MICROPROPAGATION AND PIGMENT EXTRACTION OF *Echinocereus cinerascens*

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ABSTRACT

Protocols were successfully established for *in vitro* regeneration and coloured callus production of *Echinocereus cinerascens*. Investigation mainly focused on the effects of plant growth regulators in rapid production of this endangered species and the optimum production of coloured callus. Additional assessments studied concerning the production of synthetic seeds, extraction of natural pigments and detection of somaclonal variation of the regenerants. Rapid production through direct *in vitro* regeneration gave the highest mean number of shoots, 4.37 ± 0.27, observed in MS medium supplemented with 2.0 mg/l Kinetin + 1.0 mg/l IBA which promoted the highest production of shoots after 4 months, 131 shoots in total. Nevertheless, through indirect *in vitro* regeneration, somatic embryos of *Echinocereus cinerascens* were successfully developed in two treatments of liquid medium including MS medium supplemented with 0.5 mg/l 2,4-D + 0.1 mg/l BAP + 0.5 mg/l thiamine HCl and MS medium supplemented with 0.5 mg/l 2,4-D + 0.5 mg/l BAP + 0.5 mg/l thiamine HCl, as both media promoted 100% total mean production of somatic embryos (globular, heart, torpedo-shaped and cotyledonary stage) after 4 months. Practically, the production of ideal synthetic seeds was successfully established whereby, micro shoots as the most responsive propagule were encapsulated in 3% of sodium alginate hardened in 100 mM of calcium chloride dehydrate solution for 30 minutes gave 100% of germination rate after 4 months. Complete plantlets were successfully acclimatized with the highest survival rate of 90% observed in sand, the most suitable planting substrate which possessed 3 major elements such as SiO_2, Al_2O_3 and CaO that play important roles to support the growth of *Echinocereus cinerascens*. Basically, the present study indicated that 100% production of green, yellow and pink callus was obtained after 2 months in several treatments of MS medium supplemented with 2,4-D + BAP + thiamine HCl applied in combination. Interestingly, the occurrence of dramatic changes in the

production of coloured callus was clearly observed in a conversion of green to pink callus within 2 months. Moreover, pigment extraction analysis through UV-VIS spectroscopy discovered that both regenerants and callus possessed chlorophyll a and b as the major pigment while carotenoids as the minor pigment. Meanwhile, HPLC analysis revealed individual carotenoids present in *in vitro* plantlets namely, neoxanthin, β-carotene, lutein and violaxanthin, whereas in callus were β-carotene and lutein only. Analysis of cytological studies clarified that there were no significant differences in cell organization and behaviour of *in vitro* plantlets and *ex vitro* plants. The evidence verified that *Echinocereus cinerascens* regenerated normally *in vitro* and grown vigorously after being transferred to the natural environment.

ABSTRAK

Protokol telah berjaya ditubuhkan untuk regenerasi *in vitro* dan penghasilan kalus berwarna *Echinocereus cinerascens*. Penyelidikan terutamanya difokuskan pada kesan penggalak pertumbuhan dalam penghasilan pesat species terancam ini dan penghasilan optimum kalus berwarna. Penilaian sampingan yang dikaji adalah berkenaan penghasilan biji benih sintetik, pengekstrakan pigmen semula jadi dan pengesanan variasi somaclonal regenerants. Penghasilan pesat melalui regenerasi *in vitro* secara langsung telah memberikan jumlah tertinggi purata bilangan pucuk, 4.37 ± 0.27, diperhatikan dalam media MS ditambah 2.0 mg/l Kinetin + 1.0 mg/l IBA yang menunjukkan penghasilan tertinggi pucuk selepas 4 bulan, 131 jumlah pucuk keseluruhannya. Walau bagaimanapun, melalui regenerasi *in vitro* secara tidak langsung, embrio somatic *Echinocereus cinerascens* telah berjaya dihasilkan dalam dua rawatan media cecair termasuklah media MS ditambah 0.5 mg/l 2,4-D + 0.1 mg/l BAP + 0.5 mg/l thiamine HCl dan media MS ditambah 0.5 mg/l 2,4-D + 0.5 mg/l BAP + 0.5 mg/l thiamine HCl, kerana kedua-duanya menunjukkan 100% jumlah purata penghasilan embrio somatik (peringkat berbentuk globular, hati, torpedo dan kotiledon) selepas 4 bulan. Secara praktikal, penghasilan biji benih sintetik yang ideal telah berjaya ditubuhkan di mana, pucuk mikro sebagai propagul yang paling responsif dikapsulkan dalam 3% sodium alginat dan dipejalkan dalam 100 mM larutan kalsium klorida dihidrat selama 30 minit, memberikan 100% kadar percambahan selepas 4 bulan. Anak pokok lengkap telah berjaya diaklimatasi dengan kadar kelangsungan hidup tertinggi 90% diperhatikan dalam pasir, substrat penanaman paling sesuai yang mempunyai 3 elemen utama seperti SiO_2, Al_2O_3 dan CaO yang memainkan peranan penting untuk menyokong pertumbuhan *Echinocereus cinerascens*. Pada asasnya, kajian ini menunjukkan 100% penghasilan kalus hijau, kuning dan merah jambu diperolehi selepas 2 bulan dalam beberapa rawatan media MS ditambah 2,4-D + BAP + thiamine

HCl dalam kombinasi. Menariknya, berlaku perubahan dramatik dalam penghasilan kalus berwarna yang jelas diperhatikan dalam penukaran kalus hijau kepada merah jambu dalam masa 2 bulan. Selain itu, analisis pengekstrakan pigmen melalui UV VIS spektroskopi mendapati bahawa kedua-dua regenerants dan kalus memiliki klorofil a dan b sebagai pigmen utama manakala karotenoid sebagai pigmen minor. Sementara itu, analisis HPLC mendedahkan individu karotenoid yang hadir dalam anak pokok *in vitro* iaitu neoxanthin, β-karotena, lutein dan violaxanthin, manakala pada kalus adalah β-karotena dan lutein sahaja. Analisis kajian sitologi menjelaskan bahawa tiada perbezaan yang signifikan pada organisasi dan tingkah laku sel dalam anak pokok *in vitro* dan tumbuhan *ex vitro*. Bukti-bukti ini mengesahkan bahawa *Echinocereus cinerascens* telah diregenerasi secara normal *in vitro* dan tumbuh subur selepas dipindahkan ke persekitaran semula jadi.

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2,4-D	2,4-Dichlorophenoxyacetic acid
Al_2O_3	aluminium oxide
ANOVA	analysis of variance
BAP	6-Benzylaminopurine
CA	cell area
$CaCl_2$. $2H_2O$	calcium chloride dehydrate
CaO	calcium oxide
CC	chromosome count
CO_2	carbon dioxide
CPD	critical point drying
DNA	deoxyribonucleic acid
g/l	gram per liter
HCl	hydrochloric acid
HPLC	high-performance liquid chromatography
IBA	Indolebutyric acid
KIN	kinetin/ 6-Furfurylaminopurine
kPa	kilopascal
LS	longitudinal section
mg/l	milligram per liter
$MgCO_3$	magnesium carbonate
MI	mitotic index
MS	Murashige and Skoog
NA	nuclear area
NAA	α-Naphthalene acetic acid
NaCl	sodium chloride

NaOH	sodium hydroxide
OSO_4	osmium
PGR	plant growth regulator
R^2	coefficient of determination
rpm	rotation per minute
SE	standard error
SEM	scanning electron microscope
SiO_2	silicon oxide
TS	transverse section
UV-VIS	ultraviolet visible
v/v	volume per volume
w/v	weight per volume
XRF	X-ray fluorescence

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CHAPTER 1

INTRODUCTION

Cactus species is a unique decorative plant which possesses lovers all around the world. The lovers greatly adore the beauty of the cactus species through its fantastic features which they described as ‘bizarre’, ‘weird’, ‘sculptural’, ‘architectural’, ‘exotic’, etc. Generally, some of the cactus species are known by their structures which may be quite similar to certain objects such as starfish, silver dollar, hedgehog, bunny ear and others. Besides these unique characteristics, the cactus species is also convenience to look after and can be grown indoor as interior home decoration or outdoor in the garden.

The cactus species are very well known as slow growing plants and many are found growing in the desert area that majority of the plant species cannot survive. This fascinating plant can survive in poor well-drained condition and rocky soil. Mostly, the cactus species are not tolerant of prolonged low temperature and light conditions. The species can produce a few offsets or seeds and several seeds are hard to obtain since it only produces seeds per year. Besides, the plantlets from the species are difficult to develop as they are susceptible to damping-off. Moreover, the species are keen to be disappearing from its natural habitat due to threatening by illegal cactus collectors and developers. Numerous illegal collectors harvest the cactus species for foods, medicines, forages and much more while the developers build up factories, building for industry and human benefits. Therefore, the cactus species are becoming extinct and considered as threatened plant nowadays.

In vitro plant regeneration has been achieved in numerous plant species through cell, tissue or organ culture using various types of media (George *et al*., 2008), and the applications of plant tissue culture are far reaching in many fields including plant sciences, biotechnology, agriculture, medicine, etc. Tissue culture indeed has become the cornerstone to build up further advanced researches which could lead to new

relevant findings and potential applications for the achievement of commercial goals and health benefits. *In vitro* plant regeneration is very well known as an important biotechnology tool for propagation of endangered (Coelho *et al.*, 2012; Debnath, 2004) and also offers rapid production of high quality and quantity of identical clonal plants to ensure the availability of desired and highly valued plants throughout the year. The capability of *in vitro* plant regeneration techniques to produce great numbers of plantlets de novo from dedifferentiated cell cultures suggests great chances for the achievement of genetic transformation. Successful *in vitro* plant regeneration and genetic engineering approaches have a great potential to produce superior plants which are pathogen-free or disease-resistant, and to produce plants with higher content of valuable compounds and other desirable characteristics. Currently, advanced research in plant biotechnology offers efficient procedures and a new vista for improving production of desired valuable compounds.

Plant cell and tissue cultures hold significant applications in the production of valuable pigments and bioactive compounds. These compounds are very important especially in the new discoveries of pharmaceutical products from natural resources. Through plant cell and tissue cultures, specific desirable compounds can be produced at a similar or greater rate compared to intact plants. Hence, a high market demand of natural and renewable products initiate an attention of *in vitro* plant materials as the potential factories for the production of secondary metabolites that has paved the way for a new research exploring secondary metabolite expression *in vitro* (Karuppusamy, 2009).

Therefore, this research was proposed mainly to preserve or maintain the cactus species from extinction. Besides, with regard to the matters, several objectives which significant for this research are stated and explained as below.

1. The main objective of the present study was to establish an efficient medium for *in vitro* regeneration of *Echinocereus cinerascens* which offers the production of plants in the industrial scale. Thus, unlimited planting material can be supplied throughout the years. This investigation involved several *in vitro* regeneration techniques which include direct *in vitro* regeneration through axillary shoot production and indirect *in vitro* regeneration through somatic embryo production.
2. Subsequent research is followed by the production of synthetic seeds of *Echinocereus cinerascen* since it was found that inefficient propagation of seeds in the natural habitat causes poor recovery of this cactus species. Therefore, this study aimed to examine the most suitable propagules for the creation of ideal synthetic seeds and then, to obtain the optimum medium for synthetic seed germination that may perhaps be applicable to solve the problems.
3. Furthermore, the objective of this study was to establish an efficient acclimatization protocol for *Echinocereus cinerascens* as *in vitro* plant regeneration is considered accomplish by successful acclimatization of *in vitro* plantlets. *In vitro* plantlets were transferred to various planting substrates where the observation on survival rate of plants grown vigorously in the natural environment was studied and the best planting substrate was determined. Additionally, ultrastructural analysis was done to distinguish any physiology and morphological changes of *Echinocereus cinerascens* grown *in vivo*, *in vitro* and *ex vitro*.

4. Moreover, this study also involved the extraction and detection of pigments (natural colourant) from *Echinocereus cinerascens* for future applications in health benefits. Hence, both samples either *in vitro* plantlets or coloured callus were pigment extracted and subsequently analysed to determine any valuable compounds present in the species.
5. Besides, the objective of the present study was to examine cytological behaviour of meristematic root cells of *Echinocereus cinerascens* grown *in vivo, in vitro* and *ex vitro* through the analysis of several parameters such as mitotic index, chromosome counts, cell and nuclear areas. This study offers significant findings to identify whether the plantlets produced from tissue culture are true-to-type or have been changed due to somaclonal variation by observing the cellular behaviour. In fact, the study may reveal the existence of relation between the growth performance and cellular behaviour of meristematic root cells.

CHAPTER 2

LITERATURE REVIEW

2.1 CACTUS FAMILY (CACTACEAE)

Cactus is a species of plant which is classified in the Cactaceae. "Cactus" is derived from Latin word from the Ancient Greek, "kaktos" that was originally used to describe unidentified spiny plant. In 1912, the cactus became popular and profitable since the revival of interest in cactus collections (Griffiths and Thompson, 1929). Consequently, more companies and premises in the cactus industry were established. Most of the cactus species are indigenous to Western Hemisphere but nowadays are widely spread in tropical and warm temperate areas of the world (Griffiths and Thompson, 1929).

The Cactaceae is commonly found in Mexico arid lands. Hunt (1999) reported that Mexico has 48 genera and about 570 species of cactus. Studies in the Chihuahuan Desert Region revealed the general pattern of diversity in the Cactaceae. Chihuahuan Desert Region is the world's most important centre of cactus diversity and consists of the rarest and endangered species (Hernandez *et al.*, 2008). Dinerstein *et al.* (2000) noted that, the Chihuahuan Desert Region is counted among the three more outstanding deserts in the world while Mittermeier *et al.* (2002) stated that the desert is one of the 37 wilderness areas on the planet. According to Hernandez *et al.* (2004), Chihuahuan Desert Region shelters the world's richest assemblage of cactus which comprises of 39 genera and 329 species. 58% of these plants are identified as the cactus in the genera *Mammillaria, Opuntia, Coryphantha* and *Echinocereus.* An investigation by Hernandez *et al.* (2008) suggested that the highest number of recorded species belongs to genera *Coryphantha* (11 species), followed by *Opuntia* (10 species), *Echinocereus* (8 species) and *Mammillaria* (7 species).

Cactus can be distinguished by a particular characteristic that can only be found in this plant species such as areoles, a modified axillary bud, from which wools, spines, flowers and new shoots will develop (Hay, 1978). Cactus is also known as a succulent plant that promotes fleshy green stem. Stem tissues of cactus species are able to store water which is very important for adaptation to drought and extremely high temperature, especially in the desert area. In general, stems are either globular or cylindrical in shape and other species may have segmented or flattened. Indeed, cactus species are extremely variable in stem structure or shape as illustrated in Figure 2.1. Even though the cactus species are different in shapes, but the role of stems are similar for all, to replace leaves' function. Usually, the cactus possesses spines instead of leaves in order to minimize transpiration rates. Spines act as a shield to protect the plants from heat and grazing animals. Various patterns of spines such as radial, stout, conical, bristly, comb-like, needle-like and so on are presented in Figure 2.2. Typically, spines can be divided into two groups namely, central and radial. Central spines evolve from the middle part of areole while radial spines from the outer part of the areole.

Other characteristics of cactus, it is an herbaceous perennial dicotyledonous plant. Cactus is a flowering plant that produces two leaves or cotyledons during seed germination and they are capable of growing vigorously in many seasons year after year. Flowers are hermaphrodite in most of the cactus species, usually diurnal but the columnar cactus is found nocturnal. Flowering season apparently varies depending on the species. In wild habitat, flowers are normally pollinated by insects, birds and bats. After pollination, the seeds-filled fruit will be produced.

