. On MS medium supplemented with combinations of 0.1 mg/L of BAP and 0.1 mg/L NAA, the highest average number of roots (3.031 ± 0.237) in vertical culture position were observed while in the MS medium supplemented with 0.5 mg/L of BAP and 0.1 mg/L NAA resulted the longest roots formation when cultured in horizontal position. The addition of growth regulator such as BAP in the medium increased the regeneration of complete plantlet.

The result of this study showed that plantlets which were been cultured in horizontal position gave better response in multiple shoot formation. The stem of the explants were placed horizontally and this allowed almost all the surface of stem to be in contact with the medium. This position allowed the explants to obtain the nutrient from the medium and induced more multiple shoots. In certain species, polarity of the explants in the medium influenced the regeneration process.

Table 3.2: The effect of different combinations and concentrations of hormones on *Hylocereus polyrhizus* stem explants cultured on solid MS media at pH 5.75 under 25 ± 1°C.

MS + Hormones (mg/L)	No of shoots (Mean ± SE)		Length of shoot (Mean ± SE)		No. of roots (Mean ± SE)		Length of roots (Mean ± SE)	
	Vertical	Horizontal	Vertical	Horizontal	Vertical	Horizontal	Vertical	Horizontal
Ms no hormone	NR	NR	NR	NR	NR	NR	NR	NR
0.1 BAP	$4.2 \pm 0.511_{def}$	$5.4 \pm 0.535_{fg}$	$0.615 \pm 0.078_{cdefg}$	$0.637 \pm 0.074_{cdefgh}$	NR	NR	NR	NR
0.2 BAP	$7.4 \pm 0.774_{hi}$	$8.07 \pm 0.721_{i}$	$0.857 \pm 0.073_{ghi}$	$0.797 \pm 0.078_{fghi}$	NR	NR	NR	NR
0.3 BAP	$3.67 \pm 0.568_{de}$	$7.30 \pm 0.666_{hi}$	$0.3683 \pm 0.043_{abc}$	$0.888 \pm 0.103_{ghi}$	NR	NR	NR	NR
0.4 BAP	$6.50 \pm 0.689_{gh}$	$4.93 \pm 0.377_{ef}$	$0.649 \pm 0.055_{defgh}$	$0.568 \pm 0.067_{cdef}$	NR	NR	NR	NR
0.5 BAP	$3.37 \pm 0.478_{cd}$	$3.47\pm 0.436_{cd}$	$0.359 \pm 0.031_{abc}$	$0.458 \pm 0.032_{bcde}$	NR	NR	NR	NR
0.1 KINETIN	$3.43 \pm 0.338_{cd}$	$3.90 \pm 0.379_{de}$	$1.043 \pm 0.112_{i}$	$1.447 \pm 0.114_{j}$	NR	NR	NR	NR
0.2 KINETIN	$1.63 \pm 0.237_{ab}$	$2.93 \pm 0.239_{bcd}$	$0.916 \pm 0.173_{hi}$	$1.608 \pm 0.128_{j}$	NR	NR	NR	NR
0.3 KINETIN	$2.87 \pm 0.345_{bcd}$	$1.83 \pm 0.292_{ab}$	$0.736 \pm 0.079_{efgh}$	$0.733 \pm 0.096_{efgh}$	NR	NR	NR	NR
0.4 KINETIN	$3.27 \pm 0.437_{cd}$	$4.13 \pm 0.433_{def}$	$0.735 \pm 0.107_{efgh}$	$1.498 \pm 0.106_{j}$	NR	NR	NR	NR
0.5 KINETIN	$2.10 \pm 0.216_{abc}$	$1.77 \pm 0.184_{ab}$	$0.545 \pm 0.074_{cdef}$	$0.208 \pm 0.021_{ab}$	NR	NR	NR	NR

Table 3.2, continued

0.1 BAP + 0.1 NAA	NR	NR	NR	NR	$3.031 \pm 0.237_{c}$	$2.901 \pm 0.241_{c}$	$1.186 \pm 0.082_{bc}$	$2.308 \pm 0.211_{c}$
0.2 BAP + 0.1 NAA	NR	NR	NR	NR	$1.634 \pm 0.162_{ab}$	$2.202 \pm 0.269_{b}$	$0.911 \pm 0.064_{b}$	$0.988 \pm 0.117_{bc}$
0.3 BAP + 0.1 NAA	NR	NR	NR	NR	$2.103 \pm 0.147_{b}$	$1.333 \pm 0.154_{a}$	$1.465 \pm 0.182_{b}$	$0.322 \pm 0.041_{a}$
0.4 BAP + 0.1 NAA	$2.20 \pm 0.251_{abc}$	$1.73 \pm 0.262_{ab}$	$0.442 \pm 0.059_{abcd}$	$0.367 \pm 0.079_{abc}$	$1.101 \pm 0.182_{a}$	$1.233 \pm 0.184_{a}$	$1.263 \pm 0.252_{bc}$	$0.907 \pm 0.153_{b}$
0.5 BAP + 0.1 NAA	$0.87 \pm 0.164_{a}$	$0.80 \pm 0.200_{a}$	$0.222 \pm 0.045_{ab}$	$0.177 \pm 0.053_{a}$	$2.074 \pm 0.279_{b}$	$1.704 \pm 0.199_{ab}$	$0.803 \pm 0.087_{ab}$	$2.608 \pm 0.337_{c}$

Mean values with different letters within a column are significantly different at $p < 0.05$. (NR: no response), *n = 30*

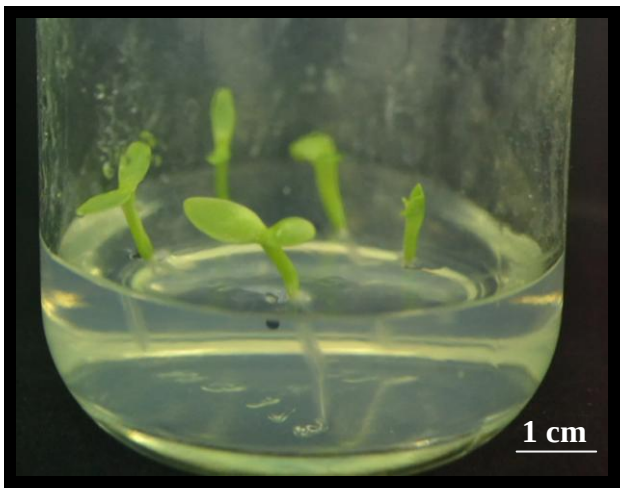

Figure 3.1.1: Germination of *Hylocereus polyrhizus* aseptic seedlings on MS medium after 1 week being cultured.

Figure 3.1.2: Development of multiple shoots from stem explants cultured horizontally on MS medium supplemented with 0.2 mg/L BAP.

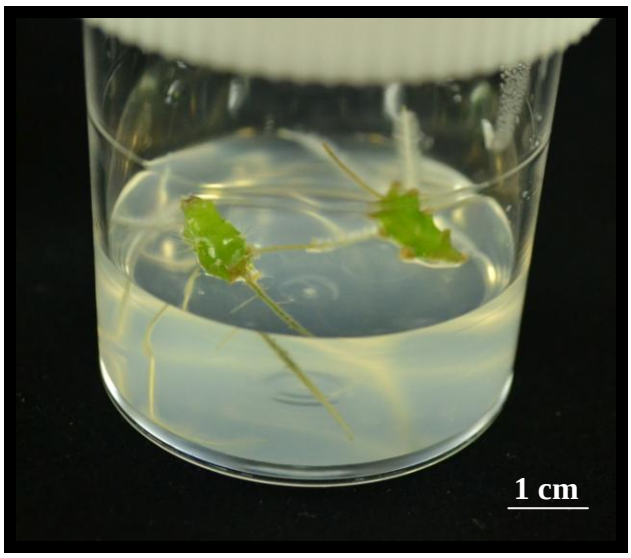

Figure 3.1.3: Formation of roots from stem explants cultured horizontally on MS media supplemented with 0.1 mg/L BAP and 0.1 mg/L NAA.

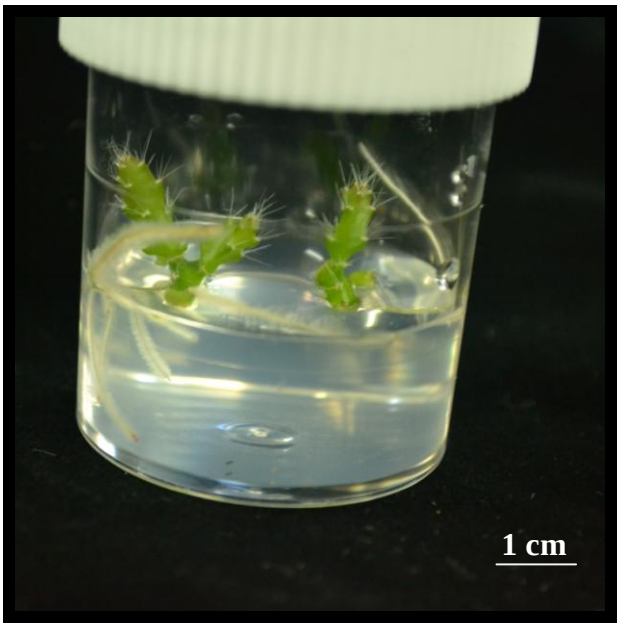

Figure 3.1.4: Development of shoots and roots from stem explants cultured vertically on MS medium supplemented with 0.5 mg/L BAP and 0.1 mg/L NAA.

3.4 SUMMARY OF RESULTS

1. An efficient protocol has been established for plant regeneration of *Hylocereus polyrhizus* through *in vitro* culture technique from stem explants.

2. Seeds of *Hylocereus polyrhizus* were germinated on MS basal medium and high frequency of multiple shoots could be induced with addition of PGRs.

3. Development of highest number of shoots (8 shoots) was observed on MS medium supplemented with 0.2 mg/L BAP when the explants were cultured horizontally with 8.07 ± 0.721 shoots.

4. The longest shoots were observed on MS medium supplemented with 0.2 mg/L Kinetin when cultured in horizontal which gave 1.608 ± 0.128 cm.

5. Formation of highest number of roots were observed on MS medium supplemented with 0.1 mg/L BAP and 0.1 mg/L NAA in vertical culture position with 3.031 ± 0.237 shoots per explant.

6. The longest roots (2.608 ± 0.337 cm) were observed on MS medium supplemented with 0.5 mg/L BAP and 0.1 mg/L NAA cultured in horizontal position.

CHAPTER 4

CALLUS INDUCTION OF *Hylocereus polyrhizus*

4.1 EXPERIMENTAL AIMS

Callus is defined as unorganized mass of undifferentiated tissues growing on media. Callus can form naturally on plants as a response to wounding and also can be induced by promoting hormones to plant cells. Callus can be multiplied and used to clone numerous whole plants. Callus cultures are initiated from a small part of an organ or tissue segment called explants on a growth supporting solidified nutrient medium under sterile conditions. Any part of the plant organ or tissues may be used as the explants. For callus induction, elevation of hormone levels and designated tissue culture media were utilized to produce callus with additions of cytokinins and auxins. The addition of other components such as vitamins and carbon sources helps callus culture to develop. Plant growth regulators are the critical media components in determining the developmental pathway in callus induction. Callus culturing is commonly performed in the dark. However, light esposure can encourage the differentiation of the callus.

Callus cultures are always used in plant genetic transformation process. Callus cells can receive the genes transfer, thus can be used to enhance crop plants traits for modern agriculture. Callus is use as starting material for the suspension culture which cells are separated and helps in the production of secondary metabolites. It is also useful for the synthesis of starting compounds that are subsequently modified to yield desirable product. The aim of this study is to investigate the production of callus by using various combinations and concentrations of hormones.

4.2 MATERIALS AND METHODS

4.2.1 Preparation of explants

Stem explants were used and obtained from 6-week-old aseptic seedlings that have been germinated *in vitro* and obtained in CHAPTER 3: *IN VITRO* REGENERATION OF *Hylocereus polyrhizus*.

4.2.2 Callus induction and medium compositions

Stem of explants were excised 3-5mm and the skin were peeled as well as the areoles. The peeled stems were cultured on MS medium supplemented with various combinations and concentration of hormones such as 2, 4-Dichlorophenoxyacetic acid (2, 4-D) and 6-benzylaminopurine (BAP). Thirty replicates of samples were used. The combinations and concentrations of hormones used for callus induction as listed below:

Table 4.1: List of combinations and concentrations of hormones for callus induction of *Hylocereus polyrhizus*

No.	Combinations and concentration of hormones
1.	MS basal (no hormone)
2.	MS + 0.1 mg/l 2,4-D
3.	MS + 0.5 mg/l 2,4-D
4.	MS + 1.0 mg/l 2,4-D
5.	MS + 1.5 mg/l 2,4-D
6.	MS + 2.0 mg/l 2,4-D
7.	MS + 1.0 mg/l BAP + 0.1 mg/l 2,4-D
8.	MS + 1.0 mg/l BAP + 0.5 mg/l 2,4-D
9.	MS + 1.0 mg/l BAP + 1.0 mg/l 2,4-D
10.	MS + 1.0 mg/l BAP + 1.5 mg/l 2,4-D
11.	MS + 1.0 mg/l BAP + 2.0 mg/l 2,4-D
12.	MS + 2.0 mg/l BAP + 0.1 mg/l 2,4-D
13.	MS + 2.0 mg/l BAP + 0.5 mg/l 2,4-D
14.	MS + 2.0 mg/l BAP + 1.0 mg/l 2,4-D
15.	MS + 2.0 mg/l BAP + 1.5 mg/l 2,4-D
16.	MS + 2.0 mg/l BAP + 2.0 mg/l 2,4-D

4.2.3 Culture conditions

All the materials involved in tissue culture procedure such as scalpel and forceps must be in aseptic conditions to prevent any contamination. The culture apparatus were autoclaved for 20 minutes. Laminar flow was exposed to UV light for at least 30 minutes and wiped with 70% ethanol before use to unsure sterility inside the chamber. All the aseptic cultures were maintained in the culture room (16 hours light and 8 hours dark photoperiod and the temperature were maintained at 25 ± 1°C). The plantlets obtained were subcultured onto the same medium at 2 months interval to maintain supply of fresh nutrient.

4.2.4 Data Analysis

Data were collected after 12 weeks. Data obtained were analysed using Duncan's Multiple Range Test (DMRT). The mean with the different letters in the same column differ significantly at p= 0.05.

4.3 RESULTS

4.3.1 Callus induction from stem explants of *Hylocereus polyrhizus*

In this study, there are two types of hormones used such as BAP and 2, 4-D in various concentrations and combinations to observe the effects on the callus induction of *H. polyrhizus*. MS basal medium without any hormone was used as a control and showed no callus formation. Skin and areoles covered the stem explants were peeled and the stem explants were placed on the medium in horizontal position.

All explants exhibited callus formation at the cut surfaces. In this study, the callus formation was well-developed in MS medium supplemented with combinations of 1.0 mg/L BAP and 0.1 mg/L 2, 4-D, 1.0 mg/L BAP and 1.0 2, 4-D and 2.0 mg/L and 1.0 mg/L 2, 4-D. The lowest percentage (27.0 ± 0.082 %) was observed when the stem explants were cultured on MS medium supplemented with 2.0 mg/L 2, 4-D. The combinations of BAP and 2, 4-D showed better responses in callus induction instead of 2, 4-D alone.

Various colours of the callus derived from stem explants were obtained such as green callus, white callus, yellow callus and light yellow callus. Green callus were observed on the MS medium supplemented with 1.0 mg/L BAP and 0.5 mg/L 2, 4-D, 1.0 mg/L BAP and 1.0 mg/L 2, 4-D, and 2.0 mg/L BAP and 1.0 mg/L 2, 4-D. The nature of the callus induced was compact on MS medium. Peeled-stem explant was found to be effective as explant source for callus induction. The success of callus formation largely relies on the selection of appropriate plant part, which is to be used as the starting material for this experiment.

Purple-colored callus was obtained when pulps of *H. polyrhizus* were cultured on MS medium without any hormone. However, the callus showed slow response and survival rates was very low. The color of the callus changed from purple into brown in color after 3 weeks. When pulps of *H. polyrhizus* were cultured on the medium with addition of any hormones, no callus was produced. The pulp changed into brown in color and no response was observed.

Table 4.2: Callus induction form stem explants of *Hylocereus polyrhizus* cultivated on MS medium with different concentrations and combinations of BAP and 2, 4-D at 25 ± 1°C with 16 hours light and 8 hours dark for 12 weeks.

Concentration of hormones (mg/L)	**Mean (%) + SE of callus formation**	**Observation**
MS no hormone	$0.00 \pm 0.000_{a}$	No callus
0.1 2, 4-D	$80.0 \pm 0.074_{bc}$	Light green callus
0.5 2, 4-D	$70.0 \pm 0.085_{bc}$	Yellow callus
1.0 2, 4-D	$90.0 \pm 0.056_{cde}$	White callus
1.5 2, 4-D	$73.0 \pm 0.082_{bc}$	Yellow callus
2.0 2, 4-D	$27.0 \pm 0.082_{bc}$	Green yellowish callus
1.0 BAP + 0.1 2, 4-D	$100.0 \pm 0.000_{e}$	White callus
1.0 BAP + 0.5 2, 4-D	$90.0 \pm 0.056_{cde}$	Green callus
1.0 BAP + 1.0 2, 4-D	$100.0 \pm 0.000_{e}$	Green callus
1.0 BAP + 1.5 2, 4-D	$80.0 \pm 0.074_{bc}$	White callus
1.0 BAP + 2.0 2, 4-D	$77.0 \pm 0.079_{bc}$	Light yellow callus

Table 4.2, continued

2.0 BAP + 0.1 2, 4-D	$73.0 \pm 0.082_{bc}$	Green callus
2.0 BAP + 0.5 2, 4-D	$93.0 \pm 0.046_{de}$	Green callus
2.0 BAP + 1.0 2, 4-D	$100.0 \pm 0.000_{e}$	Light green yellowish callus
2.0 BAP + 1.5 2, 4-D	$73.0 \pm 0.082_{bc}$	Yellow white callus
2.0 BAP + 2.0 2, 4-D	$87.0 \pm 0.063_{bcde}$	Light yellow callus

Mean values with different letters within a column are significantly different at $p < 0.05$.

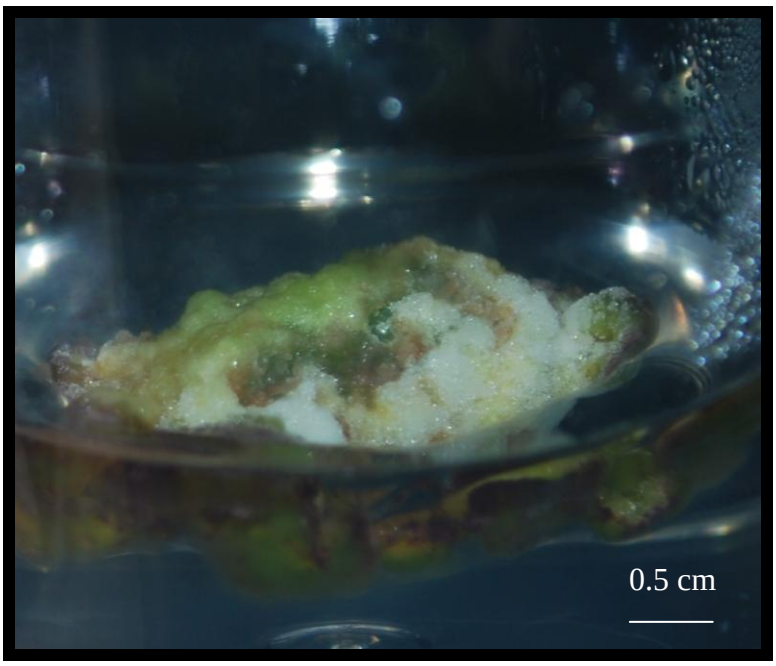

Figure 4.1.1: Callus formation of *Hylocereus polyrhizus* from stem explants on MS medium supplemented with 2.0 mg/L BAP and 1.0 mg/L 2, 4-D.