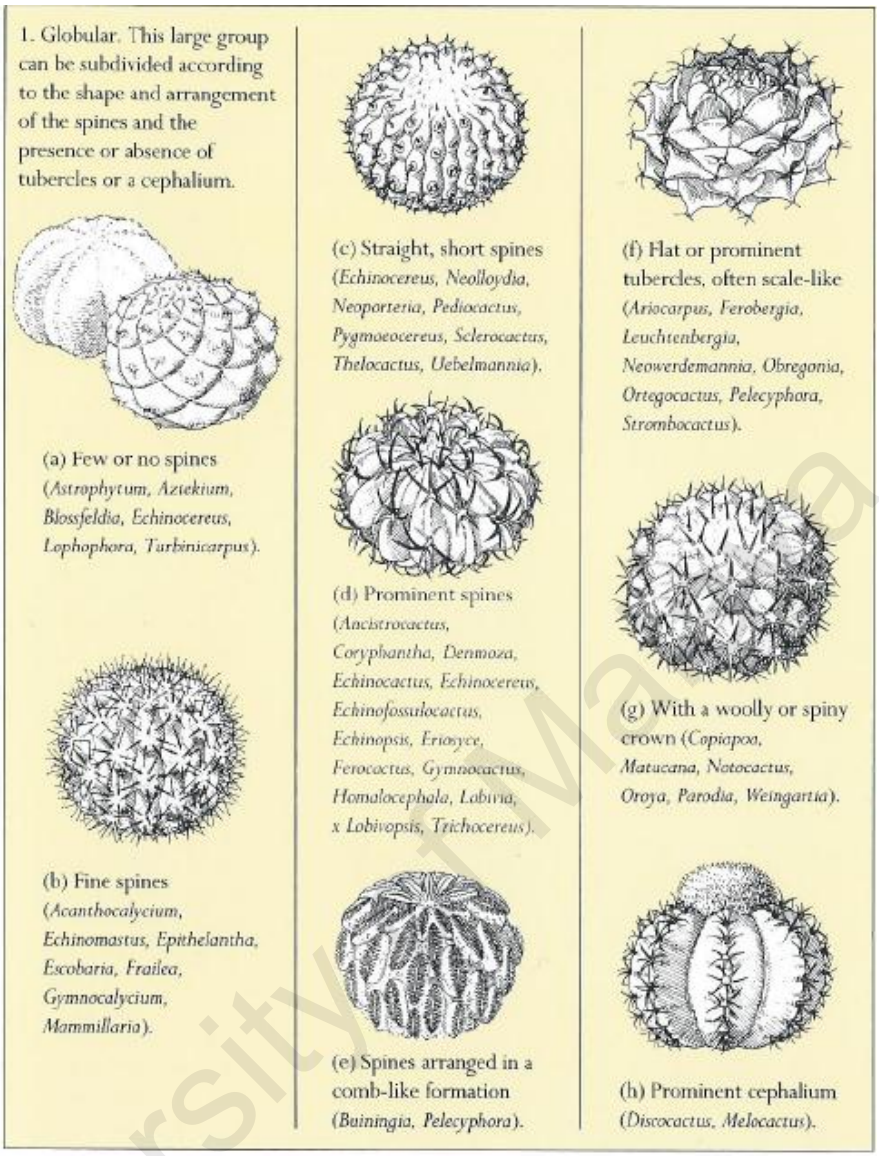


Figure 2.1: Illustration of cactus categorized in various shapes (Globular, Columnar, Clustering, Pendent, Padded or jointed, Climbing, Leaf-like and Sprawling) by Glass *et al.* (2004).

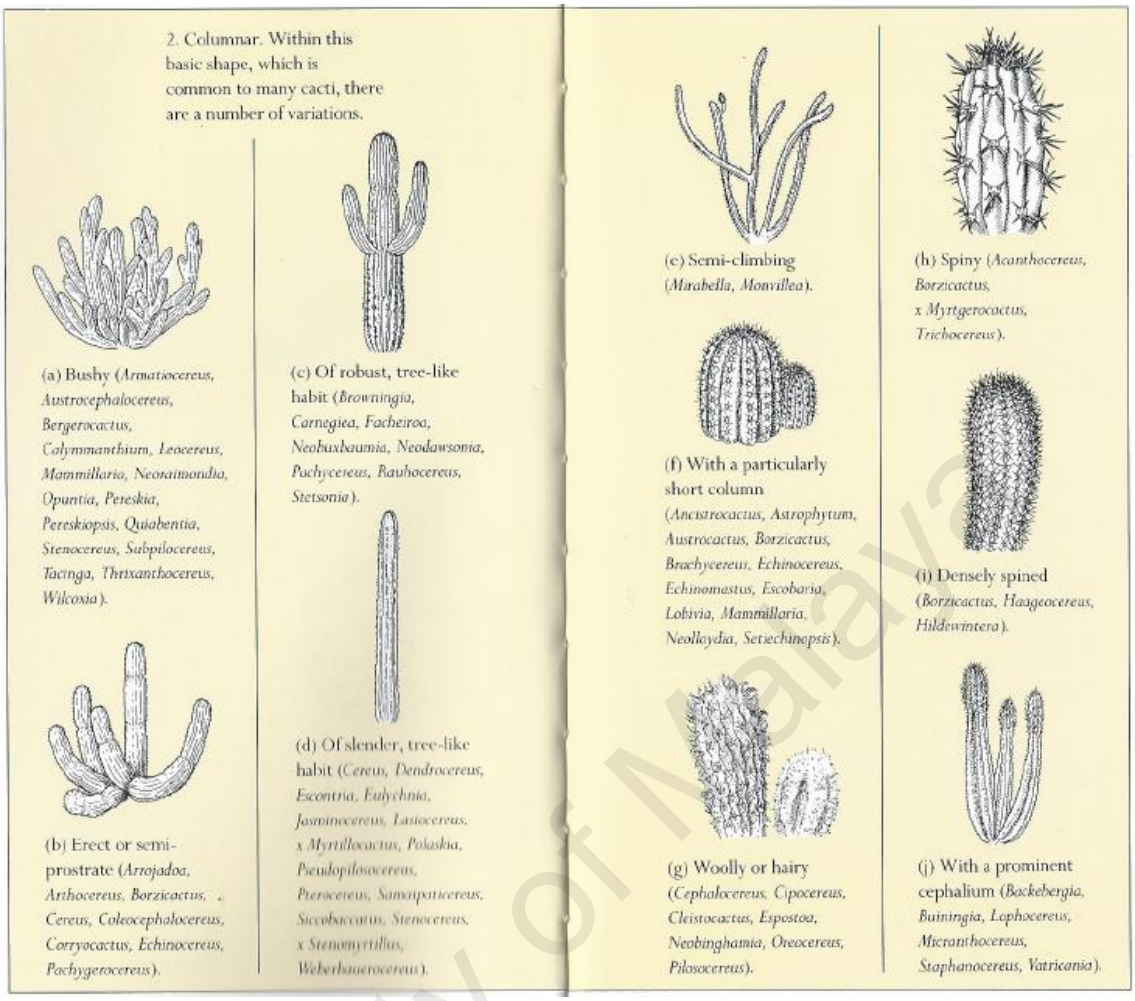
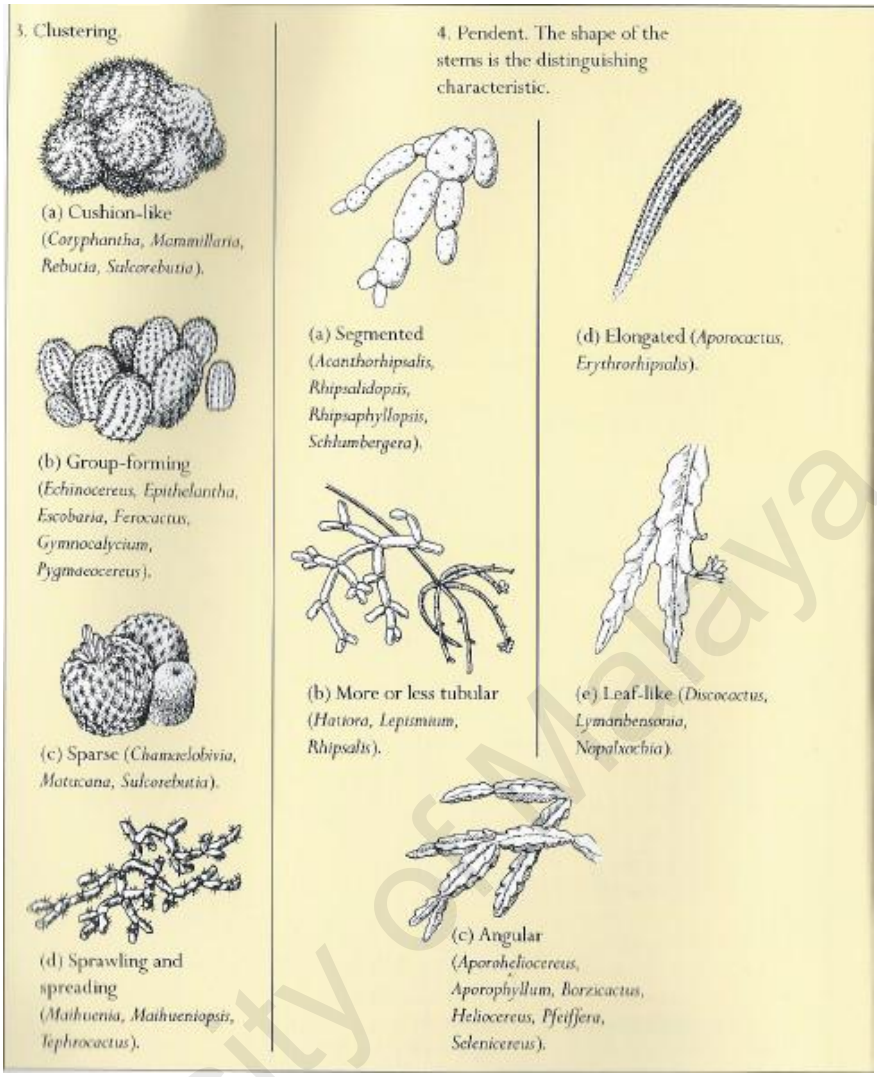


Figure 2.1: Continued



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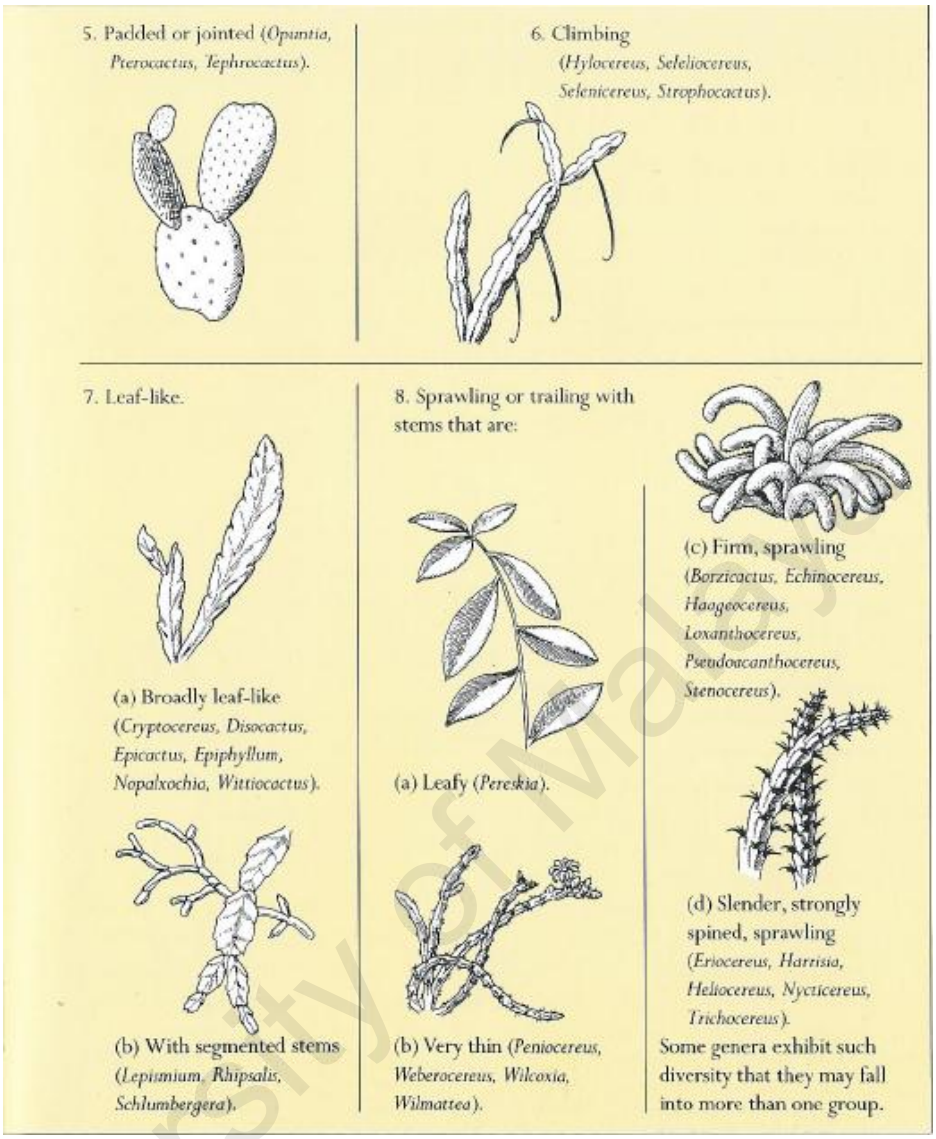

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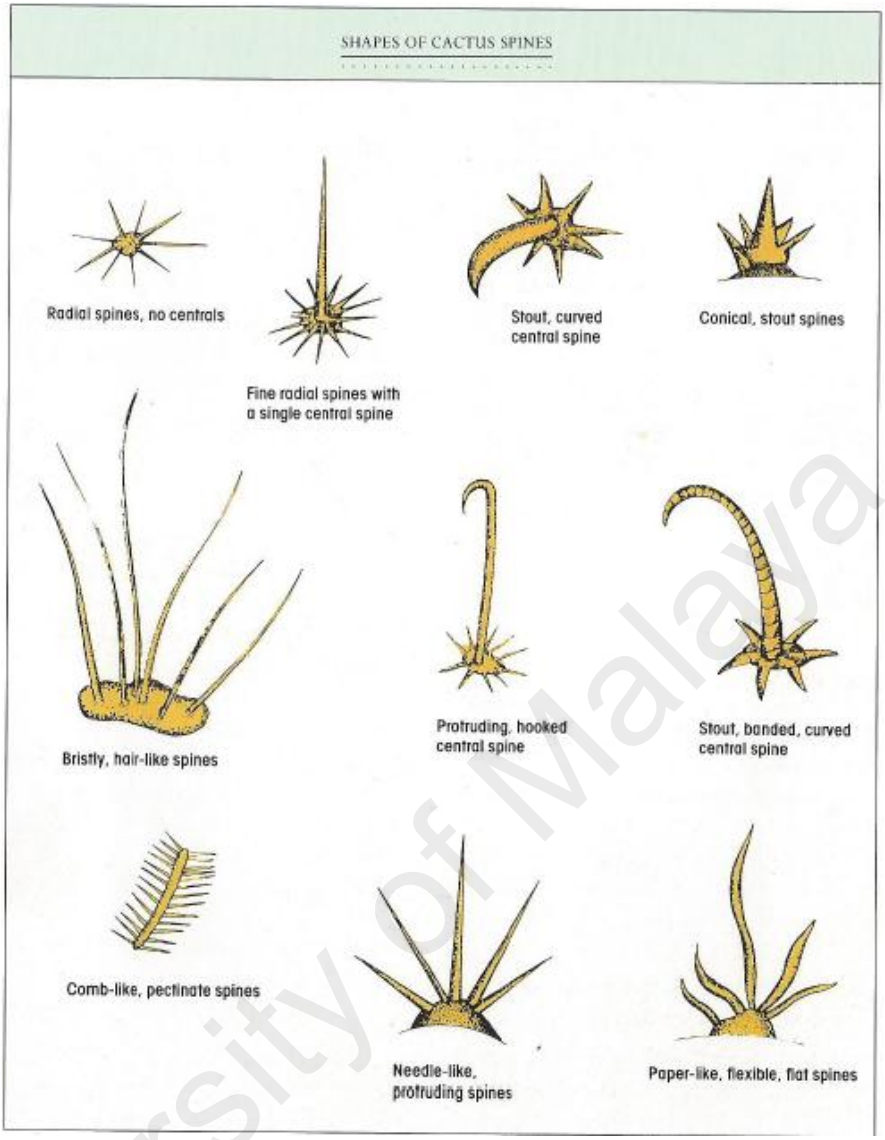


Figure 2.2: Illustration of various shapes of cactus spines by Glass *et al.* (2004).

Cactus can be divided into two categories, desert or rainforest species (Hewit, 2004). Most cultivated cactus is desert species or collectively known as xerophyte plants. These plants have either globular or columnar stems with leaves evolved to spine structures to reduce water loss. Besides, the plants consist of ribs or tubercles to support areoles and spines, while the stems are concertinaed. Cactus which belongs to rainforest species category is usually epiphytic plants. These cactus species which have climbing, sprawling or trailing stems are supported by the other plants in order to obtain maximum light for growth. Contrary to desert plants, rainforest plants prefer shady and humid condition for cultivation. Cactus plants are quite difficult to grow in cultivation since different species grows in a specific habitat.

Cactus can be cultivated by two distinct methods such as seeds or vegetative propagation. Seed propagation is the easiest way to propagate plants but the seedlings may show different characteristics from the parent plants. Most of the cactus yield seeds, however, only a few will germinate and develop into mature plants due to the unfavourable condition. Moreover, some of the species are very slow growing which may take years to germinate. Therefore, vegetative propagation is an alternative approach.

Vegetative propagation provides plants which are identical to their parents. Several vegetative propagations usually applied for cactus include stem cutting and grafting. Stem cutting should be done using a sharp and clean knife. After cutting, cactus must be placed in dry condition for a few days to form callus and roots before potting. Many *Opuntia* are propagated through this method as the stems usually fall to the ground, then rooted and eventually grow into new individual plants. Conversely, *Echinocactus*, *Echinocereus* and *Mammillaria* produces detachable branches that are easily undergo rooting after cutting (Griffiths and Thompson, 1929). Bell (2001) stated that cutting can be used to manage healthy plants and rescue infected ones.