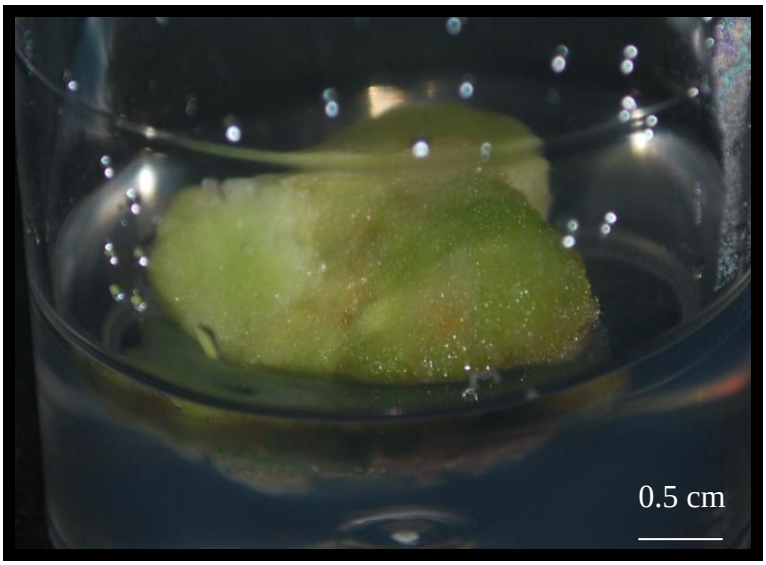

Figure 4.1.2: Callus formation of *Hylocereus polyrhizus* from stem explants on MS medium supplemented with 2.0 mg/L BAP and 0.5 mg/L 2, 4-D.

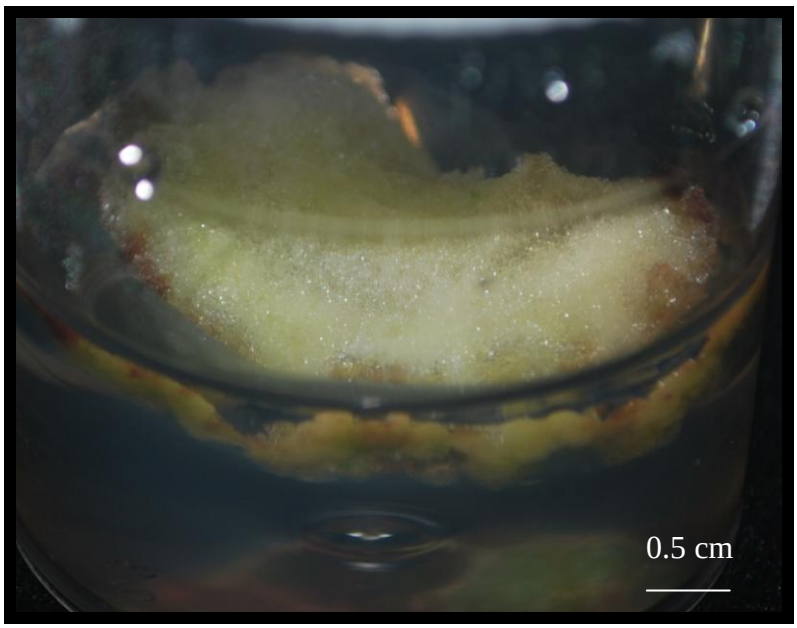

Figure 4.1.3: Callus formation of *Hylocereus polyrhizus* from stem explants on MS medium supplemented with 2.0 mg/L BAP and 1.0 mg/L 2, 4-D.

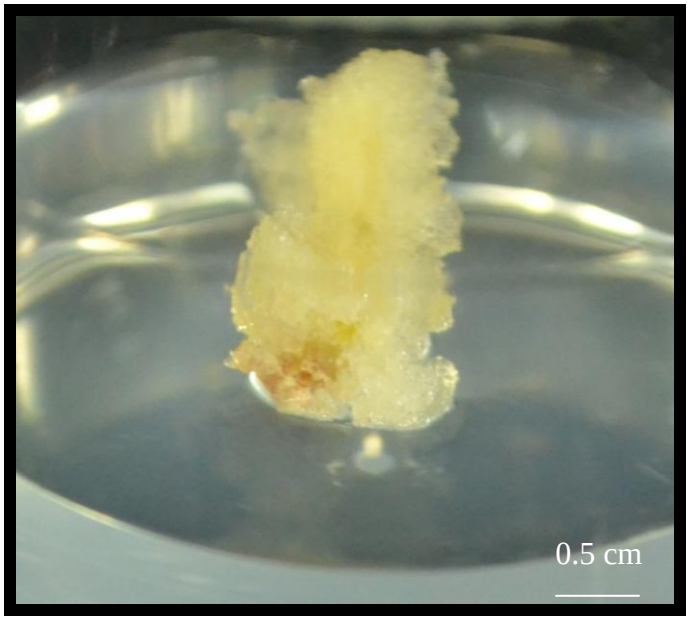

Figure 4.1.4: Callus formation of *Hylocereus polyrhizus* from stem explants on MS medium supplemented with 1.5 mg/L BAP.

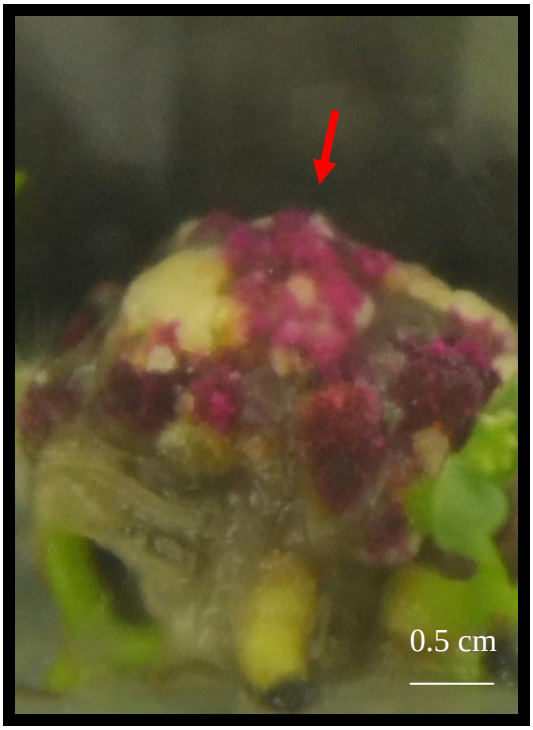

Figure 4.1.5: Purple colored callus (arrow) was obtained from pulp of *H. polyrhizus* when cultured on MS basal medium.

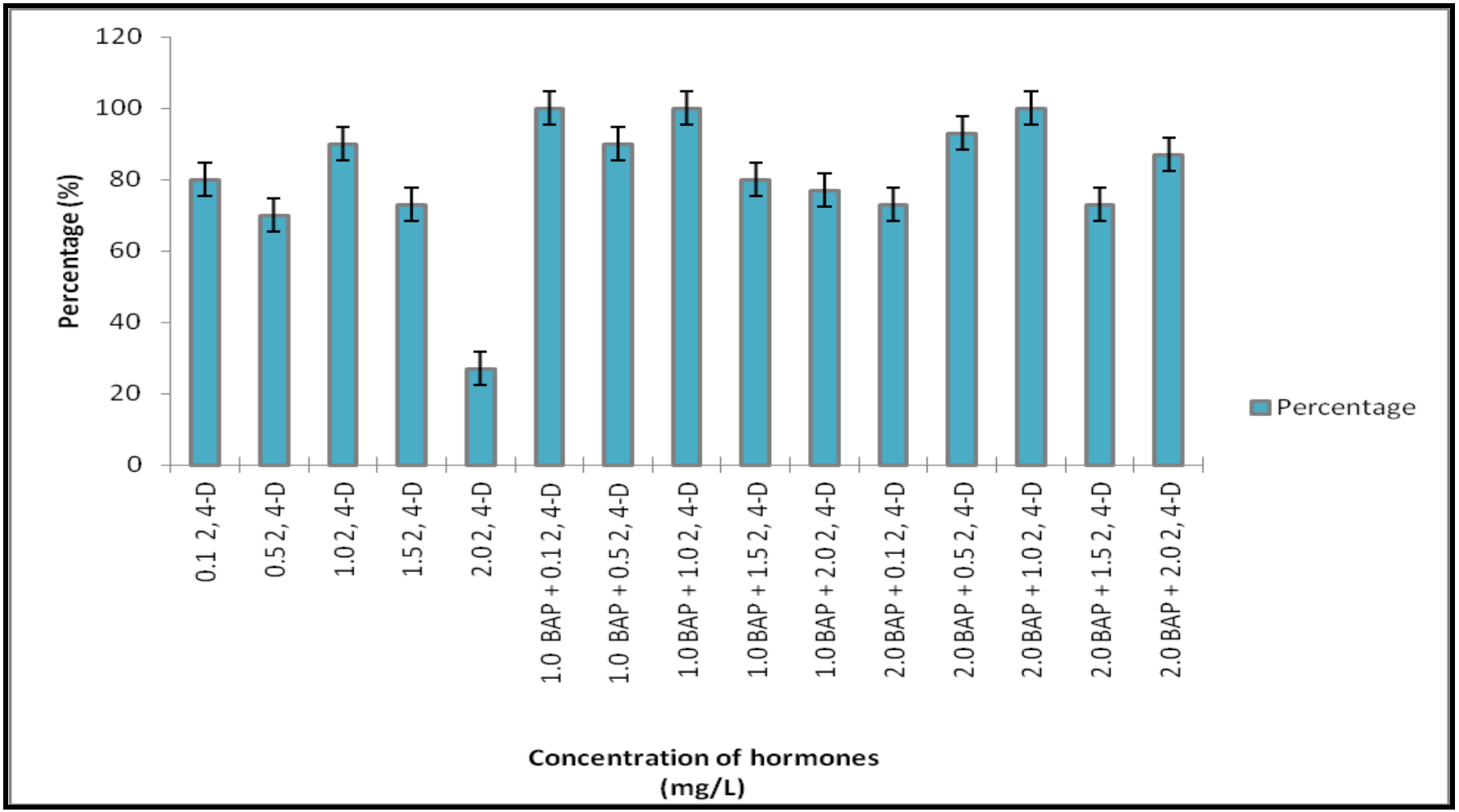

Figure 4.1.6: Percentage of callus induction from stem explants of *Hylocereus polyrhizus* cultured on MS medium supplemented with different concentrations and combinations of hormones under 16 hours dark at 25 ± 1 °C after 12 weeks.

4.4 SUMMARY OF RESULTS

1. MS medium supplemented with 2, 4-Dichlorophenoxyacetic acid and 6-benzylaminopurine managed to induce the formation of callus from stem explants. MS basal medium did not give any callus formation.

2. The combinations of hormones (2, 4-D and BAP) resulted in a better callus production than single applied hormone.

3. The highest percentage of callus production (100%) was obtained when stem explants were cultured on MS medium supplemented with 1.0 mg/L BAP and 0.1 mg/L 2, 4-D, 1.0 mg/L BAP and 1.0 mg/L 2, 4-D, and 2.0 mg/L BAP and 1.0 mg/L 2, 4-D.

4. The lowest percentage of callus formation (27.0 ± 0.082 %) was obtained when the stem explants were cultured on MS medium supplemented with 2.0 mg/L 2, 4- D alone.

CHAPTER 5

SYNTHETIC SEED OF *Hylocereus polyrhizus*

5.1 EXPERIMENTAL AIMS

Synthetic seeds are defined as artificially encapsulated somatic embryos, shoot buds, cell aggregates, or any other tissue that can be used for sowing as a seed and that possess the ability to convert into a plant under *in vitro* or *ex vitro* conditions and that retain this potential also after storage. Synthetic seed has the potential for long term storage without losing viability and also maintains the clonal nature of the resulting plants. Synthetic seeds have great potential for large scale production of plants at low cost as an alternative to true seeds. For *Hylocereus polyrhizus*, low seed viability is a problem for propagating this plant through seed and propagation via cutting is preferred. The development of synthetic seed of *Hylocereus polyrhizus* can overcome this problem thus enhanced the production of this plants. In this experiment, different encapsulation matrix was used to obtain suitable matrix endosperm for synthetic seed of this species. The most suitable concentration of alginate and calcium chloride dehydrate solution for optimum encapsulation matrix and percentage of germination synthetic seeds of this species will be identified. Besides, the viability of the seeds after certain storage periods will be observed.

5.2 MATERIALS AND METHODS

5.2.1 Sources of explants

Microshoots of the explants were obtained from the *in vitro Hylocereus polyrhizus* regenerated plants for production of synthetic seed.

5.2.2 Preparation of Culture media

5.2.2.1 Preparation of MS Stock Solution

For synthetic seed production, MS stock solutions such as macronutrient without calcium chloride, micronutrient, vitamins and irons were prepared separately in the conical flask. To prepare 1 litre of stock solution for macronutrients without calcium, the chemical compounds were added into conical flask and stirred until fully dissolved. The same procedure was repeated to prepare stock solution for micronutrients, vitamins and irons. All the stock solution was stored at 4 °C prior to use.

Table 5.1: Lists of MS medium components for synthetic seeds.

Component of MS medium (stock solution)	**Concentration (g/l)**
A) Macronutrients without calcium	
• Ammonium nitrate (NH_4NO_3)	16.5
• Potassium nitrate (KNO_3)	19.0
• Magnesium sulphate ($MgSO_4.7H_2O$)	3.7
• Potassium dihydrogen orthophosphate (KH_2PO_4)	1.7
B) Micronutrients	
• Manganese sulphate ($MnSO_4.4H_2O$)	1.56
• Zinc sulphate ($ZnSO_4.7H_2O$)	0.86
• Potassium iodide (KI)	0.083
• Cupric sulphate ($CuSO_4.5H_2O$)	0.0025
• Sodium molybdate ($Na_2MoO_4.2H_2O$)	0.025
• Cobaltus chloride ($CoCl_2.6H_2O$)	0.0025
• Boric acid (H_3BO_3)	0.062
C) Vitamins	
• Nicotinic acid	0.05
• Thiamine HCl	0.5

• Pyridoxine HCl	0.5
D) Irons	
• Sodium EDTA Ferric salt (Na_2 EDTA)	3.73
• Ferrous sulphate (Fe $SO_4.7H_2O$)	2.78

5.2.2.2 Preparation of Sodium Alginate Solution

In order to prepare the 100 ml Sodium Alginate solution, 10 ml of macronutrient without calcium, 1 ml micronutrient, 1 ml vitamin and 1 ml iron was added in the conical flask containing 50 ml of distilled water. The mixture was stirred and 3 g of sucrose and 0.1 g myo-inositol were added to the solution. While stirring, the solution was heated up before 3 g of sodium alginate was added and mixed until fully dissolved. The pH of the solution was adjusted to 5.75 and distilled water was top-upped to make the volume to 100 ml. The Sodium Alginate solution was autoclaved at 121° C (15 psi) for 20 minutes.

5.2.2.3 Preparation of Calcium chloride Dehydrate Solution

Calcium chloride dehydrate solution ($CaCl_2.2H_2O$) act as complexing agent to allow the hardening of encapsulated bead of synthetic seeds. In 500 ml conical flask, 7.351 g of $CaCl_2.2H_2O$ was added and mixed with 300 ml of distilled water. The solution was stirred until completely dissolved. Distilled water was then top-upped to make the volume to 500 ml and was autoclaved at 121° C (15 psi) for 20 minutes.

5.2.3 Culture conditions

All the apparatus involved in tissue culture procedure such as scalpel and forceps must be in aseptic conditions to prevent any contamination. The culture apparatus were autoclaved for 20 minutes. UV light was switched on for at least 30 minutes in the laminar flow chamber and wiped with 70% ethanol before use to ensure sterility inside the chamber. All the aseptic cultures were maintained in the culture room with16 hours light and 8 hours dark and the temperature was maintained at 25 ± 1°C.

5.2.4 Encapsulation of microshoots

Microshoots from 2-week-old *in vitro* regenerated plants were obtained and excised into small pieces under aseptic condition inside the laminar flow chamber. The microshoots were submerged in a sodium alginate solution and subsequently sucked through micropipette into a modified 5 ml pipette. Then, the microshoots were dropped one by one into the $CaCl_2.2H_2O$ solution. The beads contained microshoots were allowed to be hardened by allowing them to remain in $CaCl_2.2H_2O$ solution for 30 minutes and stirred gently for several times. After 30 minutes, the beads were taken out and transferred into sterile distilled water. The beads were being washed for 3 times to wash out the excess $CaCl_2.2H_2O$ solution. All beads were dried out on the sterile tissue paper before being cultured on the selected MS medium.

5.2.4 Germination of synthetic seeds on MS medium

Encapsulated beads supplemented with and without supplementation of PGRs such as BAP, Kinetin and NAA were cultured on MS basal medium. Some encapsulated beads also were stored at low temperature of 4°C for 10, 30 and 120 days before being

cultured on MS medium without any hormone to examine the seed viability in long term storage. 30 replicates of samples were used. The optimum survival percentage of the encapsulated beads was determined.

5.2.5 Germination of the Synthetic Seeds on Sowing Soil

Synthetic seeds were germinated on two types of sowing soil in two different conditions; sterile and non-sterile. Two types of sowing soils are black soil and red soil. For sterile conditions, both sowing soils were autoclaved at 121° C (15 psi) for 20 minutes. The beads were sown in sterile soil, moistened by sterile distilled water and maintained in the culture room (16 hours light and 8 hours dark photoperiod and the temperature was maintained at 25 ± 1°C). For non-sterile condition, the beads were directly sown into the black and red soil under natural environment and moistened by tap water. The most suitable sowing soil and condition were identified.

5.2.7 Data analysis

Data obtained were analysed using Duncan's Multiple Range Test (DMRT). The mean with the different letters in the same column differ significantly at p= 0.05.

5.3 RESULTS

5.3.1 Germination of encapsulated beads on different culture media

Encapsulated beads obtained from this study were supplemented with three different hormones concentrations and combinations which are BAP, Kinetin and combinations of NAA and BAP. Synthetic seeds without any hormones were also being cultured as control. All synthetic seeds were cultured on MS medium. Synthetic seed cultured on MS basal medium showed successful germination into complete plantlets. Emergence of initial shoots was observed on Day 10. However, encapsulated beads containing hormones did not form complete plantlets but only callus formation was observed. All callus turned brown in color and died after 12 weeks. No further development was observed on the synthetic seeds germination when hormones were introduced to the encapsulated beads.

Table 5.2: Effects of different culture media on germination of synthetic seeds of *Hylocereus polyrhizus*

Types of culture medium	Observations
MS basal medium	All encapsulated beads generated into complete plantlets
MS + 0.2 mg/L BAP	All encapsulated beads did not germinate into complete plantlets but only formed callus.
MS + 0.2 mg/L Kinetin	All encapsulated beads did not germinate into complete plantlets but only formed callus.
MS + 0.1 mg/L BAP + 0.1 mg/L 0.2	All encapsulated beads did not germinate into complete plantlets but only formed callus.

5.3.2 Growth of encapsulated beads after being stored at different storage period.

Encapsulated beads which were stored at different storage period (0, 10 and 120 days) were germinated in MS medium. Effects of the storage period were examined to monitor the viability of the synthetic seeds of *Hylocereus polyrhizus* at low temperature (4 °C) in the dark. Synthetic seeds which were stored for 0 day showed 100% of conversion percentage into complete plantlets. However, synthetic seeds which were stored for 10 days showed decrease in conversion percentage (40.0%). The encapsulated beads which were stored for 120 days showed no response and no emergence of shoots were observed.

Table 5.3: Germination of synthetic seeds of *Hylocereus polyrhizus* at different storage durations.

Storage durations (days)	**Conversion frequency (%)**	**Observations**
0	100.00	All encapsulated beads germinated into complete plantlets
10	40.00	Some encapsulated beads did not give any response and became contaminated
120	0.00	No response

5.3.3 Germination of synthetic seeds on Various Sowing Substrates

In the present study, synthetic seeds of *Hylocereus polyrhizus* were germinated on two types of sowing soil under two different conditions; sterile and non-sterile. Sowing substrates used were black and red soil. The soils were moistened with distilled water and encapsulated beads were sown directly after encapsulation of the microshoots. In red soil, the beads showed no response and no emergence of shoots were observed. The beads were shrinking and became contaminated around the beads. In sterile red soil, no germination of the beads was observed and the beads were shrunk. In black soil, synthetic seeds showed negative response and did not germinate into complete plantlets. The same response was also observed in sterile black soil. All beads were shrunk after 10 days and contaminated with fungi. No germination into complete plantlets was seen when the beads were sown either in sterile or non-sterile soils.

Table 5.4: Synthetic seeds germination of *Hylocereus polyrhizus* in different sowing soils.

Observations of synthetic seeds of *Hylocereus polyrhizus*			
Types of soils			
Sterile		**Non-sterile**	
Black soil	**Red soil**	**Black soil**	**Red soil**
All encapsulated beads did not give any response and beads were shrunk and contaminated	All encapsulated beads did not give any response and became shrink.	All encapsulated beads did not give any response and started to shrink after 10 days and became contaminated.	All encapsulated beads did not give any response.

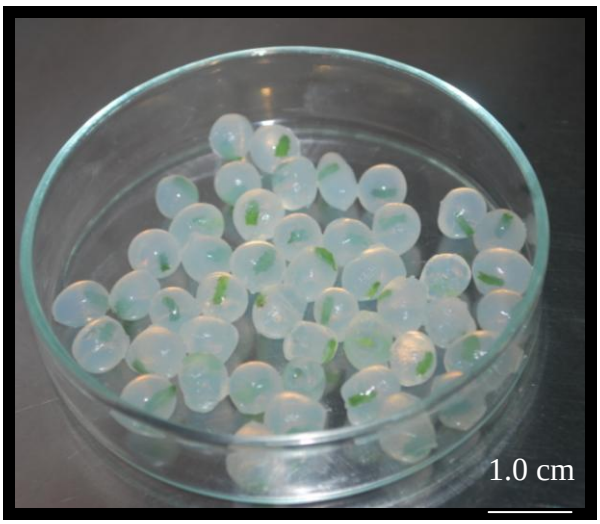

Figure 5.1.1: Synthetic seeds of *Hylocereus polyrhizus* before being stored at low temperature (4 ±1 °C).

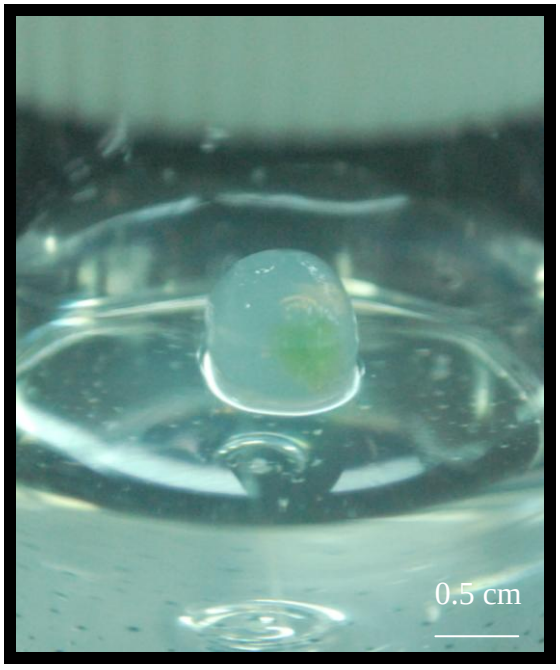

Figure 5.1.2: Encapsulated bead of *Hylocereus polyrhizus* cultured on MS basal medium on Day 0.

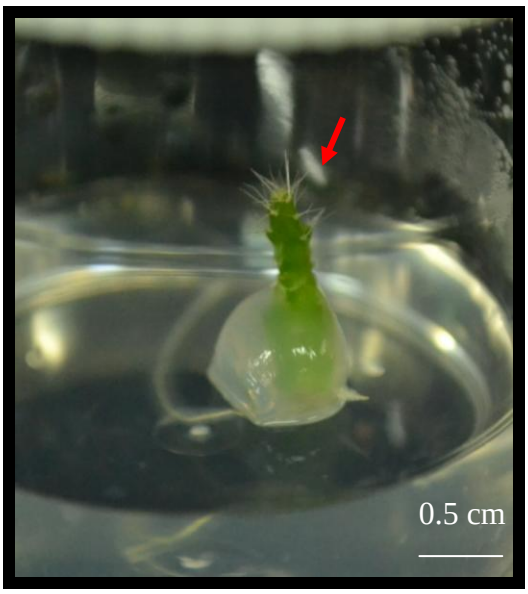

Figure 5.1.3: Emergence of shoot bud from encapsulated bead of *Hylocereus polyrhizus* cultured on MS basal medium after 10 days.

Figure 5.1.4: Multiple shoots and roots formation from synthetic seeds of *Hylocereus polyrhizus* after being cultured on MS basal medium for 8 weeks.

5.4 SUMMARY OF RESULTS

1. Synthetic seeds were successfully created from microshoots of *Hylocereus polyrhizus* using 3% (w/v) Sodium Alginate and 100 mM Calcium Chloride solution.

2. Synthetic seeds of *Hylocereus polyrhizus* were successfully germinated on MS basal medium.

3. Synthetic seeds of *Hylocereus polyrhizus* showed the best conversion frequency (100%) when being cultured directly after encapsulation process. Long term storage (120 days) decreased the viability of the seeds and totally no germination was observed.

4. Synthetic seeds of *Hylocereus polyrhizus* did not give any response when germinated on sterile and non sterile sowing soils. All beads were shrunk and eventually contaminated after 10 days.

CHAPTER 6

ACCLIMATIZATION OF *Hylocereus polyrhizus*

6.1 EXPERIMENTAL AIMS

Acclimatization is the process whereby the plants adjust and adapt to the gradual change in their environment. The plants need to have ability to survive in the natural environment conditions such as a change of temperature, humidity, photoperiod or pH upon transfer from *in vitro* to *ex vitro*. During *in vitro* regeneration, plants materials rely on sterile environment, in conjunction with defined media containing nutrients and plant growth regulators. The plants are also cultured in the limited space and ventilation, thus placed under fluorescent lighting.

The process of acclimatization allows the plants to continue surviving without the addition of plant hormones or nutrients. However, some plantlets might be easily impaired by sudden changes in environmental conditions. Acclimatization is a vital process in order to ensure the highest number of survival rate and grow vigorously when being transferred to *ex vitro* conditions (Hazarika, 2003). In this chapter, the aim of the experiment was to measure the survival rates of acclimatized plantlets using different sowing soil. Soil analysis X-ray diffractometry (XRD) and X-ray fluorescence spectrometry (XRF) also were done to observe the main components in the sowing soil used in the acclimatization process. The arrangement of these two techniques forms a potent tool for characterization of mineral composition of multiphases rock and soil samples which the individual techniques are not capable to convey. XRD is used to determine the presence and amounts of minerals species in sample, as well as identify

phases. XRD is non-destructive, fast and simple sample preparation for determination and characterization of mineral phases in particular soils.

6.2 MATERIALS AND METHODS

6.2.1 Acclimatization of *In vitro* plantlets

Well-grown *in vitro* plantlets via direct regeneration were acclimatized for further growth and development. The 12-week-old plantlets of *Hylocereus polyrhizus* from MS medium supplemented with 0.1 mg/L BAP and 0.1 mg/L NAA were used for acclimatization. Thirty (30) replicates of samples were used.

6.2.2 Effects of plantlets of *Hylocereus polyrhizus* growth on Various Substrates

The *in vitro* plantlets were taken out from the container and washed under running tap water carefully to remove all adhering agar residues. The entire excess agar from the medium must be completely removed to prevent the infection of microorganisms and lead to contamination. Plantlets were then planted on three types of sowing soils; black soil, red soil and the mixture of black soil, red soil and cocoa peat (1:1:1). The plantlets were covered with transparent plastic and placed in the culture room for 1 week. After one week, the plastic covering the plantlets were removed and transferred to shaded place or greenhouse to avoid direct sunlight. The plants were watered once a week. Lastly the plants were transferred to bigger pots before being planted on the field for further development. Percentage of survival was observed upon transfer the acclimatized *Hylocereus polyrhizus* plants to greenhouse. The substrates used in the acclimatization as below:

1. Black soil
2. Red soil
3. Black soil: Red soil: Cocoa peat(1:1:1)

6.2.3 Soil Analysis

Characterization of mineral composition in soil used for acclimatization process had been measured using X-ray diffractometry (XRD) and X-ray fluorescence spectrometry (XRF). The soil samples were ground into powder form and compressed into pallet before being analyzed. The composition of the soil samples were identified and compared thus acknowledging the most suitable compositions that allowed highest plantlets survival rates.

6.2.4 Statistical Data Analysis

Data obtained were analyzed using Duncan's Multiple Range Test (DMRT). The mean with the different letters in the same column differ significantly at p= 0.05.

6.3 RESULTS

6.3.1 Acclimatization of *Hylocereus polyrhizus* on Various Sowing Soil

Healthy and well rooted *in vitro* plantlets which were obtained from the previous study on Chapter 2 were maintained and subcultured for acclimatization process. *Hylocereus polyrhizus* plantlets were transferred to pots containing three types of sowing soils; black soil, red soil and the combinations of red soil: black soil: cocoa peat (1:1:1). All *in vitro* grown plantlets were acclimatized under similar conditions and the survival rates during acclimatization were observed.

Table 6.1 showed the highest percentage of survival for *in vitro* plants acclimatized in the combination of red soil, black soil and cocoa peat (83.3 % ± 0.069). Black soil showed 67.7 % ± 0.088 and followed by red soil 57.7 % ± 0.088. The maximum shoot length was observed from plant grown in combination soil and the minimum shoot length was observed from plant grown in red soil after 4 months.

Table 6.1: Comparison of acclimatization process of *Hylocereus polyrhizus* on various growing subtrates.

Type of soil	Percentage of survival (%)	Observations
Black soil	67.7 % ± 0.09a	Plantlets moderately survived with healthy growth. However, height of plantlets showed slow response.
Red soil	57.7 % ± 0.09a	Plantlets moderately survived with healthy growth. However, height of plantlets showed slow responses and need to water regularly to maintain the moisture of the soil.
Red soil: Black soil: Cocoa peat (1:1:1)	83.3 % ± 0.07b	Plantlets survived with healthy growth. The maximum height of plantlets were obtained and showed good response.

Mean values with different letters within a column are significantly different at $p < 0.05$.

Figure 6.1.1: Shoot formation of *Hylocereus polyrhizus* on MS medium supplemented with 0.2 mg/L BAP after 12 weeks being cultured and ready for acclimatization.

Figure 6.1.2: Plantlets of *Hylocereus polyrhizus* acclimatized on combination of red soil: black soil and cocoa peat (1:1:1) after 1 week.

Figure 6.1.3: Well grown *Hylocereus polyrhizus* plantlets on soil combinations of red soil: black soil:cocoa peat after 9 months.

6.3.2 Soil Analysis

Three types of sowing soils used in this study were sent for analysis using X-ray Diffraction (XRD) and X-ray fluorescence (XRF). XRD is used to determine the presence and amounts of minerals formed in sample, as well as identify phases of soils. Determination of multi element composition in the suitable soil used in acclimatization was analyzed by X-ray fluorescence (XRF) spectrometry.

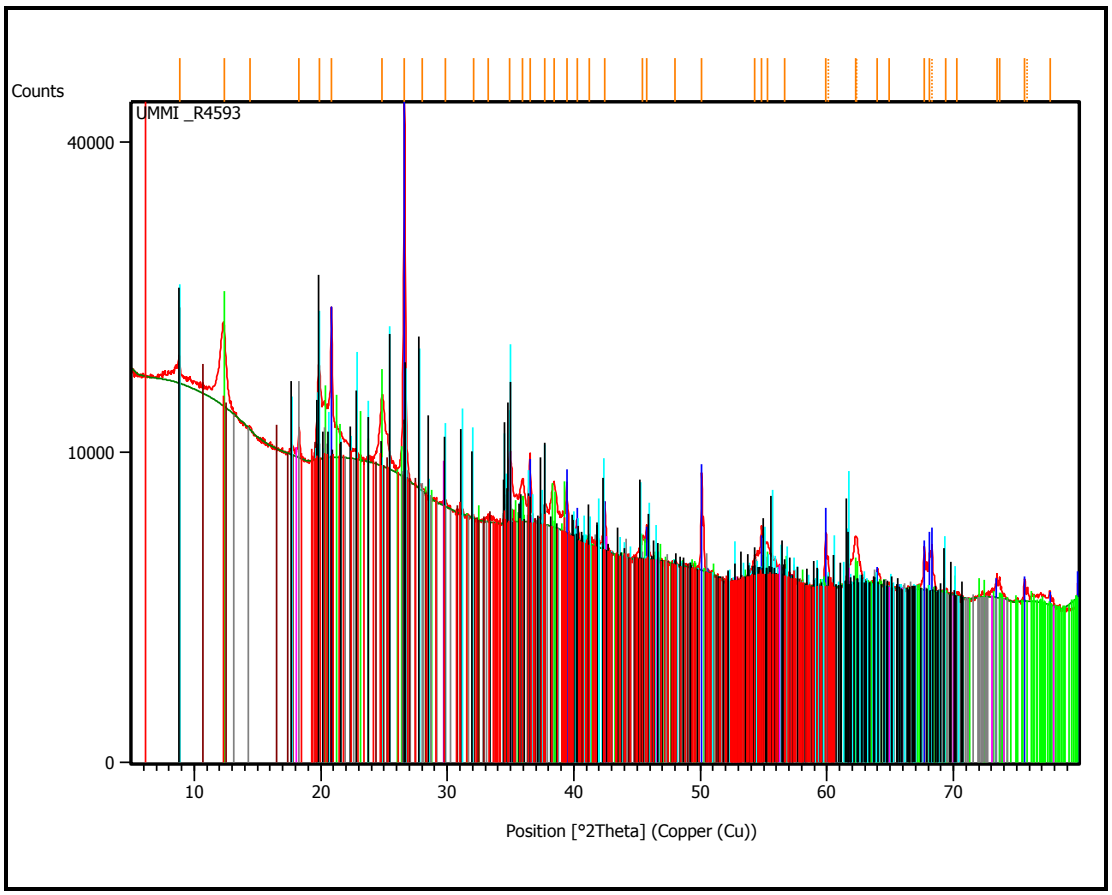

Figure 6.1.4: Graph listing all peaks of individual compound in red soil generated by X-ray Diffractometer (XRD)

Figure 6.1.4 shows the 45 phases or components that can be detected from red soil as represented by the peaks. Nevertheless, there are 8 major components or compounds presence in the red soil, as detected by X-ray Diffractometer (XRD). The peaks representing these major compounds are presented in Figure 6.1.5.

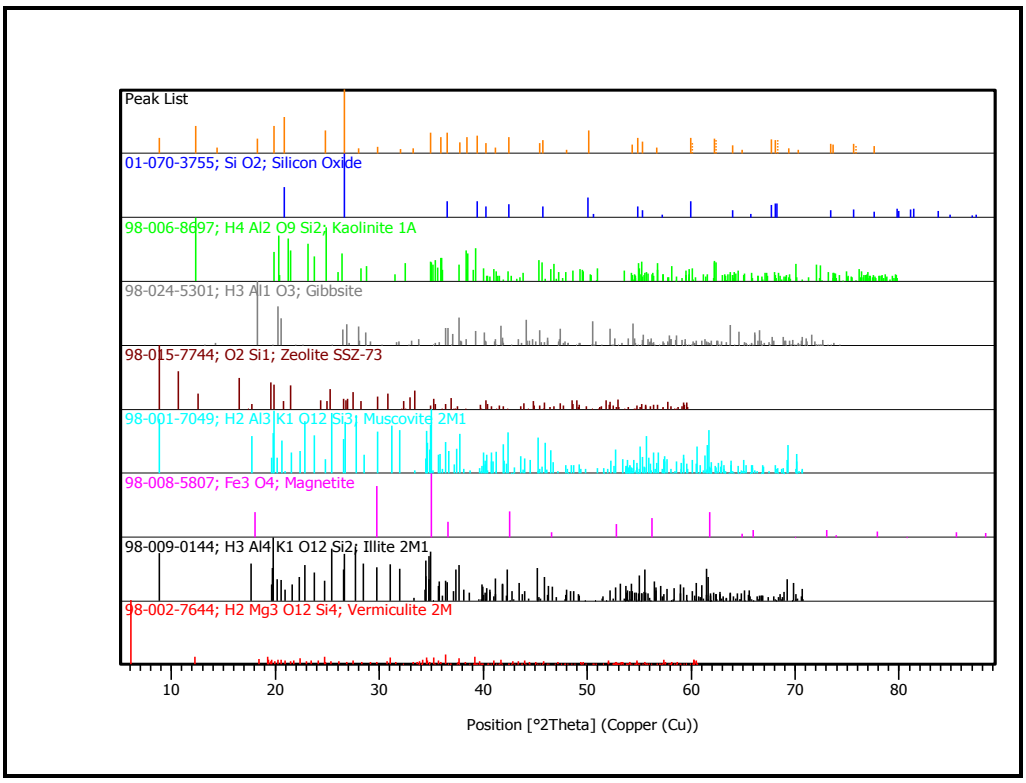

Figure 6.1.5: Graph listing the peaks of major compound in red soil generated by X-ray Diffractometer (XRD). *The identity of the reference code is stated in Table 6.2.

The major compounds in red soils are silicon oxide, kaolinite, gibbsite, zeolite, muscovite, magnetite, illite and vermiculite, as shown in Table 6.2.

Table 6.2: Major compounds in red soil as identified by X-ray Diffractometer (XRD).

Ref. Code	Score	Compound Name	Chemical Formula
01-070-3755	72	Silicon Oxide	Si O2
98-006-8697	36	Kaolinite 1A	H4 Al2 O9 Si2
98-024-5301	22	Gibbsite	H3 Al1 O3
98-015-7744	18	Zeolite SSZ-73	O2 Si1
98-001-7049	29	Muscovite 2M1	H2 Al3 K1 O12 Si3
98-008-5807	24	Magnetite	Fe3 O4
98-009-0144	21	Illite 2M1	H3 Al4 K1 O12 Si2
98-002-7644	1	Vermiculite 2M	H2 Mg3 O12 Si4

Figure 6.1.6 showed the 35 phases or components were detected from the black soil, as represented by peaks. There are only 5 major compounds that are present in the black soil, as detected by X-ray Diffractometer.