Grafting is another method for plant propagation which is quite complicated but commonly practiced in cactus. Cactus can be propagated by grafting as the plant produces mucilage that joins scion and stock together. Grafting consists of the scion, a part of cactus to be propagated and stocks to support growth, supply nutrients and for root induction as well. In grafting, the scion will be grafted on the stock. Grafting is applied for numerous reasons. The rare and desirable cactus species which are slow growing are recommended to be grafted onto the others that grow faster. In this case, the growth of grafted plant can be accelerated. Additionally, grafting can be used to rescue a few healthy tissue of diseased cactus that is impossible to grow by cutting. Besides, cactus with various desired shapes or features can be decorated and created by grafting. A very well-known cactus that has been successfully grafted is *Gymnocalycium*. *Gymnocalycium* commonly available in a wide range of colours (without chlorophyll), grafted onto green cactus and grows vigorously in the natural environment.

2.1.1 Genus *Echinocereus*

Echinocereus is one of the most fascinating and popular genus in the Cactaceae which is found abundantly in Mexico and United States. *Echinocereus* is derived from the Greek word Echinos and the Latin word cereus. Echinos refers to “porcupine” or “hedgehog” (spines spread over the plant) while cereus refers to “large wax candle” (columnar cactus). Hence, *Echinocereus* indicates the “spiny cereus”. This “spiny cereus” possesses lovely and distinctive characteristics which promote diverse range of shapes, spines, flowers and colours that have attracted many cactus lovers and collectors.

Echinocereus is usually small to medium-sized cactus. Making it suitable for growing in the vases as interior or exterior home decorations. In addition, *Echinocereus* has various shapes such as round, erect, ascending, sprawling, pendent, either branching

or not, cylindrical, thin and rambling stems which display yellowish green to dark green colours. The stems have conspicuous areoles on the ribs, are often a foot in height and two to three inches in diameter. In its habitat, the cactus species can grow up to hundreds of stems within a few years. The standard sized of the cactus species are from the diminutive *Echinocereus pulchellus, Echinocereus vidiflorus, Echinocereus chlorantus* or *Echinocereus russanthus* to the huge growing species such as *Echinocereus hempelii, Echinocereus primolanatus, Echinocereus stramineus* or *Echinocereus enneacanthus* which is able to clump in two and are more than two meters in diameter.

Other attractive characteristics of the genus include various structures of spines which can be straight, curved, twisted or hooked. Certain spine structures look fierce and some are dramatic which covering the plant stems until it is not even visible. Most of the species have moderately spines size, evenly scattered and some of the spines are colourful even though in alternating patterns. Besides, some have pectinate (comb-like) spines and some are almost spineless. All the spines are arranged on the ribs with 4 to 55 spines per areole as the areoles are in the range of 1 to 52 mm apart along the ribs. The species are well protected from predators because of numerous large and sharp spines.

Echinocereus form flower buds on the epidermis of the stem, near the stem tips and blooming completely in full sun. Flowers are usually diurnal (open during daylight) but some of the species are nocturnal (open at night). *Echinocereus* which have semi-nocturnal flowers flourish early in the morning, withers a few hours before flourishing again in the late afternoon. *Echinocereus cinerascens* generally flowers during late summer. The flowers come in a diverse range of colours such as yellow, orange, red, pink, purple and white. Many species in the genus have two toned colours, one colour toward the centre and another on the outer parts of the flower. Some of the species are

bicolour and tricolour like *Echinocereus pectinatus, Echinocereus reichenbachii* and *Echinocereus chisoensis*. The flowers can be three inches or more in diameter and are usually large in size with bright colours. These spectacular flowers undoubtedly offer greatest satisfactory to cactus lovers.

Fruits are juicy with black or dark reddish brown seeds (0.8 to 2.0 mm in size). Both fruits and seeds are edible. Seeds or plants of *Echinocereus* should be obtained from a reliable source. It is essential to maintain a pure reference collection as the field collected material is no longer available for many species of *Echinocereus*. *Echinocereus* are not self-compatible. Pollination involves two plants in order to produce seeds, either naturally or artificially. Pollination occurs in its habitat naturally or can be done artificially by transfering collected pollen from the donor plant to the stigma of another plant. The pollen can be stored for a few days by harvesting the bundles of stamens from a flower and is normally kept in a dry container at room temperature to avoid direct sunlight. Several *Echinocereus* are dioecious as male and female organ are separated. Therefore, the possibility for breeding is reduced and as a result, the seed production is low or even impossible.

Seeds can be sown directly on top of sandy soil or any mixed soil suitable for cactus in general. The seeds may take an average of 72 hours to germinate but sometimes about 14 or more days. The mixed soil has good drainage and aeration in order to sustain healthy root system and prevent fungus infection. The use of peat, soils with heavy organic content is avoided because the soils are reported to have a limited lifespan, a maximum of two to three years in pots. After two to three years, the soil will be degraded by fungi and bacteria. Cactus grows healthily in mixed soil with very low organic content and grows very well with native desert soil. *Echinocereus reichenbachii* which possesses dense and comb-like spines, is propagated by seeds and commonly known to have a fairly slow growing rate compared to those with open spination like

Echinocereus cinerascens and *Echinocereus pentalophus* which can grow faster and form fine clumps.

Other factors to be considered for cactus propagation include local climate or environment, growing condition, watering schedule. A majority of *Echinocereus* are quite cold-tolerant especially *Echinocereus triglochidatus/ coccineus* and *Echinocereus viridiflorus/ chloranthus*. These species are often subjected to snow covering, and prolonged frosts in the habitats while some species that are quite frost-tolerant and do not suffer temperature below 0 ˚C include *Echinocereus knippelianus* and *Echinocereus pulchellus.* Notable exception to *Echinocereus brandegeei, Echinocereus ferreirianus, Echinocereus sciurus, Echinocereus barthelowanus, Echinocereus grandis, Echinocereus websterianus, Echinocereus scopulorum, Echinocereus sciurus* var. *floresii, Echinocereus spinigemmatus, Echinocereus subinermis* which should be kept in a heated greenhouse with minimum temperature a few degrees above 0 ˚C. The species in *Echinocereus* also require strong sunlight to maintain healthy and vigorous growth as well as to develop beautiful spination. On the contrary, other spineless species may require some shading or half-shaded place. Light is very important for growing cactus, particularly during a winter season.

Even though cactus species can be kept completely dry throughout the dormancy period but under normal conditions, they need to be occasionally watered while young or when the day is too dry. Excess in humidity may easily cause fatality. Therefore, watering cactus species is advisable to be done once a week. Watering is mainly to enhance flowering and also helps to sustain the new growth. In order to ensure regular growth, fertilization should be done during watering at reduced rate from 0.2 to 0.5 grams per litre. Fertilization at the first watering is not allowed as during that time watering is required to develop roots for feeding.

Common pests detected for this genus are mealy bugs. In this case, diluted ethanol is usually applied via spreading to the stem while waxy mealy bugs are easily spotted. If the infection of tiny root mealy bugs is severe, the plant needs to be transferred to a new pot. Red spider mites are relatively rare in *Echinocereus* but once infested, is difficult to get rid. Minor infection can be control by spraying water while only a good miticide is suggested to be applied for a major infection.

Taxanomic Hierarchy of *Echinocereus cinerascens*

Kingdom – Plantae – Plants

Subkingdom – Tracheobiorita – Vascular Plants

Phylum – Magnoliophyta – Flowering Plants

Class – Magnoliopsida – Dicotyledons

Subclass - Caryophyllidae

Order – Caryophyllales – Cactus

Family – Cactaceae – Cactus

Genus – Echinocereus – hedgehog cactus

Species – *Echinocereus cinerascens*

2.1.2 Medicinal Properties, Valuable compounds and Other Uses

Several types of research inferred that *Echinocereus* sp. possess valuable compounds. It has been proven that *Echinocereus* sp. containing certain alkaloids (Willaman and Schubert, 1961). Further research reported that phenethylamines (N-methyl-3,4-dimethoxyphenethylamine, N,N-dimethyl-3,4-dimethoxyphenethylamine, and tetrahydroisoquinoline salsoline) have been successfully isolated from *Echinocereus merkeri* cultivated in Mexico (Agurell *et al.*, 1969; McFarlane and Slaytor, 1972). In comparison, Bruhn and Sanchez-Mejorada (1977) also indicated that *Echinocereus cinerascens* from Hidalgo, Mexico yielded similar compound viz. N,N-dimethyl-3,4-dimethoxyphenethylamine as a major alkaloid while N-methyl-3,4-dimethoxyphenethylamine was present small amounts in the samples tested. In addition,

the discovery of imidazole alkaloid, β-phenethylamines and one tetrahydroisoquinoline in both of these Mexican species is unique for *Echinocereus*.

In 1979, Robert and Bye reported that McLaughlin (personal communication, March 2, 1977) had revealed the presence of a tryptamine derivative, N,N-dimethoxytryptamine via preliminary tests on Tarahumara plants of *Echinocereus triglochidiatus*. Mexican Tarahumara Indians named *Echinocereus triglochidiatus* as "false peyote", the cactus with "high mental qualities" (Bye, 1979; Schultes and Hofmann, 1979 and 1980). Alkaloids screening through thin layer chromatography (TLC) found that *Echinocereus triglochidiatus* extracts turn to blue colour as tested with Ehrlich's reagent (Tyler and Groger, 1964; Dingerdissen and McLaughlin, 1973). This positive result indicates the presence of indoles in the extracts. Similarly, further analysis also showed positive results as the extracts tested with indoplatinic acid, Dansyl chloride and Dragendorff's reagent (Ranieri and McLaughlin, 1975). Thus, this findings verified that the extracts contain a single major non-Ehrlich positive alkaloid.

Most of the cactus species bear fruits with very refreshing and nourishing taste such as *Hylocereus* sp., *Opuntia* sp. and so on. In Mexico, *Opuntia* sp. is consumed by poorer natives while in some places, the species is grown for trade. The fruits are found to have a positive effect on the digestive systems as the cactus has large, angular and bulky seeds. Besides, the inner pulp containing seeds provides a pleasant flavour that can give the cool feeling during hot weather. Another cactus species like *Echinocereus* sp. which is generally known as a small barrel-like cactus can also bear fruits. The fruits are edible and have been consumed by people from Southwest. In addition, their fleshy stems were also eaten by Native Americans of Texas and New Mexico previously. Archaeological evidence confirmed that the ancient people collected and ate hedgehog cactus. In the past, the Navajo, Hopi Paiute and Pueblo people ate its sweet fruits. The fruits of *Echinocereus* sp. and *Mammillaria* sp. have been consumed by Navajo, Seri

and Tarahumara (Elmore, 1944; Vestal, 1952; Felger and Moser, 1985). Although *Echinocereus* fruits have sharp spines nevertheless, it can still be eaten raw like the fruits of cholla such as *Mammillaria barbata* Engelm., *M. cf. sonorensis* Craig, *Echinocereus stoloniferus* W. T. Marshall spp. *tayopensis* (Marsh.) N. P. Taylor, *E. sheeri* (Salm-Dyck) Rumpler var. *sheeri*, *E. polyacanthus* Engelm. var. *polyacanthus* and other cactus species except for *E. coccineus* englm which has been reported to be poisonous and used for heart stimulant by Navajo (Elmore, 1944).

Besides the fruits, the stems and seeds of *Echinocereus* are also edible. Seeds of *Echinocereus viridiflorus* can be eaten while the stems of certain *Echinocereus* sp. have been used as hallucinogens (Bye, 1979; Ferrigni *et al.*, 1982). Nobel (1988) stated that stems of other species of cactus have been used to make candy. In candy manufacturing, only selected cactus species is used such as barrel cactus (*Ferocactus wislizeni*) because it has a bulky soft tissue. The exterior skin and the woody ribs of the plant are cut off to obtain only soft and pulpy interior cactus. This portion will be cut into cubes and then boiled with a few changes of water before processed into cane sugar. Afterwards, the cubes are flavoured and coloured with desirable criteria.

Ethnobotany study in the Cumbres de Monterrey National Park, Nuevo León, México concluded several numerous useful species in the local folk culture belongs to the Cactaceae, Lamiaceae, Asteraceae, Agavaceae, Fabaceae, Rosaceae, Euphorbiaceae and Mimosaceae (Estrada *et al.*, 2007). The plants are either grown in the wild or cultivated for multipurpose especially for foods. Some of the genus that bear fruits which can be eaten raw or cooked include *Echinocereus, Cucurbita, Sechium, Persea, Morus, Ficus, Punica, Crataegus, Fragaria, Prunus*, *Lycopersicon* and *Physalis*. In Southern Texas, *Echinocereus enneacanthus* is very well-known as the Mexican strawberry as the fruits can be eaten raw. Meanwhile in Mexico, *Opuntia ficus-indica* which is commonly known as prickly pear cactus is either can be eaten raw as a sort of

salad or cooked as cactus vegetables. Cactus vegetables are prepared firstly by removing all the tubercles and spines. Then, the cactus will be cut according to the menus or normally into cubes before being boiled in several changes of water to get rid of mucilage. Next, the vegetables will be flavoured before ready to be served.

Investigations on common plants grow by the people were studied concerning their favourable or beneficial. Analysis of results concluded that the plants were cultivated for medicinal-food, medicinal-firewood and medicinal-ornamental. Generally, people will grow and harvest tomato (*Lycopersicon esculentum*), pea (*Pisum sativum*), prickly pears (*Opuntia ficus-indica*), pitayas (*Echinocereus* spp.) and celery (*Apium graveolens*) for food. Meanwhile, they plant chamomile (*Matricaria recutita*), rue (*Ruta graveolens*), mint (*Mentha spicata*) and aloe (*Aloe vera*) for medicine. They grow maguey (*Agave asperrima*) as beverage and syrup while funnel (*Foeniculum vulgare*) and basil (*Ocimum basilicum*) as special flavouring herbs. Pitch pine (*Pinus teocota*) and oaks (*Quercus spp*.) are normally required for firewood. Palo casita (*Cornus florida*), chilitos (*Mammillaria* spp.*)* and siempreviva (*Sedum* spp. and *Echeveria strictiflora*) are typically grown as ornamental plants as well as others that are offered for trade including wild Cactaceae (*Mammillaria* spp., *Echinocereus* spp., *Ariocarpus retusus* and *Astrophytum capricorne*), Crassulaceae (*Echeveria strictiflora*) and Agavaceae (*Agave victoriae-reginae* and *A. bracteosa*).

According to Mizrahi *et al.* (1997), many cactus species have horticultural potential as fruits and industrial crops. Srikandarajah and Serek (2004) stated that the main economic importance of cactus species in the worldwide horticultural trade is as ornamental plants. Majority of cactus species which is high in demand for florists' trade include Christmas cactus (*Zygocactus* sp.), hedgehog cactus (*Echinocereus* sp.), orchid cactus (*Epiphyllum* sp.), pincushion cactus (*Mammillaria* sp.), golden ball cactus (*Echinocactus* sp.), and also *Echinopsis* sp. Countries involved in commercial

production of ornamental cactus include Denmark, USA, Japan, Great Britain, Germany and Holland (Sriskandarajah and Serek, 2004).

2.2 PRINCIPLES OF PLANT TISSUE CULTURE

In vitro regeneration or micropropagation is a term which specifically refers to the application of tissue culture techniques for the production of plants at industrial scale where plants are grown aseptically from very small pieces of the plant's part. As reported recently, many *in vitro* regeneration protocols have been established and each varies greatly for a specific plant species. Various plant cells, tissues and organ cultures successfully grown *in vitro*, have been regenerated into individual plants. In the tissue culture system, the first basic principle is totipotency, the concept which clarifies that every single cell is capable of autonomy as it has genetic potential to develop into complete individual or entire organism. This theory has been pronounced by German plant physiologist, Haberlandt (1902) that predicted the cells, tissues and organs could be maintained indefinitely in culture. Even though he failed to exhibit any significant result to support this hypothesis, this trial is still considered to be the first attempt of plant tissue culture experiment since many researchers showed their efforts and initiatives in the investigation afterward. Thus, he is regarded as the father of tissue culture.

Nevertheless, an important breakthrough was then demonstrated by White (1934) when the root cultures of *Lycopersicon esculentum* grew continuously in medium supplemented with yeast extract. This discovery has been considered as the first successful tissue culture experiment and has become the cornerstone to develop more advanced investigations. Subsequent studies indicated similar results reported independently by Nobecourt and Gautheret in France while White in United State (1939) promoted the important role of auxin in callus proliferation. In the studies, they detected indefinite culture of callus tissue in a synthetic basal medium supplemented with auxin. However, persistent studies regarding this research discovered more callus

proliferation in medium supplemented with coconut milk which later proposed the discovery of cytokinin functions in cell divisions and also in bud formation.