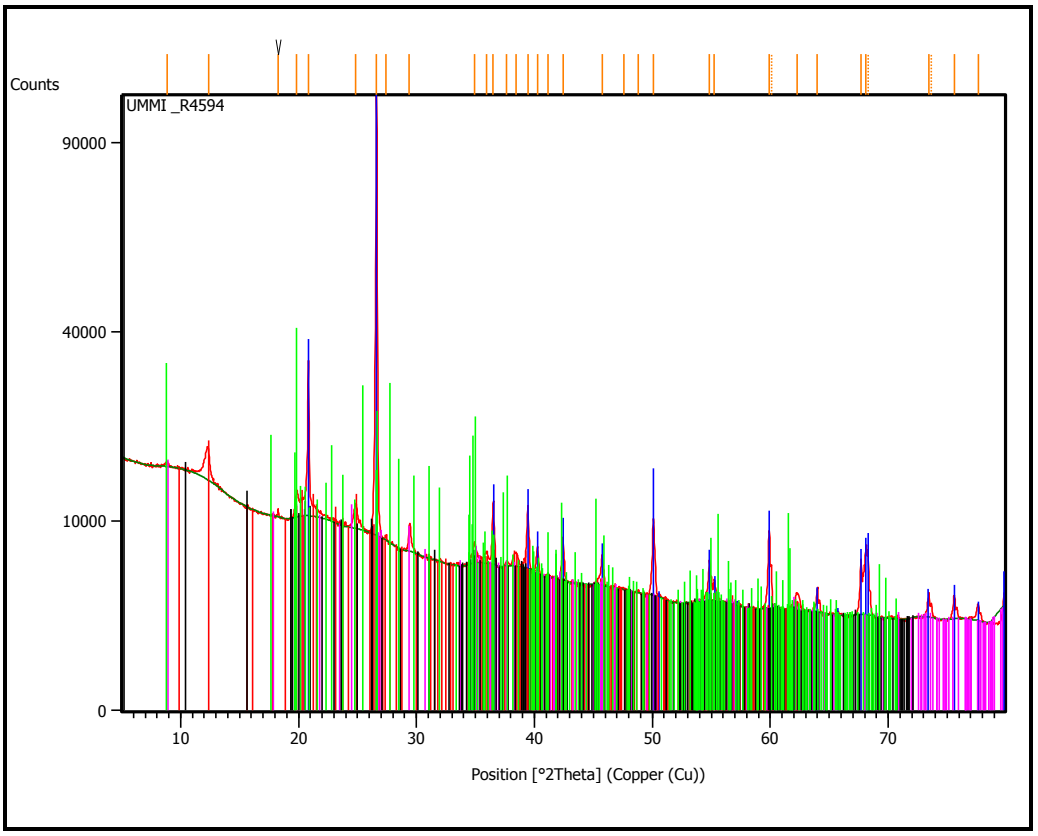

Figure 6.1.6: Graph listing all peaks of individual compound in black soil generated by X-ray Diffractometer (XRD)

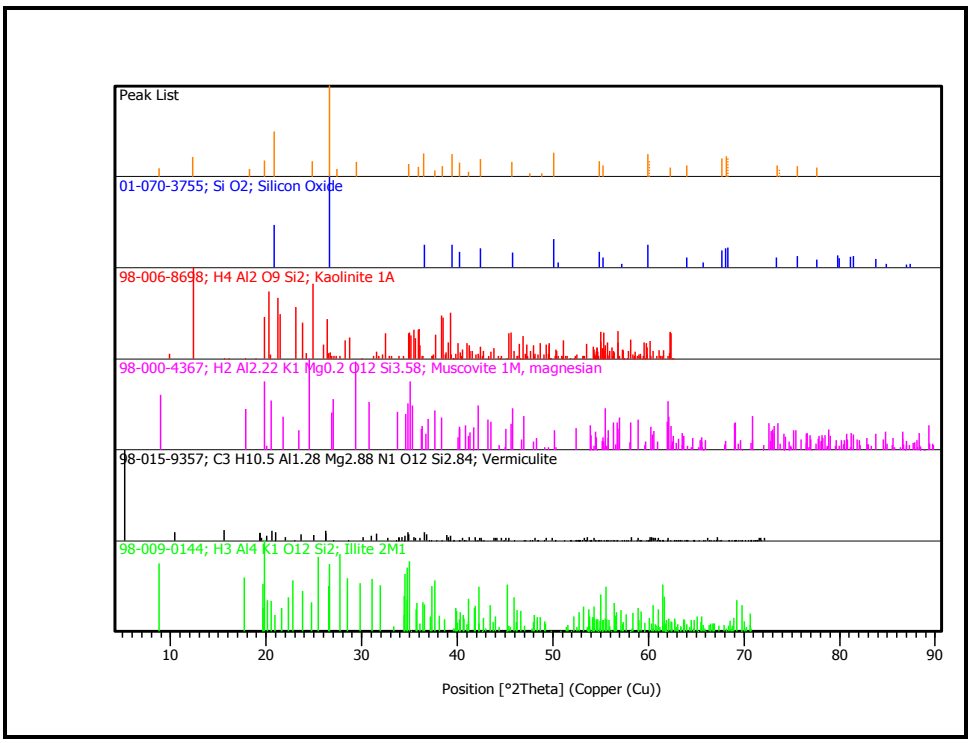

Figure 6.1.7: Graph listing the peaks of major compounds in black soil generated by X-ray Diffractometer (XRD). *The identity of the reference code is stated in Table 6.3.

The 5 major compounds in black soil are silicon oxide, kaolinite, muscovite, vermiculite and illite, as shown in Table 6.3.

Table 6.3: Major compounds in black soil as identified by X-ray Diffractometer (XRD).

Ref. Code	Score	Compound Name	Chemical Formula
01-070-3755	77	Silicon Oxide	Si O2
98-006-8698	35	Kaolinite 1A	H4 Al2 O9 Si2
98-000-4367	20	Muscovite 1M, magnesian	H2 Al2.22 K1 Mg0.2 O12 Si3.58
98-015-9357	1	Vermiculite	C3 H10.5 Al1.28 Mg2.88 N1 O12 Si2.84
98-009-0144	19	Illite 2M1	H3 Al4 K1 O12 Si2

Figure 6.1.8 showed 35 phases or components that can be detected from the combination soil, as generated by X-ray Diffractometer. However, there are only 5 major compounds presences in the soil combination.

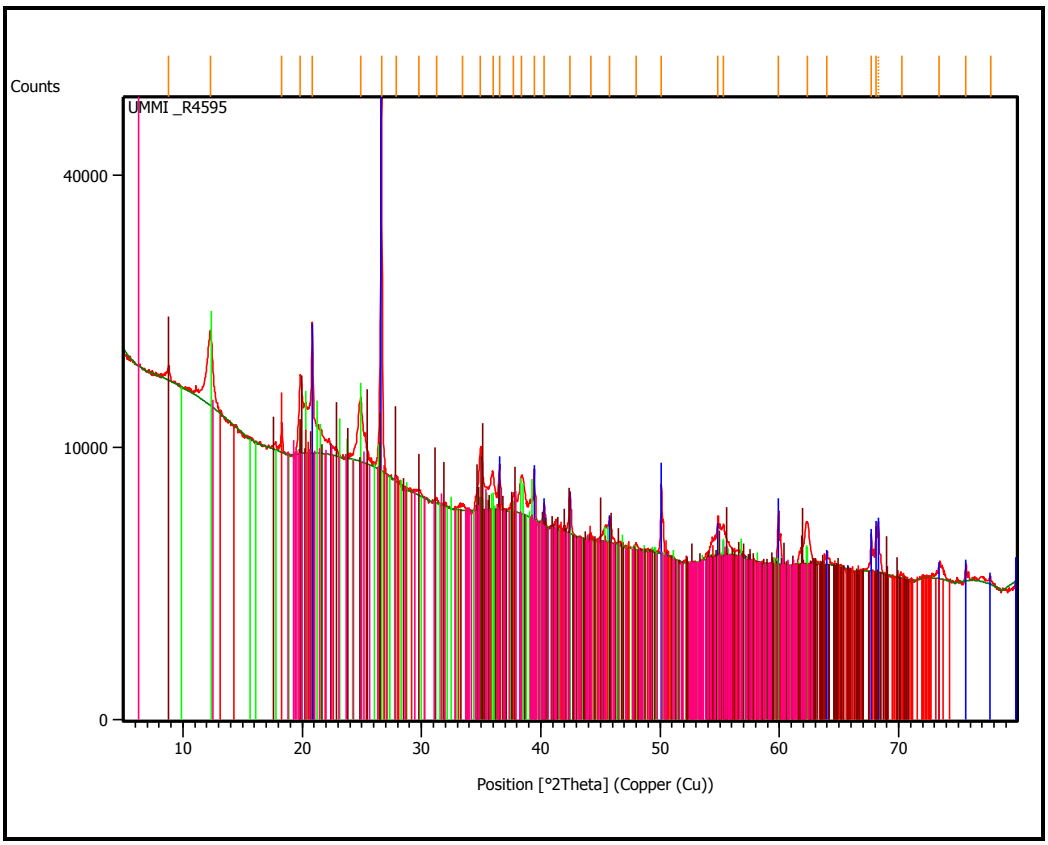

Figure 6.1.8: Graph listing all peaks of individual compound soil combination generated by X-ray Diffractometer (XRD)

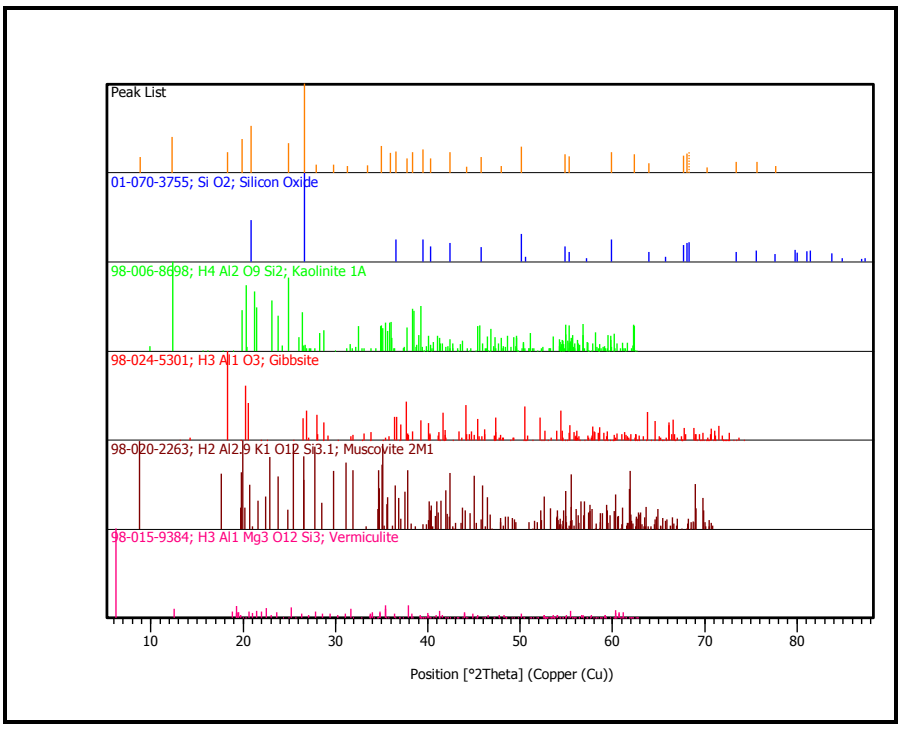

Figure 6.1.9: Graph listing the peaks of major compounds in combination soil generated by X-ray Diffractometer (XRD). *The identity of the reference code is stated in Table 6.4.

The 5 major compounds in the soil combination are silicon oxide, kaolinite, gibbsite, muscovite and vermiculite, as shown in Table 6.4.

Table 6.4: Major compounds in soil combintation as identified by X-ray Diffractometer (XRD).

Ref. Code	Score	Compound Name	Chemical Formula
01-070-3755	77	Silicon Oxide	Si O2
98-006-8698	38	Kaolinite 1A	H4 Al2 O9 Si2
98-024-5301	23	Gibbsite	H3 Al1 O3
98-020-2263	27	Muscovite 2M1	H2 Al2.9 K1 O12 Si3.1
98-015-9384	1	Vermiculite	H3 Al1 Mg3 O12 Si3

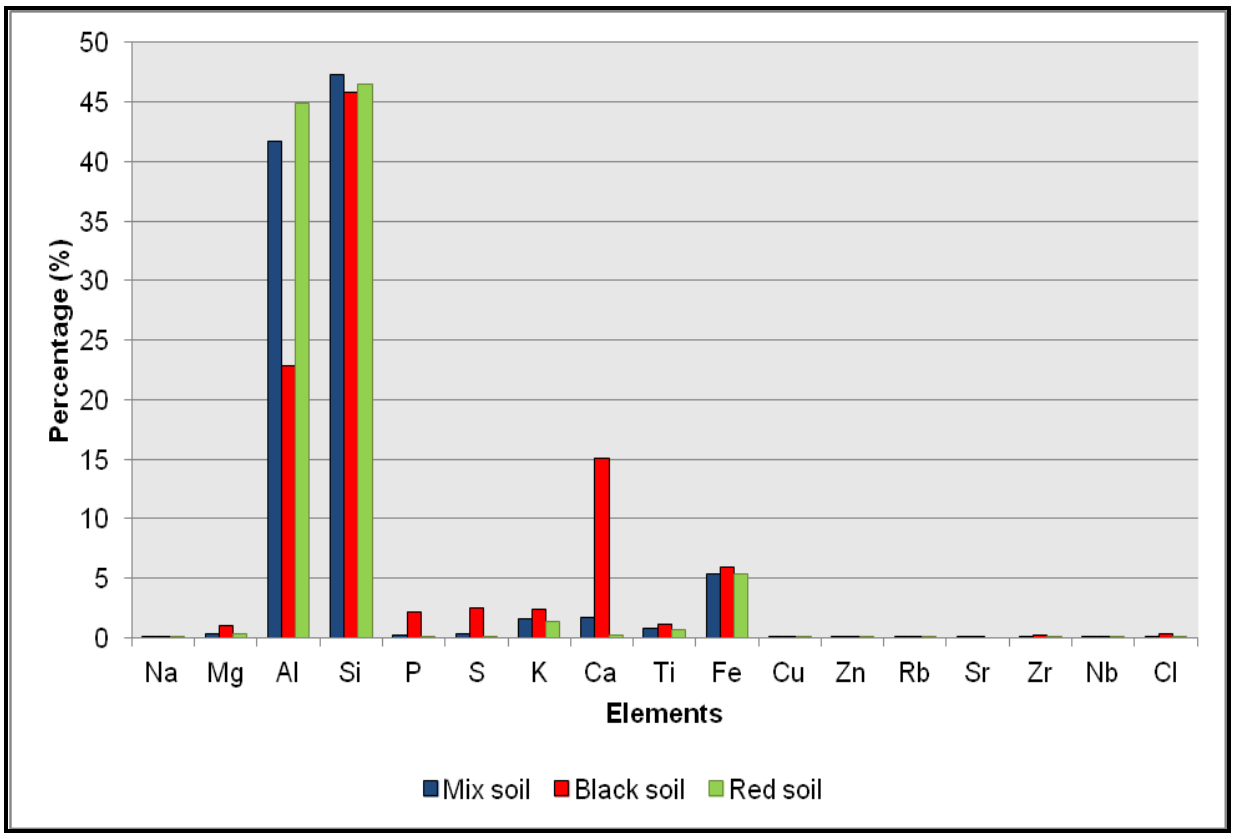

Figure 6.1.10: Graph showing the multielements presence in all the 3 types of soils analyzed by X-ray fluorescence (XRF)

Major elements presences in all types of soils are Silicon (Si), Aluminum (Al) and Ferum (Fe). Calcium can be observed more in black soil, as shown in Figure 6.1.10. Other minor elements include Sodium (Na), Magnesium (Mg), Phosphorus (P), Sulphur (S), Potassium (K), Titanium (Ti), Copper (Cu), Zinc (Zn), Rubidium (Rb), Strontium (Sr), Zirconium (Zr), Niobium (Nb) and Chloride (Cl).

6.4 SUMMARY OF RESULTS

1. Acclimatization of *Hylocereus polyrhizus* was succesful when almost all regenerated plants survived and had successfully grown into healthy plants in the field.

2. *Hylocereus polyrhizus* plantlets managed to survive on three different types of sowing soils; red soil, black soil and combination of red soil: black soil: cocoa peat (1:1:1)

3. The highest percentage of survival rate of the regenerated plants was obtained in combination of red soil: black soil: cocoa peat (1:1:1) with the percentage of 83.3 % ± 0.07.

4. Red soil contains 8 majors compounds, namely silicon oxide, kaolinite, gibbsite, zeolite, muscovite, magnetite, illite and vermiculite

5. Black soil contains 5 major compounds, namely silicon oxide, kaolinite, muscovite, vermiculite and illite.

6. Soil combination of black soil, red soil and cocoa peat (1:1:1) contains 5 major compounds, namely silicon oxide, kaolinite, gibbsite, muscovite and vermiculite.

CHAPTER 7

HISTOLOGICAL STUDIES OF *Hylocereus polyrhizus*

7.1 EXPERIMENTAL AIMS

Plant cells consist of various types of tissues like other organisms. Plant tissue can be single or complex cell type. Plant tissue system composed of dermal tissue, vascular tissue and ground tissue systems. Each plant tissue system has its own function such as provide support, nutrient formation and growth development. However, the variability in the plant cell structures can occur due to many environmental factors. Conducting histology to examine the plant structure and making comparison between plants is the effective technique to be done.

Histology is the microscopic anatomic study of cells and tissue of plants and animals by using staining to visualize the cells and tissues structures. Yeung (1999) described that histological analysis is vital tools to check and monitor the variability of the plant organization through microscopic graph in our experimental system. Variability of the morphological structures of *in vitro* specific culture can appear during the process of micropropagation and also acclimatization. In this experiment, histological examination of *in vivo* and *in vitro* grown *Hylocereus polyrhizus* plants were done to identify the differences in structures between *in vivo* and *in vitro* grown plants.

7.2 MATERIALS AND METHODS

7.2.1 Sources of explants

Stem and root explants from 12 weeks old *in vivo* and *in vitro* grown *Hylocereus polyrhizus* were used in this experiment. *In vitro* regenerated plants obtained from CHAPTER 3: *IN VITRO* REGENERATION OF *Hylocereus polyrhizus*. *In vivo* plants were obtained from seeds sown in the soil combination of black soil, red soil and cocoa peat.

7.2.2 Histological studies of *Hylocereus polyrhizus*

Stem and root explants obtained from *in vivo* and *in vitro* grown plants were excised and all samples were fixed in FAA solution containing 90 ml of 70% ethyl alcohol, 5 ml of glacial alcohol and 5 ml of formaldehyde for 48 hours. The samples were then washed with 70% alcohol for few times and dehydrated in various combinations of TBA (tertiary butyl alcohol), 95% of ethyl alcohol, 100% of ethyl alcohol and distilled water. The samples were then infiltrated in mixture of Paraffin oil and TBA and kept overnight. The samples were then placed into melted 49°C paraffin wax overnight and further transferred into 56 °C melted wax and kept for 24 hours. Embedding process was done by pouring 56 °C of paraffin wax on the paper boat. Then, the samples were sectioned (8µM) with rotary microtome (Leica, GERMANY) stained with Safranin ‘O’ and Alcian Blue and mounted on the slides using Albumin.

7.2.3 Data Analysis

The samples were viewed under Axioskop Zeiss (GERMANY) microscope attached to AxioCam MRc video camera and were then analyzed using AxioVision 4.7 software.

7.3 RESULTS

In the present study, stem and root explants obtained from 12-week-old *in vivo* and *in vitro Hylocereus polyrhizus* plants were used and analyzed. The microscopic views of cell and tissue structures were observed. A comparative study of histological traits between *in vivo* and *in vitro* grown *H. polyrhizus* was done to examine and monitor the variability appearance in the cell structure.

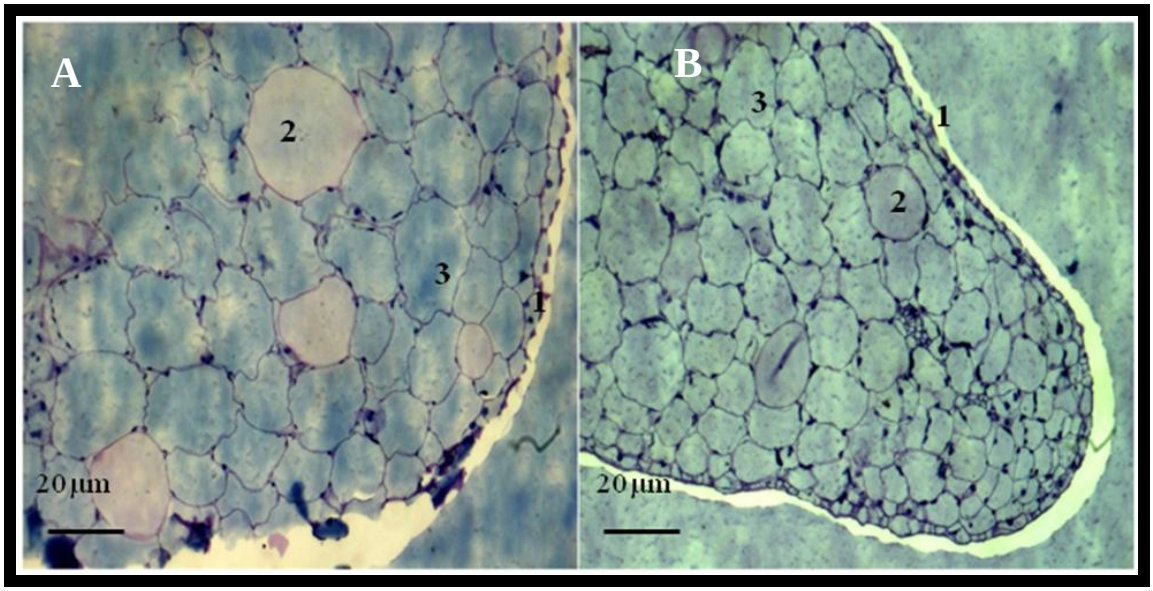

Figure 7.1.1: Longitudinal section (A) *in vivo* and (B) *in vitro* stem showing the presence of (1) Epidermis (2) Mucilage cells and (3) Cortex.

As shown in Figure 7.1.1, both *in vivo* and *in vitro* stem obtained from *H. polyrhizus* explants showed the presence of epidermis, mucilage cells and cortex. The epidermis of both *in vivo* and *in vitro* stems was uniseriate and covered by cuticles. The

cell walls of the *in vitro* plants were thinner than *in vivo* plants. Mucilage cells were observed within the cortex inner region in both plants. Mucilage cells of *in vivo* stem were more visible than *in vitro* stem.

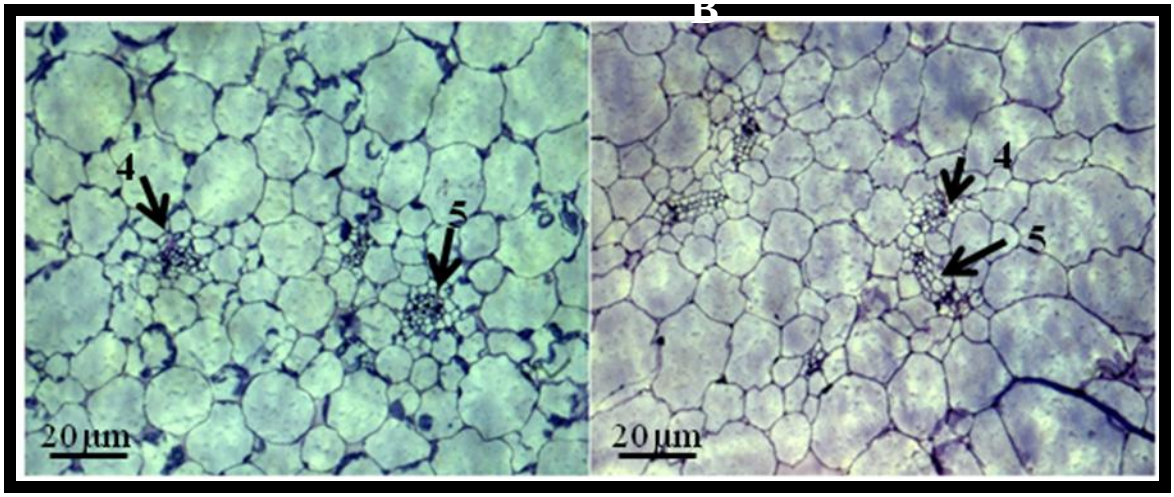

Figure 7.1.2: Longitudinal section of (A) *in vivo* and (B) *in vitro* stem showing the presence of (4) Phloem (5) Xylem.

Phloem and xylem were observed in both *in vivo* and *in vitro H. polyrhizus* vascular systems. Vascular bundles were collateral for both but smaller quantity of xylem in *in vitro* plant as shown in Figure 7.1.2.

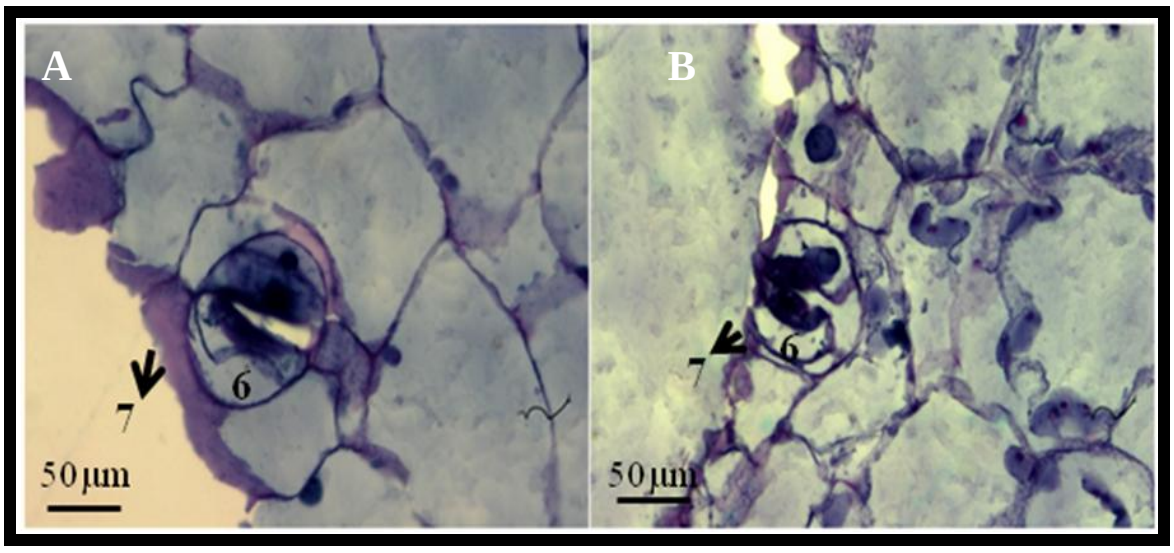

Figure 7.1.3: Longitudinal section of (A) *in vivo* and (B) *in vitro* stem showing the presence of (6) guard cell (7) cuticle layer.

As seen in Figure 7.1.3, stomata of both plants were oriented on the same level of epidermal layer. Nevertheless, several *in vitro* stomata were poorly developed and malfunctioning. The cuticle layer of *in vivo* grown *H. polyrhizus* was observed to be thicker than *in vitro* grown plants.

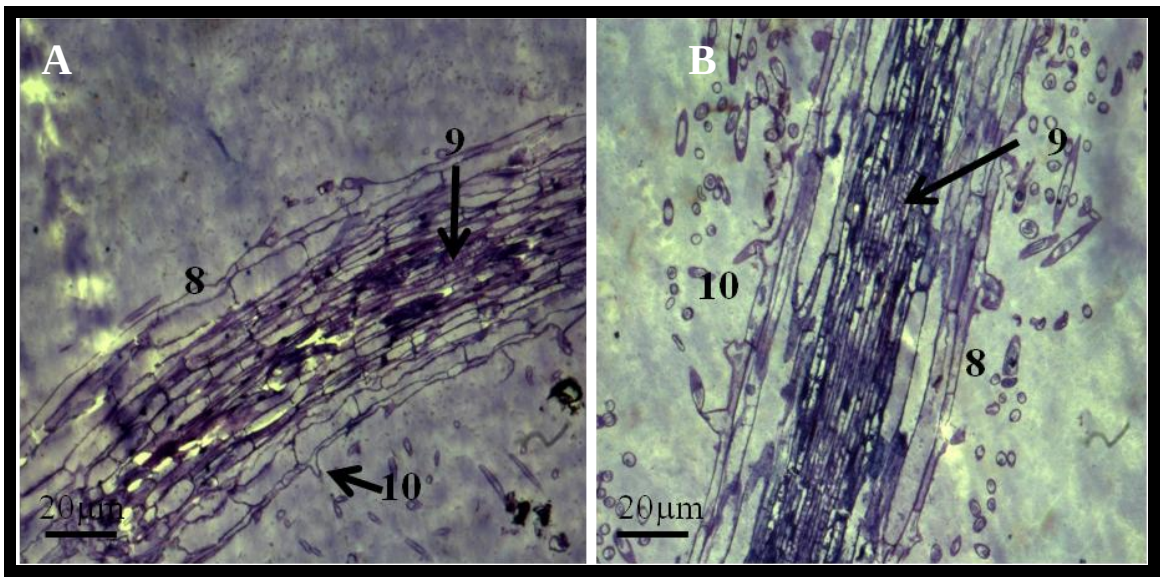

Figure 7.1.4: Longitudinal section of (A) *in vivo* and (B) *in vitro* root showing the presence of (8) Epidermis (9) Treachery element (10) Root hairs.

Figure 7.1.4 showed the epidermis, treachery element and root hairs presented in both *in vivo* and *in vitro* grown *H. polyrhizus* roots. The epidermal layer of *in vivo* and in *in vitro* roots was also uniseriate. Root hairs were observed on both kind of growth.

7.4 SUMMARY OF RESULTS

1. Comparative histological studies were performed on both *in vitro* and *in vivo H. polyrhizus* stems and roots.

2. For *in vivo* and *in vitro H. polyrhizus* stems, the presence of epidermis, mucilage cells, cortex, xylem, phloem, guard cell and cuticle layer were observed.

3. For *in vivo* and *in vitro H. polyrhizus* roots, the presence of epidermis, treachery elements and root hairs were observed.

4. Stomata for both *in vivo* and *in vitro* grown plants were positioned on the same level of epidermal layer.

5. The epidermal layer in the stems and roots were uniseriate and the vascular bundles were collateral for both.

CHAPTER 8

PIGMENT ANALYSIS OF *Hylocereus polyrhizus IN VIVO*

8.1 EXPERIMENTAL AIMS

Natural colorants from various plant pigments have been increasingly utilised as an important alternative to synthetic colorants. Natural colorants are derived from natural resources such as plants, animals and minerals. Various plant parts such as roots, leaves, twigs, stems, fruits, woods and flowers serve as natural colorant sources. Betalain, anthocyanins, carotenoids, chlorophylls and other flavonoids are good sources for harmless and free side effects natural colorants. The enhancement of stability in natural pigments needs to be considered to increase their commercial potential for consumers.

In this chapter, the main objectives were to observe the potential use of peel and pulp extracts from *H. polyrhizus* betalains pigment as natural coating and to observe the effects of pH and light on betacyanin stability through time. The betacyanin extract mixed with 20% poly (methyl methacrylate) and coated onto glass slides, were tested with different concentration of NaCl to determine its durability as natural coating material. Organic coating from plant materials are considered as eco-friendly and less harmful for consumer use.

8.2 MATERIALS AND METHODS

8.2.1 Preparation of plant material

The fruits of *Hylocereus polyrhizus* (Weber) Britton and Rose were collected from Selayang, Selangor. The whole fruits were washed and cleaned under running tap water. The peel and pulp of the fruits of *Hylocereus polyrhizus* were excised into small pieces and freeze-dried for 1 week. Dried peel and pulp were homogenized in the powder form by using mortar and pastle and stored in -20°C for further use.

8.2.2 Solvents and Reagents

Solvents and Reagents of analytical or HPLC grade were obtained from Sigma-Aldrich and Systerm.

8.2.3 Extraction of betalains pigment

The freeze-dried samples were used and weighed out for pigment extraction. Methanol (80%) and acetone (80%) were used as solvents for pigment extraction. The pigment extraction was done under room temperature. Peel and pulp of *H. polyrhizus* were mixed with solvent and dissolved by using mortar and pastel, with dry weight/volume to solvent ratio of 1:5. During pigment extraction, the mixed samples and solvent were shaken for about 2 minutes and allowed to stand for 30 minutes in order to yield maximum pigment content. Then, the treated peel and pulp were centrifuged at 3000g for 10 minutes to separate the remaining seeds, raw fibers, mesocarp fibers and mucilaginous material. After that, the supernatants were collected filtered by using 0.2 μm milipore filters (Millex®, Germany) and taken for further analysis.

8.2.4 Effect of pH on betacyanin content

The stability of betacyanin was evaluated under different pH values and was conducted according to method modified from Fong and Siow (2014). The pH of the samples were adjusted to pH 1, 3, 5, 7 and 9 using 1 M HCl (Sigma-Aldrich®, Germany) and 1M NaOH (Sigma-Aldrich®, Germany). These samples were then stored at 4°C for 24 hours and the betacyanin content of each sample were assessed.

8.2.5 Effect of light on betacyanin content

Betacyanin pigments which were extracted form pulp and peel of *H. polyrhizus* with different types of solvents were used to observe the effect of light during storage. All the samples were placed in 5 ml vials and the absorbance of pigment contents were measured using UV- Vis spectrophotometer (Lambda 25, Perkin Elmer) at 537 nm. The samples were stored under light for 24 hours in the room temperature. Samples stored in the dark were wrapped with aluminium foil and kept in the dark. The betacyanin content of each sample was assessed after 24 hours. Analysis was conducted in triplicates.

8.2.6 Determination of betacyanin content

Betacyanin pigment content was evaluated by spectrophotometric method as described by Cai and Corke (1999), with slight modification. Betacyanin pigment extracts were diluted with McIlvaine buffer (pH 6.5). McIlvaine buffer was prepared from 0.1 M citric acid and 0.2 M sodium phosphate dibasic (Jamilah *et al.*, 2011). For each sample, 0.1 mL sample was added with 2.9 mL McIlvaine buffer in cuvette prior to spectrophotometric analysis. For blank, 3.0 mL McIlvaine buffer was used. The quantification of betacyanins was carried out using the formula (Herbach *et al.,* 2007)

Bc = [(AxDFxMWx1000/εx*l*)], where, Bc is the betacyanin concentration in milligram per litre, A is the absorption value at λ_{max} (537 nm), DF is the dilution factor, MW is the molecular weight of betanin (550 g/mol), ε is the molar extinction coefficient of betanin (60000 l/molxcm) and *l* is the path length of the cuvette (1 cm). In this study, all determinations were performed in triplicates (n=3) using UV- Vis spectrophotometer (Lambda 25, Perkin Elmer) at 537 nm.

8.2.7 Preparation of betacyanin-coated glass

A mixture of resin poly (methyl methacrylate) (PMMA) with betacyanin extracts at pH 1, the most stable pigment extracts was used to prepare betacyanin-coated glass. The ratio of resin to pigment used was 1:5. The mixtures were well dissolved, coated on the glass slides (Duran Group Gmbh, Mainz, Germany) and dried for 1 hour. The coated glass slides were directly used for further durability test and the results were recorded.

8.2.8 Salt Test

Study on the effect of NaCl on betacyanin- coated glass was modified from Yaacob *et al.* (2011). Salt or NaCl was used in the experiments to mimic the sea water and observed the durability of the betacyanin-coated glass towards NaCl concentrations was observed. The betacyanin-coated glasses were dipped in 5, 10, 15 % NaCl solution for 2 hours before absorbance measurements at 537nm at 15 minutes interval.

8.2.9 Statistical Analysis

The statistical program used was SPSS version 20.0. Data were analysed using Duncan's Multiple Range Test (DMRT) and all experiments were carried out thrice. Mean with different letters in the same column differ significantly at $p < 0.05$. Standard errors (SE) of the difference between treatments were also calculated.

8.3 RESULTS

8.3.1 Effect of pH on betacyanin content

Stability of pigments from plant sources depends on several factors that could root restriction on their utilization in commercialization. Betacyanins content extracted from peel and pulp of *H. polyrhizus* using 80% methanol and 80% acetone under influenced of different pH were investigated. As shown in Table 8.1, betacyanins pigment extracted from *H. polyrhizus* pulp by using 80% acetone as a solvent at pH 1 showed the highest betacyanins content. The current result showed increasing the pH values decreased betacyanin content in the samples. The betacyanins contents in pulp were higher than the content in the peel. Apart from that, colors changing of pigment extracts also have been observed. The extracts showed purple at pH 1, red-violet at pH 3 – pH 7 and orange at pH 9, as shown in Figure 8.1.1. Betacyanin pigments are visibly changed to a yellow tint due to release of betalamic acid when being exposed to alkaline pH solution

Table 8.1: Effect of pH on betacyanin content of *H. polyrhizus* peel and pulp extracts

Solvent	Extract	pH	Betacyanin content (mg/L)
80% Methanol	Pulp	1	23.6683 ± 0.03^{b}
		3	31.7625 ± 0.01^{g}
		5	37.4825 ± 0.01^{l}
		7	37.9500 ± 0.05^{m}
		9	45.6500 ± 0.08^{r}
	Peel	1	25.0250 ± 0.09^{c}
		3	33.4033 ± 0.04^{i}
		5	41.2225 ± 0.03^{p}
		7	36.4833 ± 0.07^{j}
		9	32.7617 ± 0.03^{h}
80% Acetone	Pulp	1	50.0133 ± 0.10^{s}
		3	38.7108 ± 0.03^{n}
		5	38.9950 ± 0.10^{o}
		7	36.9414 ± 0.06^{k}
		9	41.6900 ± 0.02^{q}
	Peel	1	22.1375 ± 0.2^{a}
		3	23.9342 ± 0.02^{b}
		5	29.6358 ± 0.03^{f}
		7	26.6200 ± 0.03^{e}
		9	26.1433 ± 0.2^{d}

Mean values with different letters within a column are significantly different at $p < 0.05$.

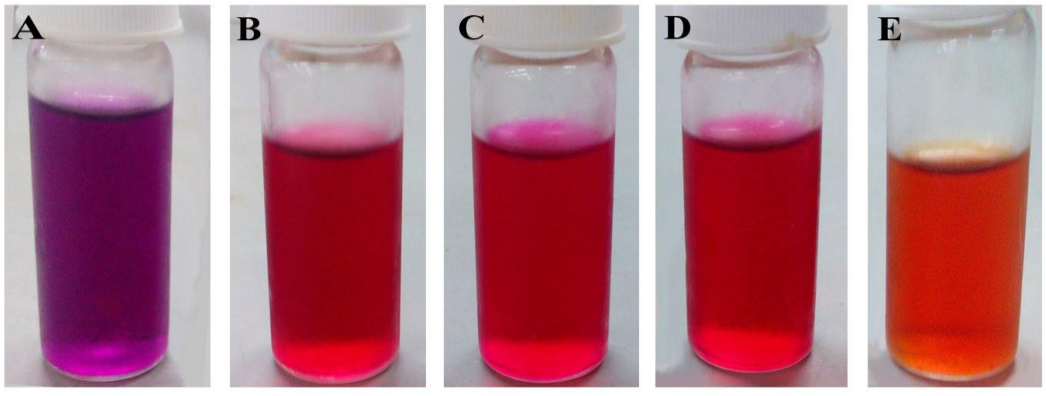

Figure 8.1.1: Color changes of betacyanin extracts with pH. (A) pH 1.0 (B) pH 3.0 (C) pH 5.0 (D) pH 7.0 (E) pH 9.0

8.3.2 Effect of light on betacyanin content

Table 8.2 shows the effect of light during storage on betacyanin content of *H. polyrhizus* peel and pulp extracts after 24 hours. Samples stored under dark conditions had a better betacyanin content compared to storage under light exposure.All samples demonstrated decreasing in betacyanin content after 24 hours storage under light condition.

Table 8.2: Effect of light during storage on betacyanin content of *H. polyrhizus* peel and pulp extracts after 24 hours

Solvent	Extract	Betacyanin content (mg/L)	
		Dark	Light
Methanol	Peel	75.5608 ± 0.01^{c}	4.6750 ± 0.00^{c}
	Pulp	75.0017 ± 0.01^{b}	8.7083 ± 0.01^{d}
Acetone	Peel	7.1042 ± 0.02^{a}	1.3750 ± 0.00^{a}
	Pulp	83.7008 ± 0.02^{d}	1.6500 ± 0.00^{b}

Mean values with different letters within a column are significantly different at $p < 0.05$

8.3.3 Effect of NaCl concentration on betacyanin-coated glass slides

The betacyanin-coated glass slides were tested to salt test (5-15% NaCl) in order to observe its durability towards NaCl concentration, as shown in Figure 8.1.2. Salt test revealed decreasing in absorbance values when glass slides coated were dipped in higher concentration of NaCl (15%). The colour of the pigment on the coated glass also became faded when subjected to high concentration of NaCl after 2 hours of treatment. The current study showed that by increasing the NaCl concentration, the durability of betacyanins coated glass decreased thus limits its application as coating material.

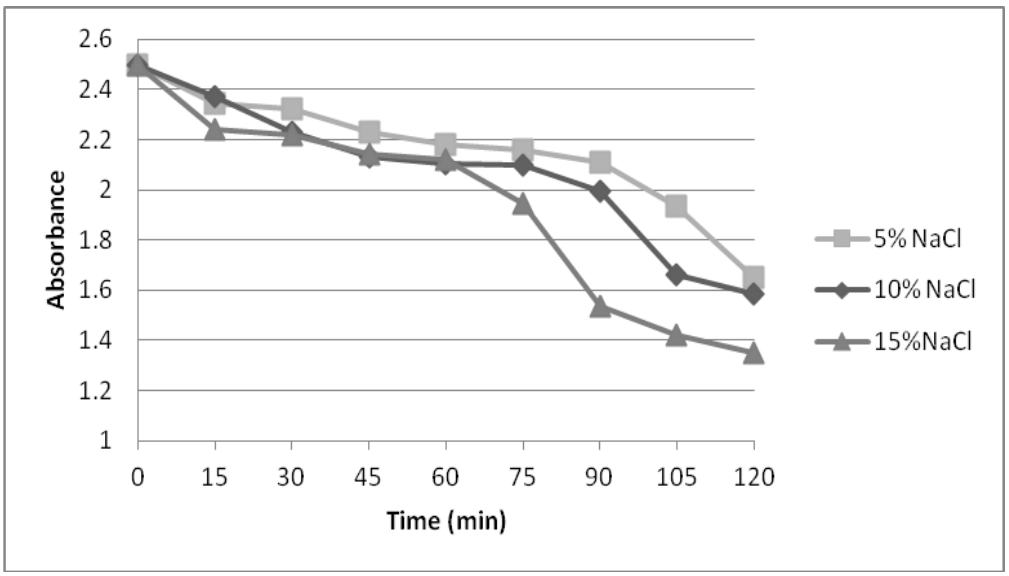

Figure 8.1.2: Effect of different NaCl concentrations on absorbance value (irradiated at 537nm, λnm) on betacyanin-coated slides.