The discoveries of auxin and cytokinin as well as their roles revealed that both shoot and root regeneration is regulated by plant growth regulators (PGRs). In 1957, Skoog and Miller proposed the concept of PGRs control organ formation which appears to be the second basic principle of tissue culture. The concept suggested that in micropropagation, shoot and root initiation were induced by the interaction between auxin and cytokinin. Interaction of the endogenous hormones and exogenous (supplemented in small quantity) PGRs influences the development and growth of explants in plant tissue culture which is unique for a species. Therefore, it is necessary to determine the exact proportion of auxin and cytokinin supplemented in growth induction medium to achieve the main objective of the study.

Commonly, auxins (Naphthaleneacetic acid, NAA; Indolebutyric acid, IBA; Indoleacetic acid, IAA; 2,4-Dichlorophenoxyacetic acid, 2,4-D) are supplemented in the media for root induction, while cytokinins (BAP, 6-Benzylaminopurine; KIN, kinetin; zeatin) are applied for shoot induction. Additional auxin at lower concentrations promotes root formation whereas at higher concentrations produces callus formation. A higher concentration of cytokinin, shoot formation was induced, thus inhibiting root formation (Pierik, 1987). As noted, auxins can inhibit cytokinin accumulation that may cause inhibition of lateral buds development, while cytokinins can inhibit some auxin functions to initiate shoot formation and sustain plant growth. Nevertheless, the interaction between auxins and cytokinins exhibits a great impact in many cell growth, cell differentiation, and organogenesis in plant tissue culture. Typically, in diverse plant species, higher proportion of cytokinin to auxin concentration demonstrate significant shoot formation (Sastri, 1963; Heide, 1965; Zenkteler, 1971; Martínez-Váquez and Rubluo, 1989; Infante, 1992).

Further research and advancement in this field suggest that shoot and root induction through direct or indirect regeneration or organogenesis also depends on the composition of media (nutrients), environmental factors, source of plant tissue and are not only regulated by the interaction of PGRs. Ideal composition of media is essential to support growth and development of a particular plant species in tissue culture. Therefore, the composition of media formulated according to the specific nutrient requirements are mainly made up of macronutrients, micronutrients, vitamins, amino acids, sugar (sucrose), organic supplements and solidifying agent (agar). Various culture media with a specific composition have been studied and established namely, Murashige and Skoog medium (MS medium), Linsmaier and Skoog medium (LS medium), Gamborg B5 medium (B5 medium), Nitsch and Nitsch medium (NN medium), Schenk and Hildebrandt medium (SH medium), woody plant medium (WPM medium) and so on.

The main physical or environmental factor for growth and maintenance of plant tissue culture is constant temperature. Normally, the cultures are kept in the culture room and maintained at 25 ± 2 ºC under 16 hours light provided by cool day fluorescent light. Besides, the relative humidity in the culture container also plays a key role to avoid dehydration of plant regenerated *in vitro*. Normal relative humidity for many plants species regenerated *in vitro* is 70%. pH of the medium is another physical factor which contributes to the vigorous growth of plant regenerated *in vitro*. In general, standard pH of the medium applied to many plant species is 5.8. Similarly, all the parameters that affects plant regeneration *in vitro* including PGRs, composition of media and environment relies on the specific plant species or source of plant tissue (type of explant).

A third major principle is the development of consecutive stages of the culture process leading to plantlet establishment. Stage I involves the establishment of explant in culture and stabilization of culture for multiple shoot development which usually achieved by manipulation of PGRs. It requires four to six weeks to complete stage 1 and to produce explants that will be transferred to stage II. Stage II includes shoot multiplication which can be repeated for several times to increase the supply of material (plantlets) to a predetermined level for subsequent stage. Stage III consists of pretransplant and *in vitro* rooting. Regenerated plantlets are subcultured in a new fresh medium for *in vitro* rooting to increase their survival rate after transplanted or acclimatized on soil in stage IV. Stage IV involves transplanting, acclimatization and *ex vitro* rooting. As the regenerated plantlets rooted *in vitro* (complete plantlets produced), they are ready to be transferred into soil for acclimatization. Initially, acclimatized plants are covered with transparent plastic bags and kept in the culture room for several days before exposed in the natural environment.

A fourth basic principle is a competency and determination. Competency is the endogenous potential of a given cell or tissue to develop into particular condition or feature according to internal cellular programming or memory. Determination is the development into specific cell, tissue or organ through a specific hormone or culture condition. In plant regeneration, competency and determination verify whether a cell or tissue will undergo embryogenesis or organogenesis and whether roots, shoots or flowers are produced.

2.2.1 Direct *In Vitro* Regeneration

In vitro plant regeneration has been achieved in numerous plant species through cell, tissue or organ culture using various types of media (George *et al*., 2008), and the applications of plant tissue culture are far reaching in many fields including plant sciences, biotechnology, agriculture, medicine and so on. Tissue culture indeed has

become the cornerstone to build up further advanced researches which could lead to new relevant findings and potential applications for the achievement of commercial goals and health benefits. *In vitro* plant regeneration is very well known as an important biotechnology tool for propagation of endangered (Coelho *et al.*, 2012; Debnath, 2004) and also offers rapid production of high quality and quantity of identical clonal plants to ensure the availability of desired and highly valued plants throughout the year. The capability of *in vitro* plant regeneration techniques to produce great numbers of plantlets de novo from dedifferentiated cell cultures suggests great chances for the achievement of genetic transformation. Successful *in vitro* plant regeneration and genetic engineering approaches have a great potential to produce superior plants which are pathogen-free or disease-resistant, and to produce plants with higher content of valuable compounds and other desirable characteristics. Nowadays, advanced research in plant biotechnology offers efficient procedures and a new vista for improving production of desired valuable compounds.

Establishment of *in vitro* plant regeneration is mostly accomplished through either direct or indirect (somatic embryogenesis) regeneration. Each of these pathways has different benefits. Direct *in vitro* plant regeneration is the production of organs directly from cultured explants while indirect *in vitro* plant regeneration is the production of organs through intermediate callus phases that are obtained from cultured explants. The explants might produce embryos from somatic cells directly through direct somatic embryogenesis (without callus formation) or indirectly (through callus phases) through indirect somatic embryogenesis.

Typically, direct *in vitro* plant regeneration is more desirable as the plants produce rapidly in large scale, uniform, and are true to type as the plant regenerated directly is transformed with physical DNA delivery. This allows manipulation of entire vegetative plant organs such as leaves, stems or others to avoid disturbance of normal

plant development and reduces *in vitro* culture condition. In contrast, indirect *in vitro* plant regeneration produces plants with high potential of somaclonal variation, associated with long callus culture period which is incompatible for further advanced research such as plant transformation, genetic engineering and so on. The appearance of somaclonal variation is due to the occurrence of chromosome or genetic changes in most of the regenerated plants in long-term culture cells (Evans and Sharp, 1986). Besides, the production of somaclonal variation increases with the duration and extent of the disorganized or undifferentiated culture (callus) phase (Karp, 1994).

Recently, a lot of successful works have been published regarding direct *in vitro* regeneration of various horticultural and ornamental plants, namely *Gerbera jamesonii* Bolus ex, Hook f. (Hasbullah *et al.*, 2008), *Ananas comosus* L. Merr (Hamad and Taha, 2008), *Rubus* hybrid cv. Black Satin (Gupta and Mahalaxmi, 2009), *Pistacia vera* L. (Tilkat *et al.*, 2009), *Dendrobium huoshanense* C.Z. Tang et. S.J. Cheng (Luo *et al.*, 2009), *Opuntia ficus-indica* (L.) Mill (Angulo-Bejarano and Paredes-López, 2011), *Populus deltoides* Bartram ex Marsh. (Cavusoglu *et al.*, 2011), *Platycerium coronarium* (Taha *et al.*, 2011), *Pistacia vera* L. (Benmahioul *et al.*, 2012), *Philodendron species* (Chen *et al.*, 2012), *Thymus lotocephalus* (Coelho *et al.*, 2012), *Ocimum basilicum* L. (Asghari *et al.*, 2012; Manan *et al.*, 2016), *Celosia cristata* (Taha and Wafa, 2012), *Nelumbo nucifera* Gaertn. (Mahmad *et al.*, 2014), *Justicia betonica* Linn. (Yaacob *et al.*, 2013), *Agapanthus praecox, Justicia betonica* and *Celosia cristata* (Yaacob *et al.*, 2014), *Fragaria vesca* L. (Yildirim and Turker, 2014), *Bixa orellana* (Mohammed *et al.*, 2015), *Echinocereus cinerascens* (Elias *et al.*, 2015), *Hylocereus* spp. (Hua *et al.*, 2015), *Viola uliginosa* (Slazak *et al.*, 2015) and *Fraxinus nigra* (Lee and Pijut, 2017). The establishment of *in vitro* regeneration systems for these species plays a central role in achieving present and future demand.

2.3 SOMATIC EMBRYOGENESIS

Somatic embryogenesis is a development process of the embryo from somatic or vegetative cell. The process involved a formation of bipolar structure including a root or shoot axis which develops from a non-zygotic cell without vascular connection with the original tissue. Somatic embryogenesis occurs through two different pathways, either directly or indirectly. Direct somatic embryogenesis occurred when the embryos are formed directly without going through the callus phase (Slater *et al*., 2003; Chen and Chang, 2004; Zhang *et al.*, 2005). In contrast, indirect somatic embryogenesis occurred when the embryos are formed through the formation of callus phase (Griga, 2002; Kim *et al.*, 2003; Conde *et al.*, 2004). Among the two pathways, direct somatic embryogenesis is likely to be more applicable for mass propagation because genetic rearrangement is limited, whereas indirect somatic embryogenesis often showed aberrant chromosome numbers in cultures (Tanaka *et al.*, 2000).

Somatic embryogenesis is preferred in certain research as an alternative strategy to propagate clones of elite cultivars for large scale production (Zimmermann, 1993; Bandyopadhay and Hamill, 2000). Indeed, somatic embryogenesis is an important vegetative propagation method because it has the possibility to scale up the production of plants via multiplication of embryogenic cells (Jime'nez, 2001). Successful propagation and identification of variant lines of *Azadirachta indica,* an elite Neem tree through indirect somatic embryogenesis have been established (Shrikhande *et al.*, 1993). Besides, the somatic embryos or the embryogenic cultures can be cryopreserved (Mathur *et al.*, 2003; Winkelmann *et al*., 2004) which is suitable for establishment of gene banks (Von Arnold *et al.*, 2002). Furthermore, somatic embryos are essential as a source of explants in the synthetic seeds production, which is practically used in commercial plant production (Gray and Purohit, 1991) especially for seedless plants. Santacruz-Ruvalcaba *et al.* (1998) indicated that somatic embryogenesis is an important

prerequisite for the use of many biotechnology tools in genetic improvement. Somatic embryogenesis helped to boost the rate of genetic improvement of commercial crop species (Stasolla and Yeung, 2003) like banana, papaya and mango that have high commercial values (Kamle, 2011).

Generally, there are three basic types of *in vitro* embryogenesis. Firstly is the somatic adventitious embryogenesis. Through somatic adventitious embryogenesis, the somatic embryos may develop from cells or callus associated with the reproduction apparatus such as pre embryonically determined cells (PEDC). The embryogenic callus and cells can be initiated from nucellus tissue of either polyembryonic or monoembryonic (Button and Kochba, 1977; Tisserat *et al*., 1979; Litz, 1987). Besides, the formation of adventitious embryos can also develop directly from single cells on the surface of immature embryos in culture or indirectly in the callus produced. Secondly is a somatic poly embryogenesis. This type of *in vitro* embryogenesis involves the transplant of highly embryogenic embryonal-suspensor mass (ESM) which leads to the formation of embryo (Gupta and Durzan, 1986; Durzan and Gupta, 1988). Further proliferation of these cells may give rise to the poly embryogenesis. ESM transplanted to appropriate culture medium may proliferate profusely and can develop directly into embryos afterward. Somatic poly embryogenesis does not involve callus, however, results from cleavage and budding of the original ESM tissue. Thirdly is an induced somatic embryogenesis. This type of somatic embryogenesis results from the induction of somatic callus and cell suspensions which subjected to specific treatment that varies for different types of plant species. Nowadays, a number of various plant species have been tested or analyzed to discover their embryogenic potential which is unique for each of plant species.

A number of studies have been established on callus induction and somatic embryogenesis of several plants such as *Syngonium podophyllum* (Zhang *et al.*, 2006), *Phyla nodiflora* (L.) Greene (Ahmed *et al*., 2011), *Gerbera jamesonii* Bolus ex, Hook f. (Hasbullah *et al*., 2011), *Brassica oleracea* var. botrytis (Siong *et al*., 2011), *Onobrychis sativa* (Mohajer *et al.*, 2012), *Phyllostachys heterocycla* (Yuan *et al.*, 2013), *Drimia robusta* (Baskaran and Van Staden, 2014), *Tapiscia sinensis* (Wang *et al*., 2014), *Solanum nigrum* (Xu *et al.*, 2014), *Rubus sanctus* and *Rubus hirtus* (Sabooni and Shekafandeh, 2017) that hold tremendous potential for genetic engineering and biotechnology applications.

2.4 SYNTHETIC SEED PRODUCTION

The discovery of somatic embryogenesis leads to the establishment concept of synthetic seed or artificial seed (Murashige, 1978). The concept offers encapsulation of somatic embryos of alfalfa and celery with natural hydrogels such as calcium alginate (Redenbaugh *et al.*, 1987; Kitto and Janick, 1985). The encapsulation methods deviated from the concept of true seed analog. They proposed that every single somatic embryo encapsulated in an artificial coat germinating and becoming a complete plant (Redenbaugh *et al.*, 1987; Sanada *et al.*, 1993). Subsequent research involves the encapsulation of somatic embryos with synthetic seed coat to make them analogs to true seed in order to provide certain aspects of complex seed environment (Fujii *et al.*, 1987). The synthetic seed coat used in the encapsulation must serve as synthetic endosperm containing carbon sources, nutrients, growth regulators, antimicrobial and so on (Redenbaugh, 1993). This synthetic endosperm which acts as food storage also helps in protecting the somatic embryos or propagules during storage of synthetic seeds.

'Synthetic seed' term was first coined by Toshio Murashige in 1970 and an area of research applying somatic embryos as functional seeds is called 'synthetic seed technology' (Gray, 1990). According to Gray, synthetic seeds are functionally defined as somatic embryos engineered to be of use in commercial plant production. However, due to rapidly growing synthetic seed technology especially in agriculture, other types of explants (vegetative parts such as shoot tips, shoot buds, axillary buds, micro shoots and other totipotent tissues) have been used instead of somatic embryos as propagules in the encapsulation process (Standardi and Piccioni, 1998). Hence, synthetic seeds are also defined as the artificial encapsulation of somatic embryos, shoot tips, axillary buds, micro shoots or other tissues that have an ability to form plant both in *in vivo* and *in vitro* condition. Danso and Ford-Llord (2003) indicated that the use of in vitro-derived axillary buds and shoot tips offer inexpensive and easily obtained propagules for

storage. Additionally, the synthetic seed technology is suggested as a high volume and low cost production technology (Ghosh and Sen, 1994).

There are two types of synthetic seeds namely, desiccated and hydrated. Desiccated synthetic seeds produced from somatic embryos are either naked or encapsulated in hydrogels using alginates or polyoxyethylene glycol (Polyox) and then followed by desiccation. Production of this type of synthetic seeds applied only in plant species whose somatic embryos are considered desiccation tolerant. The other type is known as hydrated synthetic seeds that are produced in plant species whose somatic embryos are recalcitrant and sensitive to desiccation. These synthetic seeds can be produced by encapsulating somatic embryos in hydrogel and alginate capsules. Several coating agents include sodium alginate, potassium alginate, carrageenan, sodium alginate with gelatin, sodium pectate and carboxymethyl cellulose. The method for hydrated synthetic seeds was developed by Redenbaugh *et al.* in 1984 and has been used these days as encapsulation matrix for synthetic seed production.

Recent development and advanced studies in the production of synthetic seeds have revealed their benefits and applications, especially in the agriculture field. Synthetic seed technology offers high potential application in propagation of seedless plants or plants which difficult to produce seeds, minute seed size and minimal endosperm, low seed viability, limited number of seeds, plants that have long dormancy period or long period to produce seeds. Production of synthetic seeds is commonly used to overcome plants that have difficulties in seed germination. However, further studies have recommended that it is also suitable for endangered plants, genetically modified plants, hybrid plants and polyploidy plants. Synthetic seed technology can supply unlimited seeds throughout the year where the seeds are easy to handle while in storage, easy to transport and have potential in long term storage without losing their viability. Several researches demonstrated the potential application of synthetic seed technology

for germplasm storage, transport and utilization of various clonal plant population (Ozden-Tokatli *et al.*, 2008; Naik and Chand, 2006; Singh *et al.*, 2006; Danso and Ford-Lloyd, 2003).