8.4 SUMMARY OF RESULTS

1. The red-purple pigments extracted from pulp and peel of *Hylocereus polyrhizus* were known as betacyanin, polyphenolic pigments.

2. Betacyanins pigment extracted from *H. polyrhizus* pulp by using 80% acetone as a solvent at pH 1 showed the highest betacyanins content.

3. The colour of the extracts was shown to depend on its pH, where it became purple at pH 1, red-violet at pH 3 – pH 7 and orange at pH 9.

4. Betacyanin contents decreased after 24hrs of storage under light condition. Stability of betacyanin was influenced by light.

5. The colour pigment on the coated glass faded when treated with high concentration (15%) of NaCl.

CHAPTER 9

DISCUSSION

The present work deals with *in vitro* propagation, callus induction, synthetic seeds formation and histological analysis of *Hylocereus polyrhizus* (Weber) Britton and Rose grown *in vivo* and *in vitro. H. polyrhizus* is originated from Latin America. Main countries cultivating dragon fruit are Vietnam, Colombia, Mexico, Costa Rica, and Nicaragua (Vinas *et al*., 2012). In Malaysia it is called 'buah naga'. The fruits are ellipsoidal or oblong in shape and the flesh are red to purplish-red with many tiny seeds. Red pulp of pitaya fruit had generated a lot of interest as a source of natural red colour for food colouring (Harivaindaran *et al.,* 2008), cosmetic industry and health potential for improving eyesight, preventing hypertension and combating anaemia (Raveh *et al.*, 1998; Lee, 2002; Stintzing *et al.,* 2002). Pitaya is not only utilized and valued for its fruits, but also the flowers which can be eaten as vegetables. Pitaya is also considered as potential economical crop for harsh conditions such as drought, heat, and poor soil (Mizrahi & Nerd, 1999).

Pitaya is mainly propagated by cutting (Cavalcante & Martins, 2008). However, the method is inefficient, time consuming, and susceptible to damping off. Plant tissue culture technique is a promising and an efficient method for rapid propagation of plants and to obtain healthy and pathogen free plants in a relatively shorter time and minimal space. Currently, several researchers have established protocols for propagation of pitaya (Drew & Azimi, 2002; Mohamed-Yasseen, 2002, Vinas *et al.,* 2012; Hua *et al.,* 2015). The present study is divided into several chapters that are comprised of micropropagation, induction of callus, synthetic seed development, acclimatization and histological studies of *H. polyrhizus* plants both grown *in vivo* and *in vitro*.

Chapter two in this study described *in vitro* micropropagation of *Hylocereus polyrhizus* (Weber) Britton and Rose. Various concentrations and combinations of plant growth regulators were used in this research to investigate the suitable hormones for optimum regeneration of *H. polyrhizus*. The initiation of the cultures was carried out using aseptic seedlings. Aseptic seedlings consist of young or juvenile tissues and more responsive in culture, hence increase the success rate in micropropagation. All experiments conducted in this chapter used MS (Murashige & Skoog, 1962) media as the basic media and was prepared at pH 5.8. The cultures were maintained in the culture room at 25 ± 1 °C with photoperiod of 16 hours light and 8 hours dark. Thirty replicates were prepared for all experiments and the cultures were observed every week for 12 weeks.

Murashige and Skoog (MS) medium or MS medium is frequently used in plant tissue culture and was invented by plant scientists, Toshio Murashige and Folke K. Skoog in 1962. MS basal medium contains macronutrients and micronutrients including high level of nitrate and organic additives such as agar, sugars, vitamins and growth regulators. MS medium is considered as high-salt concentration because of its K and N salt contents (Trigiano & Gray, 2011). Mauseth (1979) suggested that any high salt basal media are suitable for establishment of cactus micropropagation. MS basal medium is widely used in cacti propagation (Johnson & Emino, 1979; Clayton *et al.*, 1990; Khalafalla *et al.*,2007).

For regeneration of *H. polyrhizus*, solid media had been used. Gelrite is used as gelling agent in the media. Two (2) grams of gelrite per litre media was utilized. Gelrite produced by a tightly-controlled fermentation process, has consistent product quality

and is unaffected by the several natural conditions which affected the properties of agar. Gelrite is free from phenolic compounds but has higher ash content than agar (Scherer *et al.,* 1988). Gelrite has been considered as an alternative to agar. In general, gelrite are remarkably clear in comparison to those formed with agar. In addition, agar from different sources contains various amounts of contaminants (Debergh, 1983; Pierik, 1987; Scherer *et al.,* 1988) that may contribute to increase experimental errors.

Plant tissue culture work was done in aseptic conditions to avoid any occurrence of contaminations. Good sterilization techniques from the beginning are very important to obtain good results and avoid the experimental errors. All culture glassware, media and instruments used in handling tissues, as well as explants must be sterilised properly. All operations should be carried out in laminar airflow sterile cabinet (Chawla, 2003). Various methods and sterilisation agents such as ethanol and sodium hypochlorite were used to decontaminate the tissues from fungi, bacteria and virus. Several substances have been tested to minimize contamination and facilitate *in vitro* establishment and chlorine and ethanol-based compounds are now standard disinfection tools (Silva *et al.,* 2003). Excessive sterilization agents exposed to the plant tissues can cause the tissues to die due to toxicity. Hence, the accurate concentration of sterilants, duration of exposing the explants to the various sterilants, the consequences of using these sterilants has to be standardised. In this study, 70% ethanol, sodium hypochlorite and Tween 20 were used as sterilization agent to diminish contamination in *in vitro* cultures. Seeds of *H. polyrhizus* were washed under running tap water to remove mucilage and soak in the sterilization agent before being culture on the medium.

The selection of explants as starting materials is diverse and depends on the particular species. Many parts of explants such as leaf, stem, shoot tips, hypocotyls, flower and bulb can be used as source of explants in micropropagation. The source of explants is important in determining the morphogenetic and regenerative potential, which are significantly influenced by the physiological conditions of the donor plants (Debergh & Maene, 1981; Read, 1988). Several factors affecting responses of explants in tissue culture such as genotype, explants source, explants age and size, position of explants in culture donor plant and explants density. Therefore, choice of explants plays an important role in determining the efficiency of micropropagation (Abbasi *et al*., 2007). Stem from aseptic seedlings were used as explants in *H. polyrhizus in vitro* regeneration instead of root. Many studies evaluating efficient *in vitro* conditions for propagation of several cactus species have been conducted using *in vitro*-germinated plants, to avoid disinfection of explants, because of extreme sensitivity to common disinfection procedures and subsequent low survival rates (Pérez-Molphe-Balch & Dávila-Figueroa, 2002; Santos-Díaz *et al.,* 2003).

In this study, no direct regeneration of microshoots or adventitious shoots was observed when the explants were cultured on the MS medium without any hormones. As represented in Table 3.2, the highest average number of shoots was counted as 8.07 ± 0.721 on MS medium supplemented with 0.2 mg/L BAP when explants were horizontally cultured, while, 0.2 mg/L Kn induced the longest shoots with 1.608 ± 0.128 cm when cultured in horizontal position. MS medium supplemented with combinations of 0.1 mg/L BAP and 0.1 mg/L NAA showed the highest average number of roots (3.031 ± 0.237) in vertical culture position. In the mean time, MS medium supplemented with 0.5 mg/L BAP and 0.1 mg/L NAA resulted in the longest roots formation when cultured in horizontal position.

Low concentration of BAP (0.2 mg/L) produced more shoots from this species. Vinas *et al.* (2012) indicated that reducing the concentration of BAP, significantly reduced the apical death, and thus developed the healthy and normal plantlets. Lowest BAP concentration (0, 1 or 2 μM) produced actively growing shoots without abnormalities or apical necrosis in *Hylocereus costaricensis* [F.A.C. Weber] Britton and Rose propagation. Higher dosage of BAP inhibited the multiplication rate in varieties of turmeric (*Curcuma longa* L.) (Naz *et al.,* 2009). The involvement of BAP concentration in buds sprouting, shoot growth and proliferation have been reported in several cacti species such as *Escobaria minima*, *Mammillaria pectinifera*, *Pelecyphora aselliformis*, and *Opuntia ficus-indica* (Giusti *et al*. 2002; Khalafalla *et al*. 2007).

Kinetin has been applied for micropropagation of many ornamental plants (Jain & Ochatt, 2010). Low concentration of Kinetin induced the longest shoots formation instead of multiple shoots and this was also proved by Rafiq *et al.* (2007). Kinetin based media did not indicate any specific increment in formation of multiple shoots. Contrary to the present study, Kinetin failed to elongate the rossete of *Opuntia ficus-indica* shoots compared to those obtained in BAP medium. These findings are in agreement with those species such as *Pelecyphora aselliformis, Nealolydia lophophoroides, Aloe barbebsis* and *Turbinicapus laui* (Bustamante & Heras, 1990; Feng-Feng *et al*., 2000; Mata-Rosas, 2001). According to Gunay and Rao (1978), Kinetin was inefficient in inducing differentiation and only promoted callus in *Capsicum* (red pepper).

Cytokinins are usually known to promote buds initiation in many *in vitro* cultured organs. *In vitro* shoot propagation and reproduction are largely based on media formulations containing cytokinins as a major plant growth regulators (Mamidala &

Nanna, 2009; Hoque, 2010). Hesar *et al*. (2011) revealed the increasing of Kinetin concentration could increased the buds induction in *Matthiola incana*, an ornamental plant. He obtained the best shoot length (11.72 mm) and the highest number of nodes (4.64) when cultured in 2 mg/L Kinetin based media. Similar result was obtained by Vernosefadrani *et al*. (2009) by using medium containing 2 mg/L Kinetin and obtained the most proliferation and plantlets length on micropropagation of *Gerbera jamesonii*.

In the present study, the addition of 0.1 mg/L NAA showed positive response in promoting root formation (Figure 3.1.3). The use of NAA is generally considered to be one of the most suitable auxins for root formation in *H. polyrhizus* species. Similar with these findings, Dahanayake and Ranawake (2011) found the addition of NAA into MS medium was suitable for root formation in *Hylocereus undatus*. In contrast, Finti *et al.* (2012) reported that combinations of BAP and NAA were not effective in shoot proliferation of cacti *Opuntia ficus-indica.* According to Khalafalla *et al*. (2007), IAA was the most suitable hormone in promoting root formation for *Opuntia ficus-indica* instead of IBA and NAA which indicated 100 % rooting and the highest number (15.0 ± 1.1) of roots per rooted plantlet after two weeks of culture.

Combinations of BAP and NAA for shoots proliferation did not improve the number of shoots per explant. The efficiency of BAP for shoot formation was significantly decreased when it was combined with IAA, NAA or IBA in *Vigna mungo* L. Hepper (Saini & Jaiwal, 2005). However, the use of NAA with thidiazuron for shoot initiation was reported to be successful for *H. undatus* cultures (Mohamed-Yasseen 2002). Dahanayake and Ranawake (2011) showed that the highest number of shoot production of *Hylocereus undatus* was observed on MS medium supplemented with 2.5

mg/L BAP and 0.01 mg/L NAA. Low levels of auxin with cytokinin increased axillary bud production in some cactus species (Clayton *et al.*, 1990). However, Karrupusamy *et al.* (2006) observed that higher concentration of auxin with cytokinin produced relatively less shoot proliferation rate, on the other hand produced sensible and enormous callus at the basal portion of the nodal explants and inhibited the augmentation of multiple shoots in culture of *Vanasushava pedata*.

The interaction of auxin and cytokinin is particularly important in plant growth and developmental process. Both auxin and cytokinin act either synergistically or antagonistically to promote several significant developmental processes such as the formation and maintenance of root and shoot meristem in order to establish whole plant body. Skoog and Miller (1957) indicated that a high concentration of auxin and cytokinin ratio could induce root regeneration, whereas a low concentration of auxin promotes shoot induction. This shows the cross-talk between both hormones during *in vitro* organogenesis might have been occurred. However, the molecular mechanism of such interaction between auxin and cytokinin in the in vitro formation of meristem are remaining mostly unknown. Su *et al.* (2011) observed the antagonistic interaction between auxin and cytokinin are the key regulators for the cell differentiation and maintenance in root meristem transition zone and shoot meristem organ initiation.

Maximum number of shoots proliferation of *H. polyrhizus* was observed when the stem explants were cultured in horizontal position. In agreement with this finding, Escobar-Araya *et al.* (1986) found better results with explants horizontally oriented for micropropagation of *Opuntia amyclaea* by axillary proliferation. However, in contrast, Estrada-Luna *et al.* (2008) found that explants place vertically produced more shoots

than those placed in horizontal position in *in vitro* regeneration of the ornamental prickly pear cactus *Opuntia lanigera* Salm–Dyck. This might be possible because the shoot tips were dissected prior to subcultures and consequently eliminate the effect of apical dominance. Effects of explants orientation in culture medium also have been observed in other species such as *Glycine max* (L.) Merrill (Santarem *et al.,* 1997), *Oryza* (Mercy & Zapata, 1987), *Brassica* (Arnison *et al*., 1990) and *Musa balbisiana* 'Kluai Hin' (Kanchanapoom & Promsorn, 2011).

The orientation of the explants either vertically or horizontally cultured on the medium could trigger different effect on shoot and root formation. McClelland and Smith (1990) reported several woody plant species promotes optimum shoot formation when oriental horizontally on the medium. In agreement with the findings in this study, San-Jose *et al.* (1988) also revealed that horizontal orientation as the most effective placement of explants in *Quercus robur*. They found that the horizontal orientation could increased the proliferation capacity due to larger cotact of the explants tissue with the culture medium, hence increase the nutrient absorption. In contrast, Sharma *et al.* (2010) observed the maximum response in shoot formation when explants were placed vertically on the medium for *Murraya koenigii* (L.) Spreng. (Rutaceae), a multipurpose aromatic medicinal plant or curry tree. This might be due the channelized movement of nutrients through the vascular tissue. While, in the horizontal oriented explants, the contact of the explants with the culture medium caused excessive nutrient supply which lead callusing and phenolic exudation.

Chapter 3 in this thesis described the induction of callus from stem explants of *H. polyrhizus.* Single concentration of 2, 4-D (2, 4-Dichlorophenoxyacetic acid) and

combinations with BAP (6-benzylaminopurine) were used to determine the best hormones in inducing the callus. MS basal medium without any hormone was used as a control and showed no callus was formed. All explants exhibited callus formation at the cut surfaces. In this studies, the callus formation were well-developed in MS medium supplemented with combinations of 1.0 mg/L BAP and 0.1 mg/L 2, 4-D, 1.0 mg/L BAP and 1.0 2, 4-D and 2.0 mg/L and 1.0 mg/L 2, 4-D. The combinations between BAP and 2, 4-D was showed better in callus induction instead of 2, 4-D alone (Table 4.2). Various colours of the callus derived from stem explants were obtained such as green callus, white callus, yellow callus and light yellow callus.

2, 4-Dichlorophenoxyacetic acid (2, 4-D) is known as the main synthetic auxin used in callogenesis due to its main characteristics in stimulating cell division in the tissues of several plants (George, 1996). In the present study, the combination of 2, 4-D and BAP gave better callus induction than single application of 2, 4–D alone. Also, this is in agreement with Santiago (2003) who found that maximum callus production was achieved with the combination of 2, 4–D and BAP using leaf explants of *Piper hispidinervum.* Cytokinin is frequently added to the medium in addition to an auxin to induce the growth of callus in explants of dicotyledonous. According to Astello-Garcíal *et al.* (2013), 2, 4-D in combination with BAP, proline, biotin and casein hydrolysate was considered as the suitable medium for proliferation of callus in *Opuntia robusta*, a wild and medicinal cactus. The addition of casein hydrosylate in the medium as a source of calcium, phosphate, micronutrients, vitamins and several growth factors improved shoots and callus development (Khaleda & Al-Forkan, 2006).

This study also revealed that green callus were formed when cultured in combination media. Green callus were observed on the MS medium supplemented with 1.0 mg/L BAP and 0.5 mg/L 2, 4-D, 1.0 mg/L BAP and 1.0 mg/L 2, 4-D, and 2.0 mg/L BAP and 1.0 mg/L 2, 4-D (Figure 4.1.2). The nature of the callus induced was compact on MS medium. Similiar with these results, Angulo-Bejarano and Paredes-López (2011) also observed different colours and shapes of callus produced from cactus species, *Opuntia-ficus indica* cultured on MS medium with the addition of various concentrations and combinations of 2, 4- D and BAP. In contrast, Elias *et al.* (2015) found that single hormones medium was the best medium to produce green callus in *Echinocereus cinerascens in vitro* compared to media with hormone combinations. Green callus was induced in over 80% of explants in all treatments in which the hormones were applied singly.

The manipulation of the appropriate levels of auxin and cytokinin is crucial to define the balance growth regulators so that there is induction of callus formation in the different types of explants (Franklin & Dixon, 1994). Shaukat Ali *et al.* (2004) investigated the addition of BAP along with auxins 2, 4-D and NAA for callus induction of indica rice increased the efficiency of callus formation. Eapen and George (1990) also reported the combination of auxin and cytokinin enhanced the callus induction of *Eleusine coracana*. Similar observation was obtained in this study, whereby the combination of 2, 4-D and BAP showed better result in callus induction. In contrast, Tao *et al.* (2002) reported that low concentration of 2, 4-D alone could induce maximum number of green and compact callus of *Citrus grandis* Osbeck. Lima *et al.* (2008) also observed that the exogenous supply of the auxin 2, 4-D alone was sufficient to promote a hormonal balance in order to induce highest callus formation in leaf tissues of *C. urucuruna*.

Purple colored callus (Figure 4.1.5) was observed when pulps of *H. polyrhizus* were directly cultured on MS basal media. The formation of colored callus was probably due to the content of betacyanins pigment contained in the pulp of this spesies. Wybraniec *et al*. (2007) reported that the colour of pitaya fruit is accredited by major bioactive compound called betacyanins, water soluble pigments. Betacyanins are red-violet pigment contained in some plant species such as dragon fruit and beetroot. Betacyanins are water soluble polyphenolic compound which have many benefits such as colorants, antimicrobial and antioxidant values.

Microshoots obtained from regeneration of *H. polyrhizu*s were further used for synthetic seed development in Chapter 4. The concept of synthetic seed was introduced by Murashige in 1977. Initial experiment was done using micro shoots as propagules for encapsulation process. Encapsulated micro shoots has the capability to break the encapsulation matrix and germinate, therefore cultivate and build up as a whole plant. Besides somatic embryos, micro shoots are also ideal propagules for the construction of synthetic seeds. Microshoot was chosen as propagule for synthetic seed development in *Stevia rebaudiana* Bertoni (Ali *et al.* 2012) and *Beta vulgaris* (Rizkalla *et al.* 2012).

A wide number of encapsulating agents had been used for their capacity to produce beads such as agar, agarose, alginate, carboxymethyl cellulose, carragenan, ethylocellulose, gelrite, guargum, nitrocellulose, polyacrylamide, polyox and sodium pectate (Datta *et al*. 2001, Saiprasad 2001). In the present work, 3% (w/v) of alginate was used as gelling agent. According to Redenbaugh *et al.* (1988), Na- alginate solution could be turned into harderned Ca-alginate gel by an ion exchange reaction. The main advantages of Na- alginate are: the excellent water solubility and moderate viscosity of

Na-alginate at room temperature, easy availability at low cost, the long-term storability of the Na-alginate solution, the easy use of calcium salts for quick gellation and bead hardening at room temperature, the possibility to prepare synseeds of different hardness by changing the concentration of Na-alginate and/or the duration of the ion-exchange reaction, the absence of any kind of toxicity of the Ca-alginate matrix for explants, the possibility to mix the alginate with a nutritive medium to obtain an artificial endosperm. On the other hand, alginate produced shabby nature of the capsules because the seed coats produced are very moist, with sticky surfaces thus making the seeds adhere to each other and difficult to separate. Another limitation is due to the speedy dehydration of the alginate beads which, upon contact to air, makes the synseeds become very hard in just a few hours, preventing or making the switch of the enclosed explants into plantlets or shoots very complicated.

Calcium Chloride solution was used in encapsulation process as complexing agent to form round and firm beads. The beads were formed due to ion exchange between the Na^+ in sodium alginate with Ca^+ in Calcium chloride solution. In this study, 100Mm of CaCl was used as suitable concentration to form firm and round beads. Kikowska and Thiem (2011) reported that concentration of both gelling and complexing agents influenced the texture, size, shape of synthetic seeds and also rate of plantlets conversion. Ali *et al.* (2012) and Cheravathur *et al*. (2013) showed that 3% of sodium alginate and 100 Mm of calcium chloride was the most suitable concentration to form round, firm and isodiametric beads in *Stevia rebaudiana* and *Rhinacanthus nasutus*.

In the present work, encapsulated beads germinated in the MS without hormone showed maximum response of conversion into complete plantlets. Singh *et al.* (2006)

also found maximum percentage response for conversion of synthetic seeds on full strength MS medium without plant growth regulator in *Phyllanthus amarus*. In contrast, Verma *et al.* (2010) observed that 90% of shoots emerged from medium supplemented with BAP in *Solanum nigrum* L. Nevertheless, BAP inhibited the root formation, thus no complete plantlets were formed. Soneji *et al*., (2002) showed that germination of synthetic seeds to plantlets appeared to depend on the hormonal concentrations in bead medium. Matrix of synthetic seed mimics endosperm of natural seed. Artificial endosperm should contain nutrients to maintain survival of germplasm (Antonietta *et al.* 1999).

The addition of plant growth regulators for germination of synthetic seeds of *H. polyrhizus* did not show any shoot formation but callus was formed. Callus turned brown after several days and no complete plantlets were observed. In contrast, Remya *et al.* (2012) reported that the addition of growth regulators in the germination medium enhanced the production of shoots formation from encapsulated beads of *Aristolochia tagala*. Machii (1992) observed that the addition of growth regulators in the encapsulated beads resulted in maximum germination and shoots formation in mulberry, *Morus alba* L. Nevertheless, several workers reported high rates of germination of synseeds on nutritive medium free hormones such as *Paulownia elongata* (Ipekci & Gozukirmizi, 2003), *Cyclamen persicum* (Winkelmann *et al*. 2004), *Genista monosperma* (Ruffoni *et al*. 1994), *Geodorum densiflorum* (Datta *et al*. 2001), *Lilium longiflorum* (Standardi *et al*. 1995) and *Pelongonium horturum* (Gill *et al*. 1994).

As shown in Table 4.3, maximum percentage of germination rate was obtained when synthetic seeds of *H. polyrhizus* were directly germinated on the medium without

storage. The germination frequency decreased to 40 % by using synthetic seeds stored for 10 days and no germination was observed after 120 days of storage. All the synthetic seeds were stored in the sterile distilled water at 4 °C. In contrast with these findings, Ikhlaq *et al*. (2010) found that increasing the storage duration could increase the germination rate of synthetic seeds of *Olive* cv. Moraiolo. He observed that the germination percentage increased up to 77.5 % as compared to non-stored synthetic seeds (65% germination). Nevertheless, Zhang and Yan (2011) reported that the germination rate of synthetic seeds of *Dendrobium candidum* decreased rapidly as the storage time increased.

Suitable storage conditions with specific storage period are fundamentals to sustain synseed viability during transportation that leads to successful commercialization of synseed technology. Cold storage at 4 °C had been found to be the most suitable temperature for maintaining the viability of the synthetic seeds for long term storage in several species such as *Gerbera jamesonii*, *Mentha arvensis* L., *Morus alba* L., *C. roseus* and *Caladium bicolour* (Taha *et al.,* 2009; Islam & Bari, 2012, Machii 1992; Mehpara *et al.*, 2012; Maqsood *et al.,* 2015). According to Redenbaugh *et al.* (1993), the main problem in storing alginate capsules is the poor respiration of encapsulated tissues. Dehydration problem could occur in the synthetic seeds during the storage period. Sterilized liquid paraffin as storage solution could increase the viability of synthetic seeds in *D. Candidum* (Zhang & Yan, 2011). Kaviani (2010) reported that high level of sucrose addition in alginate matrix is necessary for cryogenic storage (encapsulation-dehydration) as sucrose increases tolerance to dehydration and improves tissue viability during embryo germination process.

Encapsulation beads were sown in two types of soils; red soil and black soil under two conditions; sterile and non sterile. All encapsulation beads showed negative response and no germination has occurred. The beads became shrunk and contaminated both in sterile or non-sterile conditions. Preece and West (2006) reported that the encapsulation beads of *Hibiscus moscheutos* were successfully developed after directly being sown into greenhouse hydroponic system, both with and without light pre-treatment. The addition of fungicide to the alginate beads of encapsulated axillary buds of mulberry (*Morus indica*) could prevent the contamination of the buds and increased the survival of buds when sown in soil (Bapat & Rao, 1990). Preece and West (2009) also indicated that the addition of 100 mg/L benomyl in the encapsulation beads of *Hibiscus moscheutos* decreased the contamination rates. Ramakrishnappa (1998) also observed that addition of fungicide (0.1% carbendazim) and bacteriocide (0.1% streptomycin) into the encapsulating gel decreased the occurrence of fungal and bacterial contamination to a minimum level and nearly 95% of the capsules remained vigorous. More studies needed to be conducted to enhance the germination frequency of encapsulation beads during hardening process on non-sterile environment, such as greenhouse or directly in the field or nursery.

To complete the process of plant regeneration or micropropagation of *H. polyrhizus*, acclimatization process was continued after the successful growth of plantlets under *in vitro* conditions. The adaptation of the *in vitro* plantlets towards natural environment is very important in order for the plant to survive. The sudden changes of environmental conditions such as temperature, humidity, pH and photoperiod could cause the plantlets unable to survive and withered. *In vitro* grown plantlets have been continuously exposed to selected unique micro environment in order to provide minimal stress and optimum conditions for plant propagation. Plantlets were

developed within the culture vessels, with low light exposure, aseptic conditions, high level of humidity and on nutritious growth medium contributed a culture-induce phenotype which cannot survive in natural environment when directly transferred in a greenhouse or field. Hazarika (2006) reported that understanding the physiological characteristics of micropropagated plants and the changes during hardening process should facilitate the development of efficient transplantation protocols, if necessary, adjusting environmental conditions. Anatomical, morphological and physiological features could be as limiting factors for the adaptation of micropropagated plants to the *ex vitro* environment (Van Huylenbroeck & Debergh, 1996).

To achieve acclimatization process, well-rooted *H. polyrhizus* plantlets were sown in three types of soils; black soil, red soil and combination soil consists of black soil: red soil: cocoa peat (1:1:1). The maximum percentage of plantlets survived in *ex vitro* environments were observed for plantlets grown in soil combination (83.3%), as represented in Table 5.1. According to Giusti *et al.* (2002) and Ramirez-Malagon *et al.* (2007), acclimatization of most cacti species is respectively easy. Vinas *et al.* (2012) found 90% of *Hylocereus costaricensis* [F.A.C. Weber] Britton and Rose *in vitro* plantlets successfully survived during acclimatization by using vermiculite plus peat moss (1:1) or perlite plus peat moss (2:1) as soil substitutes. However, 100 % of survival rates were achieved in hardening of *Opuntia* species (Finti *et al.*, 2012; Estrada-Luna *et al.*, 2008; Khalafalla *et al.*, 2007). Other cactus species such as *Echinocereus cinerascens*, *Mammillaria carmenae*, *Mammillaria prolifera*, *Astrophytum myriostigma* and *Trichocereus spachianus* also demonstrated high survival rates (80-100%) in acclimatization (Elias *et al.*, 2015; Vyskot & Jára, 1984).

The addition of cocoa peat in the soil mixture (black soil and red soil) enhanced the ability of the *H. polyrhizus* plantlets to survive during hardening process. Cocoa peat can retain the nutrients and water in the soils. Good aeration of sufficient porous substratum and adequate drainage in *ex vitro* condition influenced the rapid hardening process of regenerated plants (Dunstan & Turner 1984). Well rooted plantlets can enhance the ability of the *in vitro* plantlets to survive because roots can help plants in water balance (Díaz-Pérez, 1995). The extra uptake of the nutrients through roots during *in vitro* culture gives an advantage for roots to balance water loss cause by malfunctioning stomata. The well functional roots, adequate cuticle and the ability of controlling stomata functioning that reduces transplant shock produced by excessive water loss during acclimatization in the cactus species are important in maintaining a continuous growth (Hartmann *et al.*, 1997). Malda *et al.* (1999) also reported cacti have the useful ability to rehydrate after culture *in vitro*.

Plants with succulent stem and crassulacean acid metabolism (CAM) are easier to acclimatize compared to others because they can minimize water strain during hardening process (Malda *et al.* 1999). CAM is one of physiological adaptation in Cactaceae family which increase water-used efficiency and typically associated with semiarid plants where the seasonal water availability (Nobel, 1988; Cushman, 2001). This feature also could alter the pattern of carbon uptake and affect plant growth under artificial environmental conditions, especially in tissue culture. High humidity in the culture vessel allows the stomata of cacti to remain open during day light and maintain continuous CO_2 fixation during light and dark periods (Malda *et al.* 1999). Griffiths (1988) described that CAM plants have plasticity characteristic with regards to changes in water accessibility, due to stomatal closure and re-fixation of internal CO_2. This may

lead to improved abilities to minimize pressure during transfer from culture vessels to the *ex vitro* environment.

Brito *et al.* (2009) reported that the good quality of newly formed root system, controlled exposure to low relative humidity and high light intensity were important for a successful acclimatization in *Oleo maderensis*. Stimulating autotrophic characteristics under *in vitro* conditions may improve acclimatization ability and survival rates (Hazarika 2006; Pospíšilová *et al.* 1999). Poor root and root hair growth in tissue culture greatly affect the success of acclimatization of *in vitro* grown *Trachelospemum asiaticum* plantlets (Apter *et al.* 1993). The presence of *in vitro* roots in plantlets improved water status by increasing the water uptake uptake capacity, hence enhanced the physiological activity (Diaz-Perez *et al.* 1995). However, *in vitro* roots are considered as non functional in some plant due to these roots usually die during hardening process and can delay plant growth (Debergh & Maene, 1981). Grout and Aston (1977) reported that *in vitro* roots have poor vascular connections with shoots and resulting restricted water transfer from roots to shoots.

Acclimatization studies were continued with soil analysis using X-ray diffractometry (XRD) and X-ray fluorescence (XRF) analysis for measurement of mineral phase and multielement components. All the three types of soils (black soil, red soil and combination soil) were tested in both analyses. XRF were used to observe the multielements presence in the soils such as Si, Al, Fe, Ca, K, Mg, Na, Cu, Zn, Rb, Sr, Zr, Nb and Cl, as displayed in Figure 5.8. In XRD, the most dominant mineral and the highest content in the soil sample was *quartz* or silicon dioxide (SiO_2). In agreement with these finding, Schönenberger *et al.* (2012) also observed quartz as the most

abundant mineral in soil samples from 3 Canadian provinces; Nova Scotia (63.7%), Prince Edward Island (59.3%) and Saskatchewan (55.2%). The second most abundant mineral is *kaolinite*. Others minerals are comprised of gibbsite, zeolite, muscovite, magnetite, illite and vermiculite.

Silicon existing in the soil helps the plant acclimatized to survive and become well-developed. The suitability of the plant towards sowing soil during hardening process plays important role in order to ensure the plants are able to endure. Quartz or SiO_2 is a very resistant mineral in all terrestrial compartments and is the essential components of all silicates (e.g feldspar, quartz) and aluminium silicate minerals (Kabata-Pendias, 2010). Savant *et al.* (1999) reported that Silicon (Si) is an important element that increases the tolerance of plant against environmental stress. Silicon (Si) is also has been observed necessary for stimulation of plant growth, development and structure strength (Wallace, 1971; Richmond & Sussman, 2003). Miyake and Takashi (1978) showed deficiency of Si element inhibits the reproductive growth of tomato plants grown in culture solution. The appearance of Si in the plant soils also helps in increasing drought tolerance of plants and increasing antioxidant defence activity (Lux *et al.,* 2002; Gong *et al,.* 2005). The use of Si in fertilizer could serve as limiting agents and application in neutralization of soil acidity. An adequate Si supply can benefit plants in variety of ways, especially under stressful environment.

To compare *in vivo* and *in vitro* growth performance, a comparative study of histological traits between *in vivo* and *in vitro* grown *H. polyrhizus* was done to examine and monitor the variability appearance in the cell structure (Chapter 6). For this purpose, i*n vivo* and *in vitro* stems and roots were used and analyzed. In general, the *in*

vitro plants were slightly thinner than *in vivo* plants. The epidermis of *in vivo* and *in vitro* stem was uniseriate and covered by cuticle. The cell walls of the *in vitro* plants were thinner than *in vivo* plants. The cuticle layer of *in vivo* grown *H. polyrhizus* was observed to be thicker than *in vitro* grown plants. Mucilage cells were observed within the cortex inner region in both plants. Phloem and xylem were observed in both *in vivo* and *in vitro H. polyrhizus* vascular systems. Cortical bundles were collateral for both but smaller quantity of xylem in *in vitro* plant. Stomata of both plants were oriented on the same level of epidermal layer. Nevertheless, several *in vitro* stomata were poorly developed and malfunctioning. The epidermal layers of *in vivo* and in *in vitro* roots were also uniseriate. Root hairs were observed on both kind of growth.

The introduction of the artificial culture conditions towards micropropagated plants influence the variability occurrence in the cell structures. Several factors such as high relative air humidity, air composition and culture media content indicate the changes of the cell morphology (Paunescu, 2008). The cell wall of the regenerated plants was thinner than normal plants. Taji *et al.* (1996) demonstrated thinner cell wall allowed deposition epicuticular waxes and deprived mechanical tissue formation. The walls of epidermal cells are mostyly thin except in few taxa of *Armatocereus, Cereus, Jasminocereus* and *Mammillaria* (Loza-Cornejo & Terrazas, 2003). In Cactaceae, the epidermis act as support system for the succulent stem, water reservoir, shield the inner tissues against sunlight and grant resistance system against organisms (Gibson & Nobel, 1986). Barthlott (1981) reported that epidermal surface characters are slightly influenced by environmental conditions. Cactus epidermis usually composed of single layer of square to rectangular cells, whereas in some species consequent cell divisions lead to the construction of multiseriate epidermis (Mauseth, 2006).

Cactaceae family have the ability to withstand and well-adapted in arid and hot drylands. Capability to retain water in prolonged drought is due to the presence of water-binding capacity of mucilage (Cárdenas *et al.,* 1997). Mucilage is a complex carbohydrate with a highly branched structure and containing varied proportion of L-arabinose, D-galactose, L-rhamnose and D-xylose, as well as galacturonic acid in different proportions (Sáenz *et al.,* 2004; Paulsen & Lund, 1979; Sepúlveda *et al*., 2007). Nevertheless, the mucilage composition was found to differ among different cactus species and among different areas within a species (Trachtenberg & Mayer, 1981). Cactus mucilage has many potential uses as flocculating agent and thickening agents (Young *et al.,* 2006; Sepúlveda *et al.,* 2007). Stintzing *et al.* (2001) observed that the mucilages in *Opuntia* sp. are responsible for water retention and can be used as dietary fibre or food thickening agents. Adetunji *et al.* (2014) reported that mucilage cells of *Opuntia* sp. could serve as edible coating for prolong the shelf life of papaya fruits and able to reduce ascorbic acid content, pH and total soluble solids. Mucilage cells are commonly found in the cortex of cactus species such as *Arrojadoa bahiensis*, *Stephanocereus leucostele* A. Berger and *Brasillicereus markgrafi* Backeberg (Soffiati & Angyalossy, 2007).

Stomata malfunctioning is shown in several *in vitro* cell structures. In agreement with this finding, the culture condition using water saturated vessel could increase the transpiration rate of the plantlets, thus cause the failure of the stomata to close (Kevers *et al.,* 2004). Stoma is a small opening or tiny pore originate on the outer leaf and petiole skin, also known as the epidermis. They consist of two specialised cells, called guard cells adjoining the tiny pore and they are liable for the opening and closing of the stoma. Purpose of stoma is for gas exchange in plants. As stomata open in the existence of sunlight, carbon dioxide is taken into the plant to be used in photosynthesis, while

oxygen (product of photosynthesis) and water vapour escape from the stomata to the surrounding atmosphere through the process of transpiration. However, in cacti species, the stomata open by night, when temperature is cool, and absorb carbon dioxide which they store by chemically combining it with an organic compound containing 3 carbon atoms, producing a 4-carbon organic acid. During the day, when the stomata are closed, the carbon dioxide is released from this organic acid and used to synthesize sugars, using the energy of light. These plants do exactly the opposite of normal plants, which open their stomata by day when light is available for photosynthesis, and close them at night. The special biochemical process called crassulacean acid metabolism (CAM).

The number of xylem and phloem in *in vitro H. polyrhizus* plants less when compared with *in vivo* plantlets (Figure 7.1.2). In agreement with these findings, Cozza *et al.* (1997) also found that xylem and phloem elements decreased in micropropagated shoots of *Olea europea* L. as compared to the plants in the field. In addition, nutritional conditions influence differentiation processes to different extents in relation to growth medium and/or plant genotype. Phloem and xylem are complex tissues that perform transportation of food and water in a plant. They are the vascular tissues of the plant and together form vascular bundles. They work together as a unit to bring about effective transportation of food, nutrients, minerals and water. The cortical bundle is a vascular system which allows the transportation of water and nutrients through the voluminous cortex of the cactus. This system is unique to cacti, and helps to replace the water lost to the dry desert air. Cortical bundle phloem is involved in nutrient transfer when the cortex acts as a storage organ (Sajeva & Mauseth, 1992). They also reported that cortical bundles completely absent from subfamilies Pereskioideae but present in all Cactoideae. Cortical bundles are critically important for the evolution of a broad cortex because, even if a stem has a thick, wax-covered cuticle, it gradually loses water to dry

desert air, so epidermis, hypodermis and outer regions of cortex must be kept hydrated by some means.

The comparative histological studies between *in vitro* and *in vivo* grown *H. polyrhizus* plantlets exhibited small differences in tissue morphology. Several researchers have reported phenotypic changes in micropropagated plantlets of several species after their *ex vitro* transfer, possibly caused by sudden changes in environmental conditions (Johansson *et al.*, 1992; Noe & Bonini, 1996; Pospišilová *et al.*, 1999). Zobayed *et al.* (2001) observed in tobacco and cauliflower in *in vitro* stages at dissimilar ventilations revealed that this abnormality might be due to the acclimatization stages. In addition, abnormal and low stomata densities in regenerated carnation plants have been reported by Olmos and Hellin (1998). Poorly developed vascular bundles with undifferentiated xylem and phloem have been observed in *in vitro* plants of date palm (El-Bahr *et al.*, 2004). The specific culture conditions can cause changes in plants and lead progenies with modified characteristics, either genetic or epigenetic (Jain, 2001).

The morphological of the plants are controlled by the environmental conditions. Differentiation in histological traits occurred when the plants exposed to specific conditions either *in vivo* or *in vitro*. The structural changes in micropropagated plants were influenced by high relative air humidity, air composition and culture media content. Bitonti *et al.* (1996) reported that organ of an individual plant living under stress undergo adaptive changes which determine their more effective function. *In vitro H. polyrhizus* showed several malfunctioning stomata. Paek and Hahn (2000) described that the culture media content could influence the increased risk of stomata

malfunctioning. Ziv (1991) observed that the risk of stomata malfunctioning is increased by altered cellulose biosynthesis in the guard cell walls. Maintaining the genetic characteristics of the donor plant and a completely viable acclimatized plant yield after *in vitro* storage are the crucial fundamentals for using *in vitro* culture techniques for *ex situ* conservation purposes.

Chapter 8 of this thesis described the analysis of betacyanin pigment extracted from peel and pulp of *H. polyrhizus*. The stability of the betacyanin towards some environmental factors such as pH and light also has been evaluated. Betacyanins pigment extracted from *H. polyrhizus* pulp by using 80% acetone as a solvent at pH 1 showed highest betacyanins content (Table 8.1). The betacyanins contents in pulp were higher than the content in the peel. However, Wu *et al.* (2006) investigated the betacyanins content in the peel were higher compared to pulp component. According to Harivaindaran *et al.* (2008), heating up the water during betacyanins extraction process from *H. polyrhizus* peel cause leakage of betacyanins out from the matrix constituent more effectively. The current study showed that acetone was the best solvent to extract highest betacyanins content. However, Castellar *et al*. (2006) observed that better extraction of betalains when using water as a solvent rather than a mixture of water and ethanol in *Opuntia* fruit. Betacyanins are known more soluble in water than in non-polar solvent due to high molar absorbency index characteristic thus helps in extraction and separation processes (Strack e*t al.,* 2003). Betacyanins also can be well dissolved in polar solvent such as methanol and ethanol as well as their mixture because of it is more hydrophilic compared to anthocyanins (Schoefs, 2004).