2.5 ACCLIMATIZATION

Plantlets produced *in vitro* will be rooted in order to ensure the plantlets survival when transferring from an artificial heterotrophic environment (aseptic condition *in vitro*) to an autotrophic environment (natural environment). In this process, the plantlets are subcultured in fresh medium supplemented with low level of cytokinin but high level of auxin concentration. However, certain plant species may need to be subcultured in medium supplemented with auxin applied singly or medium free plant growth regulators which depending on the plant requirement.

Complete plantlets with well-developed roots are selected for acclimatization process. The complete plantlets are removed from the sterile condition, wash under running tap water to remove completely the agar which sticks on the roots to avoid contamination. Subsequently, the plantlets are transferred to soil or other planting substrates such as sand, vermiculite and so on. Once the plantlets are established in the planting substrates, they should be gradually exposed to a lower relative humidity (green house environment) before placed at a higher light intensity (field environment). An acclimatization process is a significant procedure to ensure a high number of *in vitro* plantlets survive and grow vigorously when transferred to soil.

In *in vitro* condition, plantlets grow under constant temperature, very high air humidity, low irradiance, very low air turbulence, variable and often insufficient CO_2 concentration, water potential dependent on medium composition, sucrose as a carbon source, growth regulators in nutrient medium and etc. This condition is totally dependent on the vessel and closure types (Solárová *et al.,* 1996) which leads to the formation of plantlets with morphology, anatomy and physiology different from naturally grown plants (Pospíšilová *et al.,* 1992; 1997; Buddensorf-Joosen and Woltering, 1994; Desjardins, 1994; Kozai and Smith, 1995; Kubota *et al.,* 1997). *In vitro* plantlets which have been transferred to soil need an adaptation process to survive

from heterotrophic to an autotrophic condition with low air humidity, high irradiance and etc. The plantlets need to be grown under a shade and gradually lowering air humidity for a few weeks before exposed to natural environment. Several plant species displayed some changes as the leaves formed *in vitro* are unable to develop further under *ex vitro* conditions. Thus, the leaves are replaced by newly formed leaves under *ex vitro* conditions (Preece and Sutter, 1991; Diettrich *et al.*, 1992).

2.6 COLOURED CALLUS INDUCTION

Callus is an amorphous mass of unorganized thin-walled parenchyma cells produced on explants *in vitro* as a result of wounding and in response to hormones, either endogenous or exogenous. According to Halperin (1969), callus comprises of unspecialized parenchyma cells. Explants from any part of plant such as leaves, stems, roots and so on which have been disinfested and excised into small pieces that are cultured on appropriate medium producing callus at the basal end of the cutting. Callus that proliferates from cells appearing at the base of the cutting can be maintained for long periods by continues subculture at three to four week intervals. Although callus appears outwardly to be uniform masses of cells, but the structures are relatively complex with considerable morphological, physiological and genetic variation within the callus.

Callus growth follows a typical logarithmic pattern. First is a slow initial cell division induction period requiring auxin. Second is a rapid cell division phase involving active synthesis of DNA, RNA and protein. Third is a gradual cessation of cell division and forth is a differentiation into larger parenchyma and vascular type cells. Initiation of cell division with subsequent callus formation requires both exogenous cytokinin and auxin in appropriate proportion depends on the explants. Generally, auxin at a moderate to high concentration is the primary hormone used to induce callus. In callus growth, cell division does not take place throughout the culture mass but is located primarily in meristematic cells on the outer layer. As a result, the inner part of callus remains undividing cells which may differ physiologically and genetically from cells on the outer layer. Consequently, variations in cell age and type may occur within this tissue culture mass.

Callus varies widely as depicted in its general appearance and in other physical features which depends on the parent tissue, callus age and growth conditions. Callus normally green, yellow, white, pink or highly coloured due to the presence of pigments. Coloured callus may be loosely packed (friable callus) or lignified with densely packed cells and hard in structure (compact callus). Even though coloured callus may not directly important in plant regeneration *in vitro* but they tend to generate a significant amount of genetic or epigenetic aberrations which may lead to somaclonal variation. This is important for the production of new and novel genotypes. Besides, it is also an alternative method for the production of valuable secondary compounds that give benefits especially for agriculture, medicine, cosmetic or food industries. These valuable secondary compounds can be obtained from induction of callus cultures *in vitro* instead of harvesting and destroying the whole plant as applied in conventional method.

Although many plant species in various families produced callus *in vitro*, however studies on coloured callus have received very little attention. A few works have been focused on coloured callus induction *in vitro* of *Ocimum basilicum* L. (Gopi and Ponmurugan, 2006), *Ocimum basilicum* L., *Ocimum sanctum* L., *Ocimum gratissimum* L., (Mathew and Sankar, 2011), *Lawsonia inermis* syn. *Lawsonia alba* (Rahiman *et al.*, 2011), *Allamanda cathartica* (Wong and Taha, 2012), *Hylocereus costaricensis* (Viñas *et al.*, 2012), *Agave* hybrid (Zhang *et al.*, 2013), *Lilium pumilum* (Jin *et al.*, 2014), *Primula forbesii* Franch.(Jia *et al.*, 2014), and *Echinocereus cinerascens* (Elias *et al.*, 2015), considering their advantages and potential application in many fields.

2.7 EXTRACTION AND DETECTION OF PIGMENT (NATURAL COLOURANT) AND BIOACTIVE COMPOUNDS

Most of the plants produce various assortments of organic or valuable bioactive compounds which also termed as natural products. The natural products promote various beneficial biological activities such as antioxidant, anticancer, antidiabetic, antimicrobial, etc. Based on their biosynthesis origin, these natural products can be divided into three major groups, namely terpenoids, alkaloids and phenolic compounds. Terpenoids including primary and secondary metabolites are derived from the five-carbon precursor isopentenyl diphosphate (IPP). Alkaloids comprised of one or more nitrogen atoms are biosynthesized principally from amino acids while phenolic compounds are formed from either through shikimic acid pathway or the malonate/acetate pathway (Hussain *et al.*, 2012).

Pigments are secondary metabolites that have been classified in different groups (Schoefs, 2004; 2002; Mortensen, 2006; Hasni, 2011) namely tetrapyrrole (e.g chlorophyll), carotenoid (e.g carotenes), flavonoid (e.g anthocyanins) and alkaloid (e.g betalains). Chlorophyll is the most abundant pigment in plants, synthesized within chloroplasts. It promotes green colour and exists as lipid soluble pigments. Carotenoids are also categorized as lipid soluble pigments but represent distinctive yellow, orange and red colours of plants. Other pigments such as anthocyanins and betalains are water soluble. Both are chemically related, but anthocyanins display blue, purple, red and orange colours while betalains are yellow, red and purple colours. Normally, plant phenolics assist in protection against ultraviolet radiation or aggression by pathogens parasites and predators, play important roles in plants' colours and flavours. Plant phenolics consist of polyphenol constituents such as flavonoids, phenolic acids, tannins and the less common stilbenes and lignans (Dai and Mumper, 2010). Flavonoids are responsible for orange, red, blue and purple colours of fruits and vegetables which can

be divided into six groups, flavones (quercetin), flavanols (catechin), flavanones (naringenin), isoflavanones (daidzein, genistein and glycitein) and anthocyanins (cyaniding-glycoside) (D'Archivio *et al.*, 2010). Phenolic acids can be divided into two classes, derivatives of benzoic acid (gallic acid) and derivatives of cinnamic acid (coumaric, caffeic and ferullic acid). Tannins that are comparable with phenolic acids can also be divided into two classes, hydrolysable tannins (gallotannins, ellagitannins) and condensed tannins (proanthocyanidins). These polyphenols can regulate the enzyme and cell receptor activities to promote their function in specific biological activities.

Several bioactives or secondary metabolites that have been produced through tissue culture system are alkaloids from *Ailanthus altissima* (Anderson *et al*., 1987), diosgenin from *Dioscorea doryophora* Hance (Huang *et al*., 1993), anthraquinone from *Cassia acutifolia* (Nazif *et al*., 2000), indole alkaloids (Monero *et al*., 1993) and catharantine (Zhao *et al*., 2001) from *Catharanthus roseus* which were produced in suspension cultures. Other compounds which were also derived from callus cultures include narigin and limonin from *Citrus* sp. (Barthe *et al*., 1987), caffeine from *Coffea arabica* L. (Waller *et al*., 1983), sterols and phenolic compounds from *Eucalypthus tereticornis* SM (Venkateswara *et al*., 1986), triterpenes from *Glycirrhiza glabra* (Ayabe *et al*., 1990), camptothecin from *Nothapodytes foetida* (Thengane *et al*., 2003).

First step involves in the consumption of phytochemical compounds is the extraction process where the plant samples are treated by milling, grinding and homogenization. The plant samples are commonly subjected to air-drying or freeze-drying before extraction. Freeze-drying is important and more practical method compared to air-drying in order to retain higher levels of phenolics in plant samples. According to Asami *et al.* (2003), freeze-dried samples of Marionberries, strawberries and corn have consistently higher level of total phenolic content compared with those air-dried. However, both of these drying processes can cause undesirable effects on the

constituents of plant samples. Proper preparation of sample extraction is needed to assure that potential active constituents are not lost, distorted or destroyed. Thus, precautions should be taken while preparing and conducting research related to medicinal compounds of plant species (Abascal *et al.*, 2005). In addition, selection of solvent extractions such as methanol, acetone, ethanol, and others should be made due to their ease of use and high efficiency in extracting desired compounds since the yield of chemical extraction depends on the types of solvent with varying polarities, extraction time and temperature, sample-to-solvent ratio as well as chemical compositions and physical characteristics of the samples. As a result, extraction procedure is unique for each of plant species. The selection of solvent extraction affects the amount and rate of polyphenols extracted (Xu and Chang, 2007). Methanol is known as more efficient solvent extraction for lower molecular weight polyphenol whereas an aqueous acetone is better solvent extraction for higher molecular weight flavanols (Metivier *et al.*, 1980; Prior *et al.*, 2001; Guyot *et al.*, 2001; Labarbe *et al.*, 1999). Meanwhile, ethanol is another preferred candidate for polyphenol extraction which is safe for human consumption (Shi *et al.*, 2005).

Next step after extraction is purification which practically performed before the identification and characterization process. Basically, plant crude extracts comprised of combinations of various types of bioactive compounds or phytochemicals with different polarities that need to be purified or separated. There are numerous separation techniques such as by TLC (thin layer chromatography), HP-TLC (high-performance thin layer chromatography), column chromatography, HPLC (high-performance liquid chromatography) and others. The compounds that have been purified and separated are subsequently subjected to analysis and identification process.

Generally, UV-VIS spectrophotometer is used to provide simple and fast screening methods to identify classes of phenolic compounds in crude plant samples. Nevertheless, this method may result in over or underestimation of sample content due to complexity of the plant phenolics and different reactivity of phenols toward assay reagents. Consequently, a modern method by high-performance chromatographic combined with instrumental analysis such as GC (gas chromatography), has been widely used especially for separation and quantification of phenolic acids and flavonoids. Other methods which also can be used for the analysis include LCMS/MS (liquid chromatography combined with mass spectrometry) and HPLC. HPLC is the most popular and reliable technique for analysis of phenolic compounds that offer a unique chance to analyse simultaneously all components of interest and their derivatives and degradation products (Sakakibara *et al.*, 2003; Downey and Rochfort, 2008). Several researches have been published the application of HPLC technique for analysis of phenolics (Stalikas, 2007; Naczk and Shahidi, 2004; Robbins, 2003; Merken and Beecher, 2000; Thabti *et al.*, 2012).

2.8 CELLULAR BEHAVIOUR

Cytology, or specifically karyology, is a research focused on cellular behaviour which includes studies on measurement of mitotic index (MI), nuclear area, cell area, nuclear DNA content, ploidy analysis, chromosome morphology and number, and others. In general, every single living organism going through cell division that plays important roles in life. Cell division enables the production and development of a fully grown organism, function in renewal and repair or replacing die cells.

Cell division process is an important part of the cell cycle. Cell cycle is defined as the life of a cell from the time it is first formed from a parent cell until its own division into two daughter cells. The parent cell can pass identical genetic material to the daughter cells through cell division to ensure the production of genetically identical daughter cells. In this event, the daughter cells are produced by nuclear division (mitosis) and then followed by cellular division (cytokinesis). Mitosis and cytokinesis are included in mitotic phase, which is the shortest part of the cell cycle (Rost *et al.*, 1984). Most of the time in the cell cycle is interphase. During interphase, the cell is in a growth stage, copies its chromosomes in preparation for cell division. Howard and Pelc (1953) stated that interphase coupled with mitosis is termed as the 'cell cycle'.

Mitosis can be divided into four stages namely, prophase, metaphase, anaphase and telophase. Prophase indicates the beginning of mitosis that can be observed by the appearance of replicated chromosomes that coil and condense. This event followed by the migration of chromosomes (sister chromatids) to the midline of the cell, oriented between the two centrosomes which indicates that the cell is in metaphase. Anaphase begins with the separation of centromeres results in the separation of chromosomes (sister chromatids). The sister chromatids which is now called as chromosomes move in opposite directions toward the centrosomes. As the two sets of chromosomes reach opposite poles of the cell, they begin to uncoil. Now, the cell is in Telophase. Telophase

ends with cytokinesis when the cell plate divides the cytoplasm into two which leads to the formation of two daughter cells.

Interphase can be divided into three subphases such as G1 phase (first gap), S phase (synthesis) and G2 phase (second gap) as reported by Howard and Pelc (1953). The G1 phase (pre-synthetic phase) follows mitosis, is a period of growth and metabolic activity. The cell prepares itself metabolically to go through DNA synthesis which involves accumulation and synthesis of specific enzymes to control DNA synthesis and production of DNA base unit. The S phase follows G1, is a period of DNA synthesis. During this time, the DNA is duplicated. Incomplete preparation events in G1 may interrupt the S phase. The G2 phase (post-synthetic phase) follows S and leads to the next mitotic division. According to Van't Hof (1985), the cell cycle refers to the passage of cell from the time it is first formed (first division) until its own divides (second division), starting Mitosis phase proceeds to G1 phase, onto S phase, then to G2 and finally to Mitosis phase again.

There are two major potential control points in the cell cycle. Firstly is the initiation of replication (the G1/S phase transition). Secondly is the initiation of division (the G2/M transition). Cell cycle length is determined primarily by the cell growth rate. Van't Hof (1985) also reported that there is a linear correlation between cell cycle duration and DNA content. However, previous studies on the duration of mitotic phases in a Triticale and its parent found that duration of prophase and other mitotic phases were not completely parallel to the cell's DNA content (Kaltsikes, 1971). Further researches by Sunderland (1973) proposed several events that would affect the cell's DNA content including abnormalities in spindle fibres and chromosome breakage. Abnormalities in spindle fibres may disturb the separation of chromosome while chromosome breakage may influence the chromosome count within a nucleus which then alters the structural arrangements between the chromosomes. Continuous DNA

replications accompanied by lack of mitosis and chromosome breakage may lead to an increase of chromosomal material and so on. Besides, he also found that repeated DNA replication with the lack of mitosis resulted in an increase of DNA content and chromosome girth, without affecting the chromosome number.

Figure 2.3: Intact plant of *Echinocereus cinerascens* growing in their natural habitat. (Retrieved from http://www.nelocactus.org/lista-echinocereus.html)

CHAPTER 3

IN VITRO REGENERATION OF *Echinocereus cinerascens*

3.1 EXPERIMENTAL AIMS

An efficient micropropagation system offers a useful alternative for rapid shoot production, which is convenient for conservation and commercial production of plants species. Tissue culture techniques have been practically applied for micropropagation of cactus species, which are very well known as slow growing plants with a low rate of seed production. In micropropagation, each species responds differently to the conditions for multiplication. Therefore, it would be necessary to ascertain the optimum micropropagation system for each cactus species (Hubstenberger *et al.*, 1992). Since micropropagation through axillary buds proposes the greatest probability of maintaining genetic traits, this research focused on the production of plantlets through the main meristematic tissues in cactus stem, called areoles, that will produce axillary shoots. Most of the plantlets propagated through axillary buds *in vitro* displayed higher genetic stability and uniformity with the parent plants. Production of shoots directly from meristematic structures promises higher genetic stability (Peschke and Phillips, 1992).