Betacyanins pigments are more stable in acidic condition rather than alkaline condition. Woo *et al.* (2011) reported that betacyanins pigments are more stable on acidic pH between pH 3-7 and beyond this range, pigment could be degraded. Herbach *et al.* (2006) found that heated dragon fruit juice under pH 4 achieved maximum stability of betacyanins pigment content. Under acidic conditions, decarboxylation of betanin and also recondensation of betalamic acid with *cyclo*-dopa 5-O-β-glucoside were observed for regeneration of betacyanins (Huang & von Elbe, 1987; Azeredo, 2009; Schwartz & von Elbe, 1983). The pigment extracts demonstrated color changing, where its showed purple at pH 1, red-violet at pH 3 – pH 7 and orange at pH 9 (Figure 8.1.1). Alkaline pH solution triggers the betalains pigments changed into yellow tint due to release of betalamic acid. According to Stephen Nottingham (2005), discolouration of betalains in *Beta vulgaris* does not affect pH range of 3.5-7. Devi *et al.* (2012) reported that betacyanins pigments of *Basella alba* are more stable and in red hue under neutral and weak acidic condition, while under strong alkaline conditions, the colour of pigments change into yellow due to typical alkaline-acidic reaction. Heating and high illumination intensity leads to discolouration of betalains. Degradation of betacyanins are influenced by pH and known to undergo hydrolytic cleavage (Stintzing & Carle, 2007). Schwartz *et al.* (1981) found that hydrolytic cleavage could degrade betanin and yielded biogenetic precursor yellow betalamic acid and the colorless cyclo-dopa 5-O-β-glucoside. Other than that, deglucosylation could produce aglycone accompanied by bathrochomic shift. Cold storage of heated pigment also could regenerate betanin by recondensation of those hydrolysis products associated with recuperate of pigment colour.

As represented in Table 8.2, samples stored under dark conditions had higher betacyanin content compared to storage under light exposure. Betacyanin contents

decreased after 24 hours storage under light conditions. Stability of betacyanin was influenced by light. According to Jackman and Smith (1996), the effect of light could excite the electron of betalains chemophores to a high energy state, resulting higher reactivity or lowered activation energy of the molecule. Betacyanins are known as light sensitive pigments and tend to degrade due to light absorption in the visible light and the ultra violet range of betalains molecule (Herbach *et al.*, 2006; Cai *et al.*, 2005). The presence of oxygen and light exhibits a synergistic effect on degradation of betalains pigment. These two conditions caused 28.6% reduction on half life of betanin in beet juice (von Elbe *et al.*, 1974). The addition of 1 % ascorbic acid retained betacyanins content in *H. polyrhizus* juice from detrimental effects of light exposure (Herbach *et al.*, 2006). Attoe and von Elbe (1985) also confirmed the presence of ascorbic acid as antioxidant increased the stability of betalains. Antioxidants supplementation capable of protecting the betalains from deformation by removing dissolved oxygen in pigment solution.

The absorbance values decreased when the coated glass subjected to higher concentration of NaCl (15%). The colour of the pigment on the coated glass also became faded when exposed to high concentration of NaCl after 2 hours of treatment. The current study showed the durability of betacyanin coated glass decreased in high NaCl concentration, thus limits its application as coating. The utilization of ascorbic acid as co-pigment to enhance the stability of betacyanin pigment and protect the pigment from discoloration should be a good use in natural colorant application. Yaacob *et al.* (2011) also made similar observation, where glass slides coated with anthocyanins pigments extracted from *Agapanthus praecox* decreased in absorbance values when exposed to high concentration of NaCl.

CHAPTER 10

CONCLUSION

An optimum protocol of *in vitro* regeneration for mass propagation of *Hylocereus polyrhizus* (Weber) Britton and Rose plant was successfully established. Callus induction from stem explants of *H. polyrhizus* by using various concentration and combination of plant growth regulators was observed. Stem explants were used as explant sources for regeneration and callus induction. For *in vitro* plant regeneration studies of *H. polyrhizus*, stem explants were cultured horizontally and vertically. Both positions successfully induced the multiple shoots. The growth of the new shoots was observed after 1 week being cultured on MS medium supplemented with various concentrations and combinations of hormones. The highest average number of shoots was counted as 8.07 ± 0.721 on MS medium supplemented with 0.2 mg/L BAP with explants horizontally cultured. Despite the fact that, 0.2 mg/L Kn induced the longest shoots with 1.608 ± 0.128 cm, when cultured in horizontal position, MS medium supplemented with combinations of 0.1 mg/L BAP and 0.1 mg/L NAA showed the highest average number of roots (3.031 ± 0.237) when cultured vertically. In the mean time, MS medium supplemented with 0.5 mg/L BAP and 0.1 mg/L NAA resulted in the longest root formation when cultured in horizontal position.

Development of callus can be done by manipulating plant growth regulators. Various concentrations and combinations of 2, 4 –D and BAP were used in this study to promote the growth of callus. Skin and areoles of the stem explants were removed and placed in horizontal position to enhance the callus development. In this study, the callus formation were well-developed in MS medium supplemented with combinations of 1.0 mg/L BAP and 0.1 mg/L 2, 4-D, 1.0 mg/L BAP and 1.0 2, 4-D and 2.0 mg/L and 1.0

mg/L 2, 4-D. The lowest percentage (27.0 ± 0.082 %) was observed when the stem explants were cultured on MS medium supplemented with 2.0 mg/L 2, 4-D. The combinations of BAP and 2, 4-D showed better callus induction instead of 2, 4-D alone. Various colours of the callus derived from stem explants were obtained such as green, white, yellow and light yellow callus. Green callus was observed on the MS medium supplemented with 1.0 mg/L BAP and 0.5 mg/L 2, 4-D, 1.0 mg/L BAP and 1.0 mg/L 2, 4-D, and 2.0 mg/L BAP and 1.0 mg/L 2, 4-D. The nature of the callus induced was compact on MS medium.

Synthetic seed technology offers another alternative method for mass propagation of plant species. In this study, synthetic seed technology was successfully studied and established for this species. Synthetic seeds were important in germplasm conservation, and have benefits such as easy handling, long term storage, and preserve endangered plant species. In the present study, synthetic seeds germinated on MS basal medium showed highest germination frequency (100%). The emergence of initial shoots was observed on Day 10. The addition of hormones such as BAP, Kinetin and NAA in the encapsulated beads resulted in callus formation, however, no complete plantlets was generated. No further development was observed and all callus turned brown in colour and died after 12 weeks. Viability of synthetic seeds of *H. polyrhizus* decreased with increasing of storage period. No response and emergence of shoots were observed after 120 days of storage period. In germination of synthetic seeds, all encapsulated beads either in sterile or non sterile black soil and red soil gave negative responses with fungal infection. More research requires to be done in this aspect to enhance the germination of synthetic seeds directly in the soil.

Acclimatization process is very important to ensure the survival of the regenerated plantlets. For acclimatization studies, three different types of soils were used. Overall, all regenerated plants adapted well when transferred to the soil. The highest percentage of survival *in vitro* was obtained from plants acclimatized in the combination of red soil, black soil and cocoa peat (83.3 % ± 0.07). Black soil showed 67.7 % ± 0.09 survival rates and followed by red soil 57.7 % ± 0.09. The maximum shoot length was observed with plant grown in soil combination and the minimum shoot length was observed in plants grown in red soils after 4 months. Soil analysis for all the three types of soils were examined by using X-ray diffractometry (XRD) and X-ray fluorescence spectrometry (XRF). In XRF, the multielements presence in the soils were Si, Al, Fe, Ca, K, Mg, Na, Cu, Zn, Rb, Sr, Zr, Nb and Cl. In XRD, the most dominant mineral and the highest content in the soil sample was *quartz* or silicon dioxide (SiO_2). The second most abundant mineral was *kaolinite*. Other minerals were comprised of gibbsite, zeolite, muscovite, magnetite, illite and vermiculite.

The micromorphological differences between *in vitro* and *in vivo* grown plantlets were observed via histological analysis. *In vivo* and *in vitro* stems and roots were used and analyzed. In this study, the minimal difference in plant organization and cell structure can be observed between *in vivo* and *in vitro H. polyrhizus* plantlets. The artificial conditions that have been applied during tissue culture process for *in vitro* plantlets influenced some microphological differences in the plant cell structures. In this study, *in vivo* and *in vitro H. polyrhizus* stems and roots were used and analyzed to identify any variability on their micromorphological cell structures. Based on observation, *in vitro* plants were slightly thinner than *in vivo* plants. The epidermis of *in vivo* and *in vitro* stems was uniseriate and covered by cuticle layer. Cuticle layer presence in *in vivo* plants was thicker than regenerated plants. The cell wall of *in vitro*

plants was thinner than *in vivo* grown plant. Mucilage cells were observed within the inner cortex region in both plants. A cortical bundle contains xylem and phloem was observed in both and collateral. The orientation of stomata of both plants was observed on the same level of epidermal layer. However, several stomata were poorly developed and malfunctioning. For *in vivo* and *in vitro* roots, the epidermal layer was also uniseriate and root hairs were present in both.

In order to observe the stability of betacyanins pigment from *H. polyrhizus* towards several environmental factors, UV visible spectrophotometric analysis was used. Betacyanin pigment extracted from pulp of *H. polyrhizus* using 80% acetone as solvent showed highest betacyanins content at pH 1.0. Light was also found could degrade the pigment since the betacyanins content decreased after exposed to light in 24 hours. In addition, the *H. polyrhizus* betacyanins were able to mix well with PMMA resin and coated onto glass slides. This proved that betacyanins pigment can be used as natural coating in decorating application. The salt test on betacyanins coated glass slides showed decreasing in absorbance values and colour faded when NaCl concentration increased.

The findings in this thesis provide a useful protocol for *in vitro* plant regeneration especially of cactus species (*H. polyrhizus* (Weber) Britton and Rose). Plant tissue culture system is unique in obtaining, maintaining, and mass propagating of specific pathogen-free plants. The other potential plant tissue culture researches such as plant genetic studies and transformation can be done for improvement of agronomical traits, including disease resistance, insect tolerance, better nutritional values, and other desirable qualities. Apart from that, further study on ability of *H. polyrhizus*

betacyanins pigment to maintain the colour in harsh condition can enhance its utilization as natural colorants.

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APPENDIX I

Formulations of MS media (Murashige & Skoog, 1962)

Components	**Details**	**Content**
Macronutrient	$MgSO_4.7H_20$	370 mg/L
	KH_2PO_4	170 mg/L
	KNO_3	1900 mg/L
	NH_4NO_3	1650 mg/L
	$CaCl_2.2H_2O$	440 mg/L
Micronutrient	H_3BO_3	6.2 mg/L
	$MNSO_4.H_2O$	15.6 mg/L
	$ZnSO_4.7H_2O$	8.6 mg/L
	$NaMoO_4.2H_2O$	0.25 mg/L
	$CuSO_4.5H_2O$	0.025 mg/L
	$CoCl.6H_2O$	0.025 mg/L
	KI	0.83 mg/L
Iron	$FeSO_4.7H_2O$	27.8 mg/L
	Na_2EDTA	37.3 mg/L
Vitamin	Thiamine HCl	0.5 mg/L
	Pyridoxine HCl	0.5 mg/L
	Nicotinic acid	0.05 mg/L
	Myo-inositol	100 mg/L
Sucrose		30 g/L
Gelrite		2 g/L
pH		5.75

APPENDIX II

CONFERENCES AND EXHIBITIONS ATTENDED

1. **Razak, U.N.A.A.** and Taha, R.M. (December, 2013). Callus Induction, *In vitro* Regeneration and Acclimatization of *Hylocereus polyrhizus* (Red-fleshed Dragon Fruit). Paper presented at 5th GLOBAL SUMMIT ON MEDICINAL & AROMATIC PLANTS at Marriott Resort & Spa, Miri, Sarawak, Malaysia.

2. **Razak, U.N.A.A.** and Taha, R.M. (December, 2014). The effects of BAP, NAA and Kinetin on micropropagation and histological studies of *Hylocereus polyrhizus* (Weber) Britton & Rose for early detection of somaclonal vaiation. Poster presented at 19th ANNUAL BIOLOGICAL SCIENCES GRADUATE CONGRESS at National University of Singapore, Singapore.

3. Taha, R.M., Yaacob, J.S., Salleh, A., Manan, A.A., Mohamed, N., Mahmad, N., and **Razak, U.N.A.A.** (October, 2013). Floral Scientific Handicraft and Indoor Garden Derived from *In vitro* Techniques. Participated in BIOMALAYSIA & BIOECONOMY ASIA PACIFIC 2013 at Persada Johor International Convention Centre, Johor Bahru. (Bronze Award).

APPENDIX III

LIST OF PUBLICATIONS

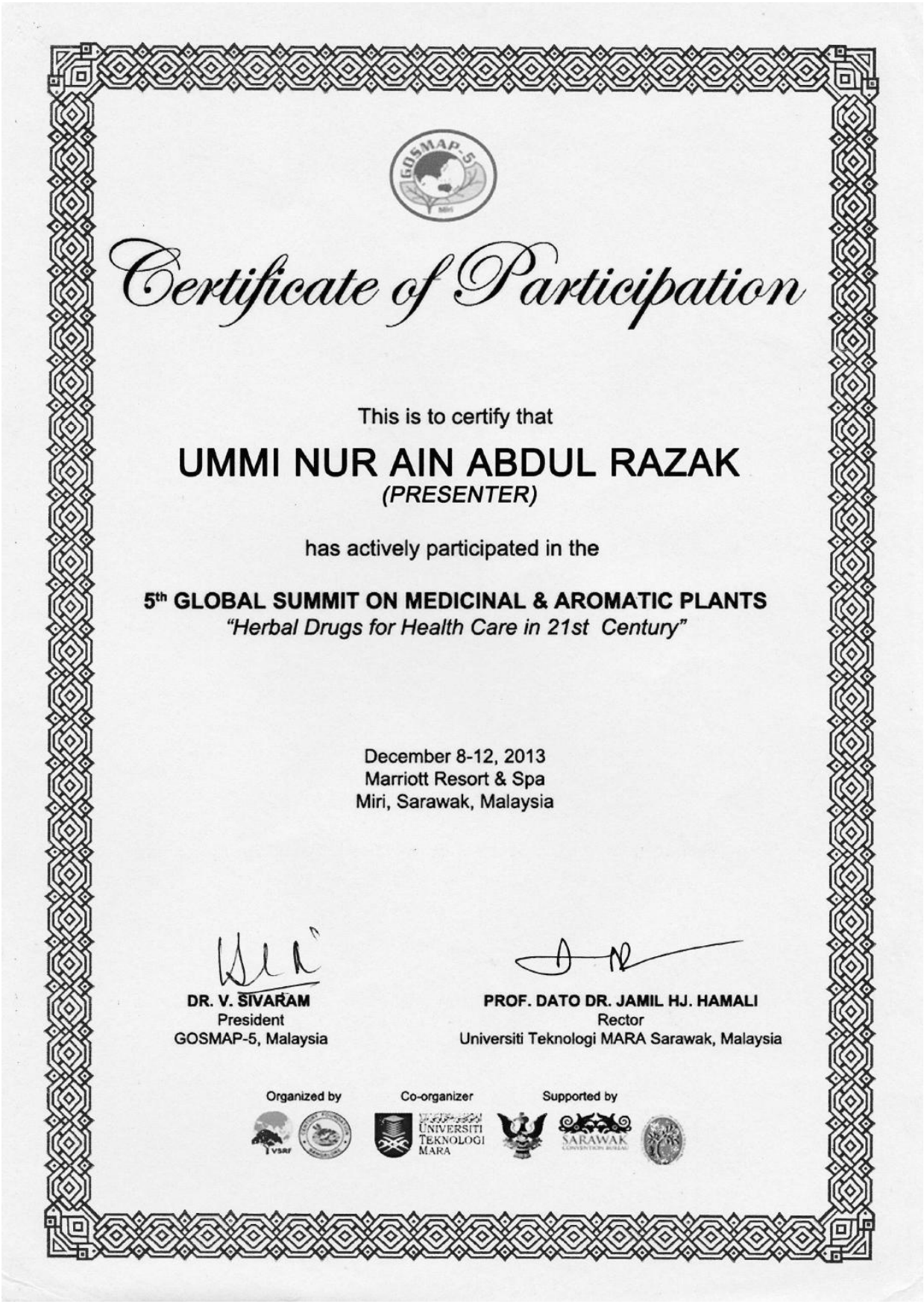
1. **Ummi Nur Ain Abdul Razak,** Rosna Mat Taha, Siti Aisha Nailla Che Musa and Normadiha Mohamed. 2016. Detection of Betacyanins Pigments from *Hylocereus polyrhizus* (Weber) Britton & Rose Fruit Pulp and Peel for Possible Use as Natural Coating. *Pigment & Resin Technology*. **46(4).** (Accepted)

2. **Ummi Nur Ain Abdul Razak**, Rosna Mat Taha, Normadiha Mohamed and Hashimah Elias. 2016. The effects of plant growth regulators on micropropagation and histological studies of *in vivo* and *in vitro* grown of *Hylocereus polyrhizus* (Weber) Britton & Rose – An edible cacti. *Sains Malaysiana (*In Review)

3. Normadiha Mohamed. Rosna Mat Taha, **Ummi Nur Ain Abdul Razak**, Hashimah Elias and Sakinah Abdullah, 2016. The Role of PGRs in the Development of *In Vitro* Flowering, Histology and Ultrastructural Studies in *Impatiens balsamina* L. cv Dwarf Bush. *Planta Daninha.*

4. Normadiha Mohamed, Rosna Mat Taha, **Ummi Nur Ain Abdul Razak**, Hashimah Elias and Nor Azlina Hasbullah. 2017. Micropropagation and and cellular behaviour changes during *in vitro* flowering of *Impatiens balsamina* L. cv Dwarf Bush. *Planta Daninha.*

5. Siti Aisha Nailla Che Musa, Rosna Mat Taha, **Ummi Nur Ain Abdul Razak**, Nordiyanah Anuar and Abdul Kariem Arof. 2016. Influence of various extraction parameters on pigments production from Cucumis melo L. for potential coating technology. *Pigment & Resin Technology.*



Certificate of Participation

This is to certify that

UMMI NUR AIN ABDUL RAZAK

(PRESENTER)

has actively participated in the

5th GLOBAL SUMMIT ON MEDICINAL & AROMATIC PLANTS

"Herbal Drugs for Health Care in 21st Century"

December 8-12, 2013
Marriott Resort & Spa
Miri, Sarawak, Malaysia

DR. V. SIVARAM
President
GOSMAP-5, Malaysia

PROF. DATO DR. JAMIL HJ. HAMALI
Rector
Universiti Teknologi MARA Sarawak, Malaysia

Organized by Co-organizer Supported by


NUS
National University of Singapore
DBS
Department of Biological Sciences
Certificate of Participation
This is to certify that
Ummi Nur Ain Abdul Razak
Participated in the 19th Annual Biological Sciences Graduate Congress
conducted on
12-14 December 2014
PROFESSOR WANG SHU
Deputy Head, Graduate Studies
ASSOCIATE PROFESSOR HENRY MOK
Assistant Head, Graduate Studies
13 DECEMBER 2014
DATE

BioInnovation Awards 2013

Bronze Award

presented to

PROF. DR. ROSNA MAT TAHA,
DR. JAMILAH SYAFAWATI YAACOB,
AZANI SALEH, AZIEMAH ABDUL MANAN,
NORMADIHA MOHAMED, NORAINI MAHMAD,
NORLINA RAWI, UMMI NUR AIN ABDUL RAZAK

FLORAL SCIENTIFIC HANDICRAFT AND INDOOR GARDEN
DERIVED FROM *IN VITRO* TECHNIQUES

YBHG DATO' DR MOHD NAZLEE KAMAL
Chief Executive Officer
Malaysian Biotechnology Corporation Sdn Bhd

DR WAN MANSHOL BIN W. ZIN
Organising Chairman of BioInnovation Awards 2013
Malaysian Association of Research Scientists, MARS