Several cactus species that have been successfully propagated through axillary buds *in vitro* include *Mammillaria carmenae* (1.0 mg/l NAA + 2.0 mg/l BAP), *Mammillaria prolifera* (low concentrations of NAA + BAP, 0.5-1.0 mg/l), *Astrophytum myriosigma* and *Trichocereus spachianus* (5.0 mg/l IAA + 0.5 mg/l Kn) (Vyskot and Jara, 1984). In 1998, Perez-Molphe-Batch *et al*. successfully established micropropagation of 21 Mexican cactus species by using axillary shoot proliferation. These Mexican cacti include *Coryphantha clavata, Coryphantha radians, Echinocactus platyacanthus, Echinofossulocactus sp., Mammillaria candida, Mammillaria craigii, Mammillaria uncinata* and *Stenocactus coptonogonus* (optimum shoots produced on MS medium supplemented with 1.0 mg/l BA), *Astrophytum myriostigma,*

Cephalocereus senilis, Coryphantha durangensis, Echinocereus dubis, Echinocereus pectinatus and *Mammillaria sphacelata* (optimum shoots obtained on MS medium supplemented with 1.0 mg/l BA + 0.01 mg/l NAA), *Ferocactus hamatacanthus, Ferocactus histrix, Ferocactus latispinus, Mammillaria formosa* and *Mammillaria obscura* (optimum shoots promoted on MS medium with the addition of 1.0 mg/l BA + 0.1 mg/l NAA), *Nyctocereus serpentinus* (yielded optimum shoots on MS medium with 2.0 mg/l BA). Besides, three species of columnar cactus, *Carnegiae gigantean, Pachycereus pringlei* and *Stenocereus thurberi* were also found to generate shoots from axillary buds when cultured on MS supplemented with 2.0 mg/l BAP and 1.0 mg/l BAP, respectively (Perez-Molphe-Batch *et al*., 2002).

Explant responses are mostly influenced by the types of nutrients and plant growth regulators in the medium applied. Nevertheless, the responses especially for optimum plant regeneration relies on the most responsive starting culture material (explant), the optimum medium, culture condition and so on. Regenerative ability is unique for not only plant species or varieties, but for each individual plant, since it is a characteristic of genotype (Bregitzer, 1992; Lemaux *et al*., 1998; Barro *et al*., 1999; Jacobsen *et al*., 1999; Filippov *et al*., 2006).

In this chapter, the experiment aim was to investigate particular effects of plant growth regulators (PGRs) such as BAP, NAA, IBA and Kinetin applied singly or in combinations for *in vitro* regeneration of *Echinocereus cinerascens.* Stem explants were cultured in various treatments (MS medium supplemented with PGRs applied singly or in combinations) to induce shoots and roots which subsequently grown up to produce plantlets. Parameters examined included the mean number of shoots per explant, mean (%) explants producing shoots and callus, mean shoot height and diameter. Analysis on the parameters recorded will determine the optimum medium for establishment of direct *in vitro* regeneration of *Echinocereus cinerascens*.

3.2 MATERIALS AND METHODS

3.2.1 Source of Explants

Echinocereus cinerascens seeds which were imported from Singapore were germinated *in vitro* on MS basal medium. The seeds were germinated as early as one week, however the highest germination rate was observed on the fourth week of culture. After two months of culture, aseptic seedlings grown vigorously were selected and cultured on fresh MS medium to ensure enough explant sources (plantlets supply) for further studies. Age (6-month-old plantlets) and size (6 cm height and 1 cm diameter) of explant sources used in the studies were standardized to minimize error which could influence the respond of the explants.

3.2.2 Sterilization of Seeds

The seeds were first disinfected with dettol solution for a few minutes. Then, the seeds were washed thoroughly under running tap water approximately 1 hour before surface sterilized with a serial descending concentrations of commercial clorox (70, 50, 30, 10% (v/v)) for 5 minutes each, followed by 3 time rinses with sterile distilled water. 2 to 3 drops of Tween 20 were added while 70% of clorox was applied. The seeds were further sterilized by 70% ethanol (v/v) for 1 minute before rinsed 3 times with sterile distilled water in the laminar flow cabinet. Sterilized seeds were dried on sterile tissue paper and ready to be cultured on MS basal medium afterward. This sterilization procedure was found significant to minimize bacterial and fungal infections and to ensure the aseptic seedlings produced free from contamination.

3.2.3 Preparation of Culture Media

Generally, MS (Murashige and Skoog, 1962) medium was the most preferred medium for *in vitro* regeneration or micropropagation of plants. Most of the researchers approved that MS medium composition (Appendix III) consisted macronutrients, micronutrients, irons and vitamins which are essential and applicable for many plants

species. Nowadays, the medium was commercially prepared in powder form and could be purchased from the suppliers such as SIGMA Chemical Company, Sigma-aldrich, St. Louis, USA and etc.

MS basal medium was prepared for seed germination and as a control while MS medium supplemented with different concentrations of PGRs applied singly or in combinations were modified according to the objectives of the studies. To prepare 1 litre MS basal medium, 800 ml of distilled water was filled into 1 litre conical flask. Then, 4.4 g/l of MS medium including vitamin (commercial powder) and 30 g/l of sucrose were added in the conical flask. The conical flask was placed on the hot plate and the medium solution was stirred constantly with magnetic stirrer to allow the chemicals homogenized or dissolved in the solution. Next, the conical flask was refilled with distilled water up to 1 litre before the medium solution pH was adjusted to 5.7 with either 1.0 N sodium hydroxide (NaOH) or 1.0 N hydrochloride acid (HCl). Subsequently, the medium solution was solidified with 8 g/l agar technical (Agar No.3) and autoclaved for 20 minutes at temperature of 121 °C, pressure of 104 kPa (15 psi). After autoclaved, sterilized medium was transferred to the laminar air flow cabinet and dispensed 20 ml into each of 60 ml sterile universal container.

For MS medium fortified with PGRs, stock hormones (PGRs) were prepared. To prepare 1 mg/ml stock hormone, 100 mg of hormone (PGR) in powder form was dissolved in a few drops of NaOH or HCl. After that, 100 ml of distilled water was added as a clear solution was obtained. Synthetic hormones were added in the medium before the medium was autoclaved. In contrast, natural hormones were filter sterilized with 0.22 µm sterile membrane filters (Milipore filter), then added in the medium after autoclaved and cooled at room temperature. In this study, only synthetic hormones were applied singly or in combinations to identify the optimum medium for direct regeneration of this species.

3.2.4 Culture Conditions

All apparatus essential for culturing such as forceps, scalpels, jam jars, conical flask and etc. were cleaned and wrapped with aluminium foils before being autoclaved for 20 minutes to sterile the culture apparatus. UV light in the laminar flow cabinet was turned on for 15 minutes to provide aseptic culture conditions. Culture work was in the laminar flow cabinet as UV light was turned off and hot bead sterilizer reached temperature of 250 °C. The laminar flow cabinet surfaces were disinfected with 70% ethanol before culturing. Additionally, forceps and scalpels were sterilized with hot bead sterilizer and then, dipped in 70% ethanol or sterile distilled water to be cooled before the explants were sectioned into desired sizes. Explants that have been sectioned into small pieces were cultured in the media prepared. All cultured explants were allowed to develop and maintained in the culture room at 25 ± 2 °C under 16 hours light provided by cool daylight or fluorescent light (36W), PHILIPS.

3.2.5 Plant Regeneration *In Vitro*

The effects of NAA, BAP, IBA and KIN applied singly and in combinations were studied to obtain the optimum medium in direct regeneration of the species *in vitro*. Throughout this study, mean number of shoots produced, mean explants produced shoots, mean explants produced callus and mean shoots size were observed. Explants (stems) used should be uniform and were excised into square size (0.5 cm x 0.5 cm in average) to ensure 4-6 areoles on each explant. Areoles are the structures on cactus stem where shoots or branches will emerge. The explants were cultured in universal sterile containers containing 20 ml of selected media. Thirty explants were cultured and tested for each treatment. Observations were done every week and data were recorded monthly.

3.2.5.1 Effects of NAA and BAP Applied Singly and in Combinations on Plant Regeneration *In Vitro*

All treatments or concentrations of NAA and BAP applied singly and in combinations tested throughout this study were prepared as listed below;

1. MS basal (medium without hormone/ control)
2. MS + 0.5 mg/l NAA
3. MS + 1.0 mg/l NAA
4. MS + 1.5 mg/l NAA
5. MS + 2.0 mg/l NAA
6. MS + 0.5 mg/l BAP
7. MS + 1.0 mg/l BAP
8. MS + 1.5 mg/l BAP
9. MS + 2.0 mg/l BAP
10. MS + 0.5 mg/l NAA + 0.5 mg/l BAP
11. MS + 0.5 mg/l NAA + 1.0 mg/l BAP
12. MS + 0.5 mg/l NAA + 1.5 mg/l BAP
13. MS + 0.5 mg/l NAA + 2.0 mg/l BAP
14. MS + 1.0 mg/l NAA + 0.5 mg/l BAP
15. MS + 1.0 mg/l NAA + 1.0 mg/l BAP
16. MS + 1.0 mg/l NAA + 1.5 mg/l BAP
17. MS + 1.0 mg/l NAA + 2.0 mg/l BAP
18. MS + 1.5 mg/l NAA + 0.5 mg/l BAP
19. MS + 1.5 mg/l NAA + 1.0 mg/l BAP
20. MS + 1.5 mg/l NAA + 1.5 mg/l BAP
21. MS + 1.5 mg/l NAA + 2.0 mg/l BAP
22. MS + 2.0 mg/l NAA + 0.5 mg/l BAP

23. MS + 2.0 mg/l NAA + 1.0 mg/l BAP
24. MS + 2.0 mg/l NAA + 1.5 mg/l BAP
25. MS + 2.0 mg/l NAA + 2.0 mg/l BAP

3.2.5.2 Effects of IBA and Kinetin Applied Singly and in Combinations on Plant Regeneration *In Vitro*

All treatments or concentrations of IBA and KIN applied singly and in combinations tested throughout this study were prepared as listed below;

1. MS basal (medium without hormone/ control)
2. MS + 0.5 mg/l KIN
3. MS + 1.0 mg/l KIN
4. MS + 1.5 mg/l KIN
5. MS + 2.0 mg/l KIN
6. MS + 0.5 mg/l IBA
7. MS + 1.0 mg/l IBA
8. MS + 1.5 mg/l IBA
9. MS + 2.0 mg/l IBA
10. MS + 0.5 mg/l KIN + 0.5 mg/l IBA
11. MS + 0.5 mg/l KIN + 1.0 mg/l IBA
12. MS + 0.5 mg/l KIN + 1.5 mg/l IBA
13. MS + 0.5 mg/l KIN + 2.0 mg/l IBA
14. MS + 1.0 mg/l KIN + 0.5 mg/l IBA
15. MS + 1.0 mg/l KIN + 1.0 mg/l IBA
16. MS + 1.0 mg/l KIN + 1.5 mg/l IBA
17. MS + 1.0 mg/l KIN + 2.0 mg/l IBA
18. MS + 1.5 mg/l KIN + 0.5 mg/l IBA
19. MS + 1.5 mg/l KIN + 1.0 mg/l IBA

20. MS + 1.5 mg/l KIN + 1.5 mg/l IBA
21. MS + 1.5 mg/l KIN + 2.0 mg/l IBA
22. MS + 2.0 mg/l KIN + 0.5 mg/l IBA
23. MS + 2.0 mg/l KIN + 1.0 mg/l IBA
24. MS + 2.0 mg/l KIN + 1.5 mg/l IBA
25. MS + 2.0 mg/l KIN + 2.0 mg/l IBA

3.2.6 *In Vitro* Rooting

Shoots propagated from axillary buds grew into plantlets within 4 months. Subsequently, plantlets were separated and subcultured in MS basal medium for rooting. This would enable plantlets to undergo elongation process to become complete plantlets. Complete plantlets which had fulfilled the criteria of 6 cm in height and healthy fibrous roots were selected for acclimatization.

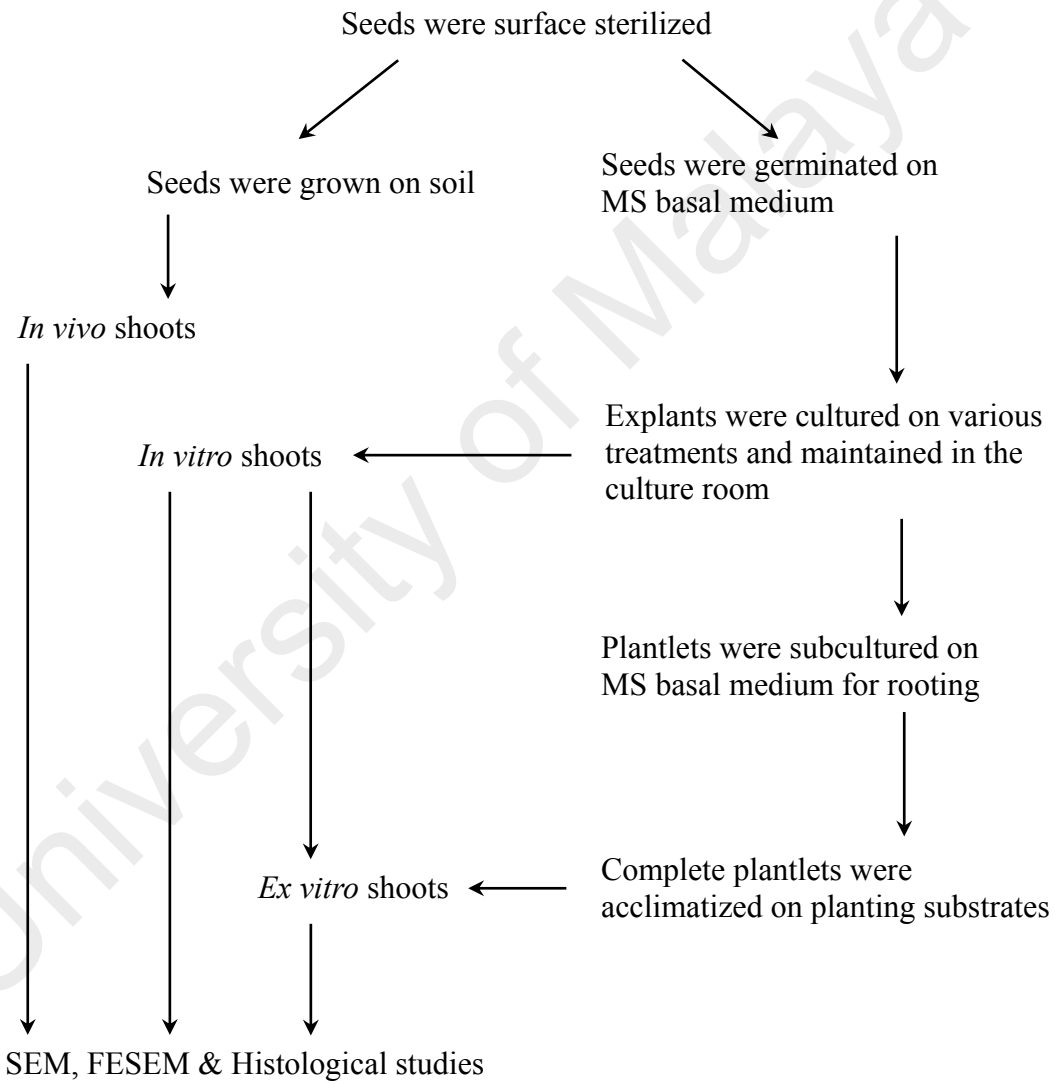
3.2.7 Microscopic Studies

In vitro regenerated plantlets were subjected to ultrastructural and light microscopy analysis to study further details on their morphology and anatomy. In this study, anatomical structure of shoots grown *in vivo* (2 months of seedlings grown on soil), *in vitro* (2 and 4 months of plantlets grown on MS basal medium) and *ex vitro* (4 months of plants grown on soil) were analysed. Both *in vivo* and *in vitro* samples were kept in the culture room (25 ± 2 °C) whereas the *ex vitro* sample was kept outdoor in shade (30 ± 1 °C). Comparisons between all the samples tested were done by light microscope (for histological studies), scanning electrons microscope (SEM) and field emission scanning electron microscope (FESEM). Observations including analysis on epidermis surfaces, shoot apical structures, starch grains, vascular bundles, stomata structure, stomata density and sizes of stomata opening. Standard procedures used in preparation of samples for SEM, FESEM and histological studies were attached in the Appendix IV.

3.2.8 Data Analysis

Data obtained in *in vitro* regeneration were statistically analyzed using Duncan's Multiple Range Test (DMRT). Means with different letters in the same column differ significantly at p=0.01. In microscopic studies, the data were analyzed using independent samples T-test (p=0.01).

3.2.9 Experimental Outline



3.3 RESULTS

Formation of callus from stem explants of *Echinocereus cinerascens* could be observed on the cut surfaces as early as the first week of culture (Figure 3.1). However, the initiation of shoots (Figure 3.2) from axillary buds could obviously be detected after a month on proliferation medium. After 4 months of culture, the shoots were developed into plantlets. Therefore, the production of shoots *in vitro* was recorded until 4 months, after which the plantlets were transferred to rooting medium.

3.3.1 Effects of NAA and BAP Applied Singly and in Combinations on Plant Regeneration *In Vitro*

Overall, the results confirmed that a greater mean number of shoots was produced in media with combinations of hormones as compared to media in which hormones were applied singly. Mean number of shoots produced per explant was statistically analysed and the results showed that each explant produced 3 shoots when cultured on MS medium supplemented with higher concentrations of BAP alone (1.5 and 2.0 mg/l BAP) (Table 3.1) and in several combinations of hormones with higher concentrations of BAP (0.5 mg/l NAA + 2.0 mg/l BAP, 1.0 mg/l NAA + 2.0 mg/l BAP, 1.5 mg/l NAA + 2.0 mg/l BAP, 2.0 mg/l NAA + 1.5 mg/l BAP) (Table 3.2). However, the highest mean number of shoots per explant was 3.07 observed in treatment with a combination of 2.0 mg/l NAA and 1.5 mg/l BAP (Figure 3.3). This result verified that MS medium supplemented with the combination of hormones of 2.0 mg/l NAA and 1.5 mg/l BAP was the optimum medium for shoot formation or *in vitro* regeneration of this species.

In the control, 80% of explants produced shoots. In single-hormone treatments, the percentage of explants that produced shoots in treatments with NAA only was significantly lower than the control, whereas the percentage of explants that produced shoots in the treatments with BAP only was either the same or greater than the control

(Table 3.1). However, when both hormones were applied in combination, the percentage of explants producing shoots were more varied and no conclusion could be reached (Table 3.2).

Shoot sizes were analysed and the results verified that most of the shoots produced about 1.0 cm height and diameter as they reached 4 months old. The maximum mean shoot height recorded was 1.43 cm in MS medium supplemented with 2.0 mg/l BAP (Table 3.3) and in the control, while 1.13 cm height was observed in MS medium with the addition of 0.5 mg/l NAA + 1.0 mg/l BAP (Table 3.4). The maximum mean shoot diameter recorded was 1.28 cm, observed in MS fortified with 1.5 mg/l NAA + 2.0 mg/l BAP (Table 3.4), which showed no significant difference with 1.25 cm observed in MS fortified with 2.0 mg/l BAP (Table 3.3). It is interesting to note that when the hormones were applied singly, the mean shoot height and shoot diameter in treatments with NAA only were significantly lower than the control, while those in treatments with BAP only were similar to the control (Table 3.3). However, when the hormones were applied in combination it was difficult to detect a clear pattern in their effect on shoot height and diameter.

In contrast to the shoot production data, data on callus production showed that all treatments added with plant growth regulators produced callus more readily than the control (83%), with 90% callus formation observed in some of the treatments with hormones applied singly (Table 3.1) and 100% callus formation for all treatments with both hormones in combination (Table 3.2).

Analysis on production of shoots monthly revealed that treatments with BAP produced higher number of shoots after 1 month (Figure 3.4) compared to treatments with NAA only and combinations of BAP and NAA (Figure 3.5) as well. Treatments with BAP only could produce 79 shoots after 1 month and then increased slightly up to 83 shoots after 4 months in MS medium supplemented with 1.5 mg/l BAP (Figure 3.4)

whereas treatments with NAA , with the shoots emerging after 2 months, could produce one to six shoots only after 4 months.

Although treatments with combinations of BAP and NAA produced shoots after 1 month, the production of shoots was greatly increased after 2 and 3 months (Figure 3.5). It was found that MS medium supplemented with 1.5 mg/l NAA + 2.0 mg/l BAP produced 32 shoots after 1 month, which subsequently increased with 52 shoots to give 84 shoots after 2 months. Meanwhile, MS medium supplemented with 0.5 mg/l NAA + 2.0 mg/l BAP that produced 28 shoots after 2 months, increased 61 shoots to give 89 shoots after 3 months. Nevertheless, the highest production of shoots was observed on MS medium supplemented with 2.0 mg/l NAA + 1.5 mg/l BAP which showed 15 shoots produced after 1 month and finally increase up to 92 shoots after 4 months. Similarly, MS medium supplemented with 0.5 mg/l NAA + 2.0 mg/l BAP could also produce higher number of shoots with slightly different, 91 shoots after 4 months (Figure 3.5).

Table 3.1: Effects of different concentrations of single hormones on the mean number of shoots per explant, mean number of explants producing shoots and callus (%) after 4 months.

MS + Hormone (mg/l)	Mean no. of shoots per explant	Mean no. of explants produced shoots (%)	Mean no. of explants produced callus (%)
control	$0.93 \pm 0.11_{c}$	$80.00 \pm 0.07_{a}$	$83.00 \pm 0.07_{b}$
0.5NAA	$0.20 \pm 0.09_{d}$	$17.00 \pm 0.07_{b}$	$93.00 \pm 0.05_{a}$
1.0NAA	$0.20 \pm 0.09_{d}$	$17.00 \pm 0.07_{b}$	$93.00 \pm 0.05_{a}$
1.5NAA	$0.03 \pm 0.03_{d}$	$3.00 \pm 0.03_{b}$	$100.00 \pm 0.00_{a}$
2.0NAA	$0.03 \pm 0.03_{d}$	$3.00 \pm 0.03_{b}$	$100.00 \pm 0.00_{a}$
0.5BAP	$2.43 \pm 0.23_{a}$	$100.00 \pm 0.00_{a}$	$100.00 \pm 0.00_{a}$
1.0BAP	$1.50 \pm 0.22_{b}$	$80.00 \pm 0.07_{a}$	$90.00 \pm 0.06_{a}$
1.5BAP	$2.77 \pm 0.19_{a}$	$100.00 \pm 0.00_{a}$	$100.00 \pm 0.00_{a}$
2.0BAP	$2.57 \pm 0.16_{a}$	$100.00 \pm 0.00_{a}$	$100.00 \pm 0.00_{a}$

Data represents mean value ± standard error (SE) with 30 explants in each treatment. Means with different letters in the same column are significantly different at p=0.01according to Duncan's multiple range test (DMRT).

Table 3.2: Effects of different concentrations and combinations of hormones on the mean number of shoots per explant, mean number of explants producing shoots and callus (%) after 4 months.

MS + Hormone (mg/l)	Mean no. of shoots per explant	Mean no. of explants produced shoots (%)	Mean explants produced callus (%)
control	$0.93 \pm 0.11_{de}$	$80.00 \pm 0.07_{ab}$	$83.00 \pm 0.07_{b}$
0.5N + 0.5B	$1.47 \pm 0.18_{cd}$	$93.00 \pm 0.05_{ab}$	$100.00 \pm 0.00_{a}$
0.5N + 1.0B	$2.33 \pm 0.21_{ab}$	$100.00 \pm 0.00_{a}$	$100.00 \pm 0.00_{a}$
0.5N + 1.5B	$2.60 \pm 0.24_{abc}$	$100.00 \pm 0.00_{a}$	$100.00 \pm 0.00_{a}$
0.5N + 2.0B	$3.03 \pm 0.21_{a}$	$97.00 \pm 0.03_{ab}$	$100.00 \pm 0.00_{a}$
1.0N + 0.5B	$0.23 \pm 0.09_{de}$	$20.00 \pm 0.07_{d}$	$100.00 \pm 0.00_{a}$
1.0N + 1.0B	$2.00 \pm 0.24_{abcd}$	$100.00 \pm 0.00_{a}$	$100.00 \pm 0.00_{a}$
1.0N + 1.5B	$2.10 \pm 0.29_{abcd}$	$83.00 \pm 0.07_{ab}$	$100.00 \pm 0.00_{a}$
1.0N + 2.0B	$2.87 \pm 0.30_{ab}$	$100.00 \pm 0.00_{a}$	$100.00 \pm 0.00_{a}$
1.5N + 0.5B	$0.23 \pm 0.09_{de}$	$20.00 \pm 0.07_{d}$	$100.00 \pm 0.00_{a}$
1.5N + 1.0B	$1.67 \pm 0.40_{bcd}$	$57.00 \pm 0.09_{c}$	$100.00 \pm 0.00_{a}$
1.5N + 1.5B	$2.33 \pm 0.39_{abc}$	$87.00 \pm 0.06_{ab}$	$100.00 \pm 0.00_{a}$
1.5N + 2.0B	$2.93 \pm 0.41_{a}$	$100.00 \pm 0.00_{a}$	$100.00 \pm 0.00_{a}$
2.0N + 0.5B	$1.03 \pm 0.22_{de}$	$53.00 \pm 0.09_{c}$	$100.00 \pm 0.00_{a}$
2.0N + 1.0B	$2.03 \pm 0.29_{abcd}$	$80.00 \pm 0.07_{ab}$	$100.00 \pm 0.00_{a}$
2.0N + 1.5B	$3.07 \pm 0.57_{a}$	$73.00 \pm 0.08_{bc}$	$100.00 \pm 0.00_{a}$
2.0N + 2.0B	$1.97 \pm 0.19_{abcd}$	$93.00 \pm 0.05_{ab}$	$100.00 \pm 0.00_{a}$

Data represents mean value ± standard error (SE) with 30 explants in each treatment. Means with different letters in the same column are significantly different at p=0.01according to Duncan's multiple range test (DMRT). N, NAA; B, BAP.

Table 3.3: Effects of different concentrations of single hormones on the mean height and diameter of shoots after 4 months.

MS + Hormone (mg/l)	Mean shoot height (cm)	Mean shoot diameter (cm)
control	$1.43 \pm 0.15_{a}$	$0.75 \pm 0.05_{c}$
0.5NAA	$0.12 \pm 0.05_{c}$	$0.09 \pm 0.03_{d}$
1.0NAA	$0.08 \pm 0.03_{c}$	$0.08 \pm 0.03_{d}$
1.5NAA	$0.01 \pm 0.01_{c}$	$0.01 \pm 0.01_{d}$
2.0NAA	$0.01 \pm 0.01_{c}$	$0.01 \pm 0.01_{d}$
0.5BAP	$0.93 \pm 0.08_{b}$	$0.93 \pm 0.06_{b}$
1.0BAP	$0.97 \pm 0.09_{b}$	$0.92 \pm 0.07_{b}$
1.5BAP	$1.33 \pm 0.09_{a}$	$1.10 \pm 0.04_{a}$
2.0BAP	$1.43 \pm 0.09_{a}$	$1.25 \pm 0.05_{a}$

Data represents mean value ± standard error (SE) with 30 explants in each treatment. Means with different letters in the same column are significantly different at p=0.01according to Duncan's multiple range test (DMRT).

Table 3.4: Effects of different concentrations and combinations of hormones on the mean height and diameter of shoots after 4 months.

MS + Hormone (mg/l)	Mean shoot height (cm)	Mean shoot diameter (cm)
control	$1.43 \pm 0.15_{a}$	$0.75 \pm 0.05_{ef}$
0.5N + 0.5B	$0.91 \pm 0.09_{bcde}$	$0.75 \pm 0.07_{ef}$
0.5N + 1.0B	$1.13 \pm 0.08_{b}$	$1.02 \pm 0.07_{bcd}$
0.5N + 1.5B	$0.88 \pm 0.07_{bcde}$	$0.73 \pm 0.06_{ef}$
0.5N + 2.0B	$1.07 \pm 0.04_{bc}$	$1.07 \pm 0.03_{bcd}$
1.0N + 0.5B	$0.18 \pm 0.07_{g}$	$0.17 \pm 0.06_{h}$
1.0N + 1.0B	$0.75 \pm 0.06_{de}$	$0.67 \pm 0.04_{f}$
1.0N + 1.5B	$0.87 \pm 0.06_{bcde}$	$0.97 \pm 0.04_{cd}$
1.0N + 2.0B	$1.03 \pm 0.02_{bcd}$	$1.17 \pm 0.04_{abc}$
1.5N + 0.5B	$0.28 \pm 0.11_{fg}$	$0.25 \pm 0.09_{h}$
1.5N + 1.0B	$1.10 \pm 0.08_{b}$	$1.05 \pm 0.04_{bcd}$
1.5N + 1.5B	$0.92 \pm 0.05_{bcde}$	$1.02 \pm 0.04_{bcd}$
1.5N + 2.0B	$1.08 \pm 0.03_{b}$	$1.28 \pm 0.05_{a}$
2.0N + 0.5B	$0.46 \pm 0.03_{f}$	$0.45 \pm 0.02_{g}$
2.0N + 1.0B	$0.72 \pm 0.05_{e}$	$0.90 \pm 0.06_{de}$
2.0N + 1.5B	$0.98 \pm 0.03_{bcde}$	$1.20 \pm 0.05_{ab}$
2.0N + 2.0B	$0.78 \pm 0.05_{cde}$	$0.98 \pm 0.06_{bcd}$

Data represents mean value ± standard error (SE) with 30 explants in each treatment. Means with different letters in the same column are significantly different at p=0.01according to Duncan's multiple range test (DMRT). N, NAA; B, BAP.

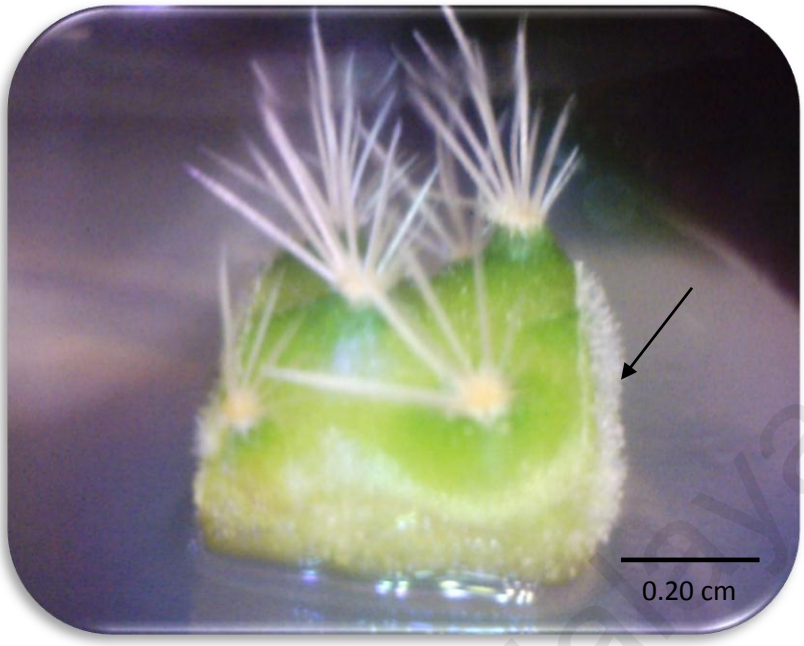


Figure 3.1: Stem explant of *Echinocereus cinerascens* (0.5 cm x 0.5 cm size with 4-6 areoles) produced callus on the cut surfaces after 1 week of culture on proliferation medium.

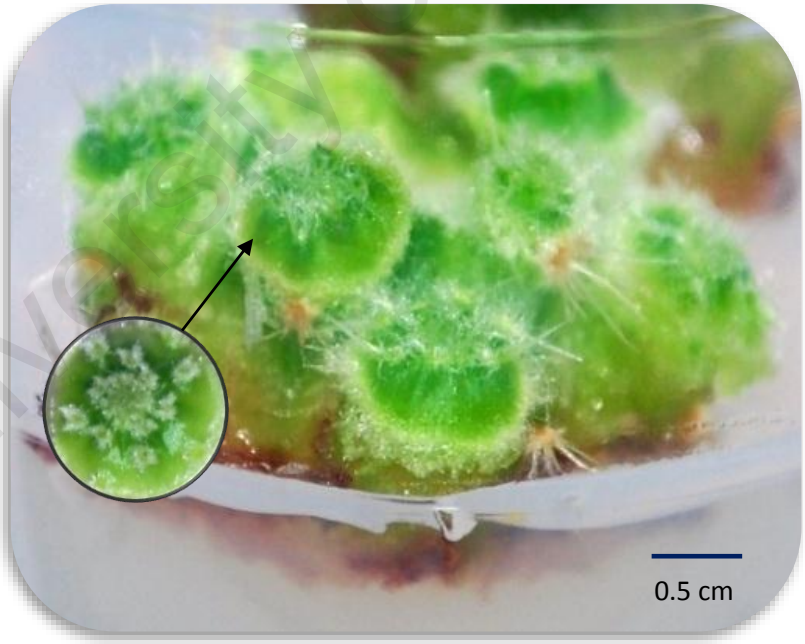


Figure 3.2: Production of shoots from stem explant of *Echinocereus cinerascens* could be seen clearly after 1 month of culture on proliferation medium.

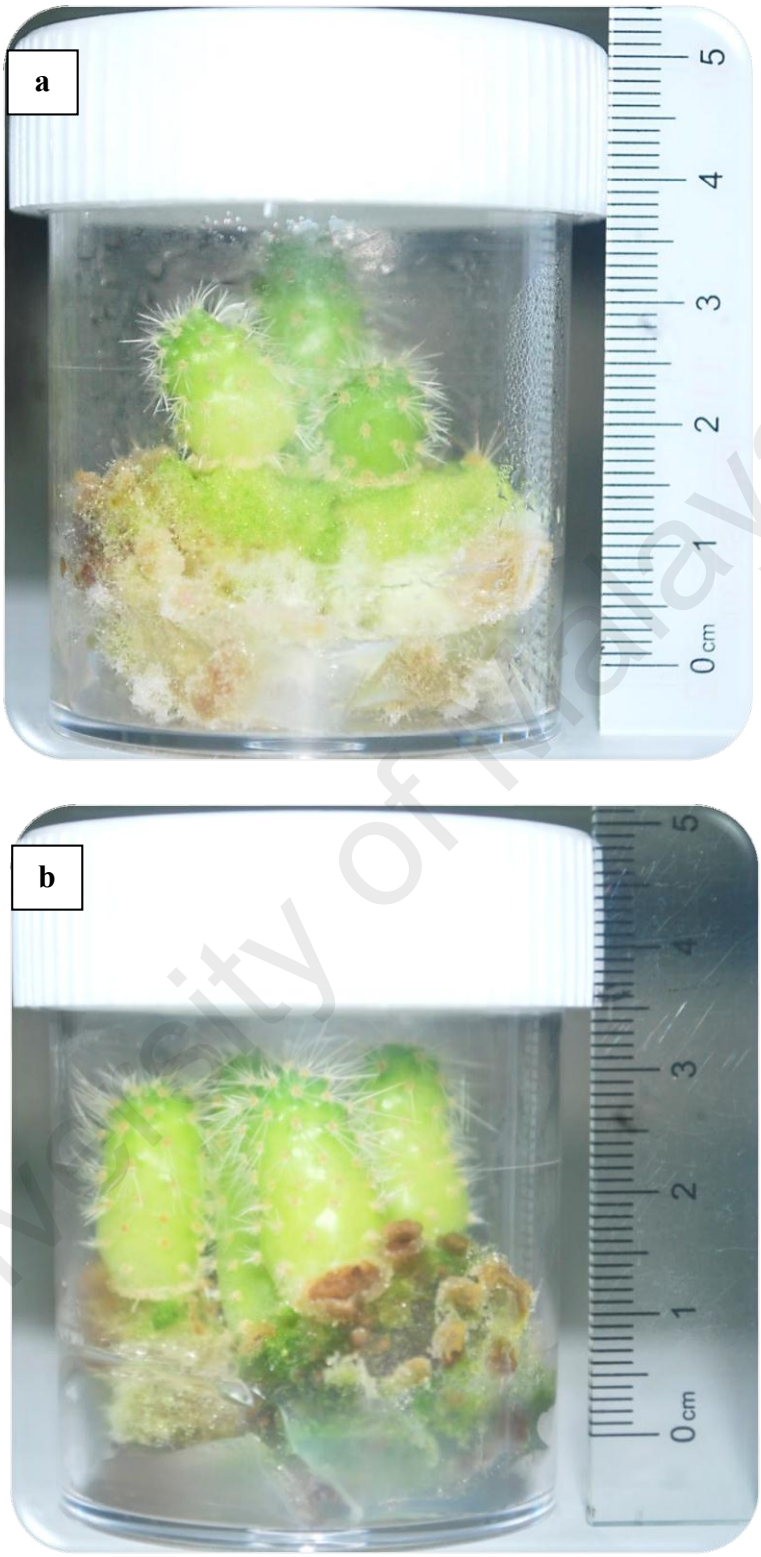


Figure 3.3: Production of (a) 3 shoots per explant, (b) 4 shoots per explant incorporated with callus after 4 months of culture on MS medium supplemented with 2.0 mg/l NAA + 1.5 mg/l BAP.

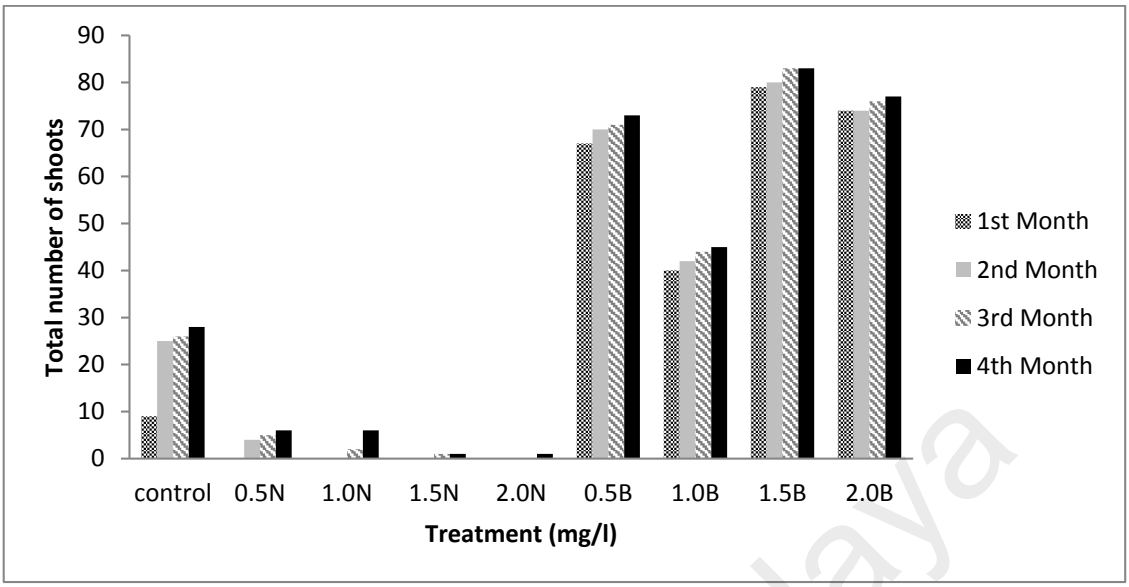


Figure 3.4: Total number of shoots produced in different concentrations of single hormones observed monthly. N, NAA; B, BAP.

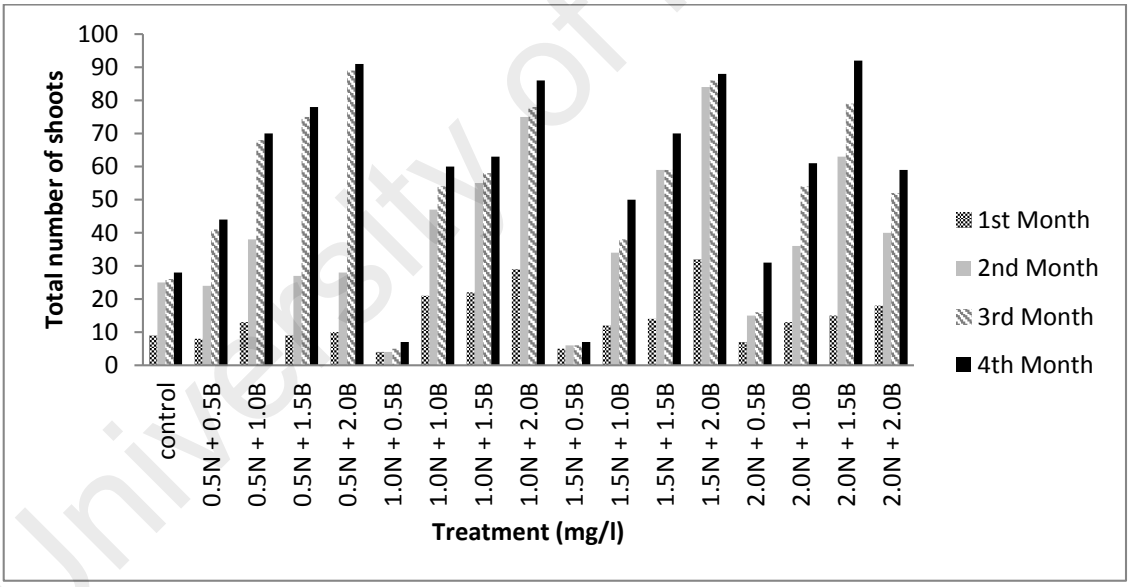


Figure 3.5: Total number of shoots produced in different concentrations and combinations of hormones observed monthly. N, NAA; B, BAP.

3.3.2 Effects of IBA and Kinetin Applied Singly and in Combinations on Plant Regeneration *In Vitro*

Investigations indicated that greater number of shoots produced in media with higher concentrations of single hormone, 1.5-2.0 mg/l Kinetin and nearly all media with combinations of hormones, Kinetin and IBA. MS medium supplemented with 2.0 mg/l Kinetin produced higher mean number of shoot, 3.80 (Table 3.5) while the highest mean number of shoots, 4.37 produced in MS medium supplemented with 2.0 mg/l Kinetin + 1.0 mg/l IBA (Table 3.6). The result verified that MS medium supplemented with 2.0 mg/l Kinetin + 1.0 mg/l IBA was the optimum medium established for *in vitro* regeneration of this species (Figure 3.6). Interestingly, it could be seen clearly that mean number of shoots increased gradually in media combinations of Kinetin and IBA with the increasing concentrations of Kinetin. Statistical analysis verified that explant produced 1 shoot in MS medium supplemented with 0.5 mg/l Kinetin + IBA, 2 shoots in MS medium supplemented with 1.0 mg/l Kinetin + IBA, 3 shoots in MS medium supplemented with 1.5 mg/l Kinetin + IBA and 4 shoots in MS medium supplemented with 2.0 mg/l Kinetin + IBA (Table 3.6). In contrast, media with IBA alone showed mean number of shoots almost similar to the control, with no different statistically (Table 3.5).

However, percentage of explants produced shoots in media with IBA alone was significantly lower than the control. Unlike in the media with IBA alone, 100 % explants produced shoots in media with 1.5-2.0 mg/l Kinetin alone (Table 3.5), which showed significantly higher than the control. Similar result was observed as the explants were subjected to media with combinations of 1.0-2.0 mg/l Kinetin and 0.5-2.0 mg/l IBA (Table 3.6).

Generally, the shoots size produced in media with Kinetin alone and media with combinations of Kinetin and IBA were 1.0 cm in height and diameter. Nevertheless, most of the media including media with IBA alone produced shoot with height lower than the control, 1.43 cm. Several media which produced shoot size higher than the control were MS medium supplemented with single hormone, 2.0 mg/l Kinetin (Table 3.7), while in hormone combinations of, 0.5 mg/l Kinetin + 0.5 mg/l IBA, 1.0 mg/l Kinetin + 0.5 mg/l IBA, 1.5 mg/l Kinetin + 0.5 mg/l IBA, 2.0 mg/l Kinetin + 0.5 mg/l IBA and 2.0 mg/l Kinetin + 1.0 mg/l IBA (Table 3.8). Medium with single hormone that produced greater shoot height, 1.7 cm was MS medium supplemented with 2.0 mg/l Kinetin (Table 3.7) whereas the media with hormone combination that produced the greatest shoot height, 2.72 cm was MS medium supplemented with 2.0 mg/l Kinetin + 0.5 mg/l IBA (Table 3.8). In contrary, the greatest shoot diameter, 1.3 cm was observed in medium with single hormone, 2.0 mg/l Kinetin (Table 3.7) as the medium in hormone combination, 2.0 mg/l Kinetin + 1.5 mg/l IBA could only produce 1.13 cm diameter of shoot (Table 3.8).

Production of callus could be observed in both treatments either media with hormones applied singly or in combination. Treatments with single hormone such as MS medium supplemented with 1.5-2.0 mg/l Kinetin and 1.0-2.0 mg/l IBA showed 100% production of callus (Table 3.5). Similarly, all treatments with hormones in combination produced 100% callus which showed significantly different with control (Table 3.6).

Analysis production of shoot monthly indicated that media added with Kinetin alone produced higher number of shoots after 1 month and the shoots increased with the increasing concentration of Kinetin (Figure 3.7). Higher number of shoots was observed in medium with 2.0 mg/l Kinetin that produced 112 shoots after 1 month and slightly increased on the following months to give 114 shoots after 4 months. Conversely, media

which added with IBA alone produced less number of shoot after 1 month but then moderately increased after 2 months (increased 12 shoots) and 4 months (increased 14 shoots) (Figure 3.7). After 1 month, medium added with 1.0 mg/l IBA produced 1 shoot only could give up to 13 shoots after 2 months while medium added with 1.5 mg/l IBA which produced 13 shoots after 3 months could give 27 shoots after 4 months.

Production of shoots monthly in media added with combination of Kinetin and IBA correspond with the control as both showed higher shoots increased after 2 months (Figure 3.8). In the control, explants produced 9 shoots after 1 month, followed by 16 shoots increase afterwards to give 25 shoots after 2 months. Nevertheless, MS medium supplemented with 2.0 mg/l Kinetin + 1.0 mg/l IBA produced 36 shoots after 1 month, increased greatly with 88 shoots and yielded up to 124 shoots after 2 months. Subsequently, shoots that slightly increased on the following months gave 131 shoots after 4 months, which showed the highest production of shoots monthly.

Table 3.5: Effects of different concentrations of single hormones on the mean number of shoots per explant, mean number of explants producing shoots and callus (%) after 4 months.

MS + Hormone (mg/l)	Mean no. of shoots per explant	Mean no. of explants produced shoots (%)	Mean no of explants produced callus (%)
control	$0.93 \pm 0.11_{d}$	$80.00 \pm 0.07_{ab}$	$83.00 \pm 0.07_{abc}$
0.5KIN	$0.93 \pm 0.20_{d}$	$60.00 \pm 0.09_{b}$	$67.00 \pm 0.09_{c}$
1.0KIN	$1.67 \pm 0.25_{c}$	$73.00 \pm 0.08_{ab}$	$73.00 \pm 0.08_{bc}$
1.5KIN	$3.07 \pm 0.16_{b}$	$100.00 \pm 0.00_{a}$	$100.00 \pm 0.00_{a}$
2.0KIN	$3.80 \pm 0.23_{a}$	$100.00 \pm 0.00_{a}$	$100.00 \pm 0.00_{a}$
0.5IBA	$0.83 \pm 0.11_{d}$	$73.00 \pm 0.08_{ab}$	$90.00 \pm 0.06_{ab}$
1.0IBA	$0.77 \pm 0.13_{d}$	$63.00 \pm 0.09_{b}$	$100.00 \pm 0.06_{a}$
1.5IBA	$0.90 \pm 0.11_{d}$	$77.00 \pm 0.08_{ab}$	$100.00 \pm 0.00_{a}$
2.0IBA	$0.77 \pm 0.13_{d}$	$63.00 \pm 0.09_{b}$	$100.00 \pm 0.00_{a}$

Data represents mean value ± standard error (SE) with 30 explants in each treatment. Means with different letters in the same column are significantly different at p=0.01according to Duncan's multiple range test (DMRT).

Table 3.6: Effects of different concentrations and combinations of hormones on the mean number of shoots per explant, mean number of explants producing shoots and callus (%) after 4 months.

MS + Hormone (mg/l)	Mean no. of shoots per explant	Mean no. of explants produced shoots (%)	Mean no. of explants produced callus (%)
control	$0.93 \pm 0.11_{de}$	$80.00 \pm 0.07_{b}$	$83.00 \pm 0.07_{b}$
0.5K + 0.5I	$1.67 \pm 0.19_{d}$	$100.00 \pm 0.00_{a}$	$100.00 \pm 0.00_{a}$
0.5K + 1.0I	$1.47 \pm 0.20_{d}$	$93.00 \pm 0.05_{ab}$	$100.00 \pm 0.00_{a}$
0.5K + 1.5I	$0.50 \pm 0.16_{e}$	$30.00 \pm 0.09_{d}$	$100.00 \pm 0.00_{a}$
0.5K + 2.0I	$1.03 \pm 0.24_{de}$	$57.00 \pm 0.09_{c}$	$100.00 \pm 0.00_{a}$
1.0K + 0.5I	$2.73 \pm 0.21_{c}$	$100.00 \pm 0.00_{a}$	$100.00 \pm 0.00_{a}$
1.0K + 1.0I	$2.50 \pm 0.16_{c}$	$100.00 \pm 0.00_{a}$	$100.00 \pm 0.00_{a}$
1.0K + 1.5I	$2.83 \pm 0.17_{bc}$	$100.00 \pm 0.00_{a}$	$100.00 \pm 0.00_{a}$
1.0K + 2.0I	$2.70 \pm 0.19_{c}$	$100.00 \pm 0.00_{a}$	$100.00 \pm 0.00_{a}$
1.5K + 0.5I	$3.67 \pm 0.21_{ab}$	$100.00 \pm 0.00_{a}$	$100.00 \pm 0.00_{a}$
1.5K + 1.0I	$2.90 \pm 0.28_{bc}$	$100.00 \pm 0.00_{a}$	$100.00 \pm 0.00_{a}$
1.5K + 1.5I	$2.97 \pm 0.24_{bc}$	$100.00 \pm 0.00_{a}$	$100.00 \pm 0.00_{a}$
1.5K + 2.0I	$2.93 \pm 0.17_{bc}$	$100.00 \pm 0.00_{a}$	$100.00 \pm 0.00_{a}$
2.0K + 0.5I	$4.00 \pm 0.19_{a}$	$100.00 \pm 0.00_{a}$	$100.00 \pm 0.00_{a}$
2.0K + 1.0I	$4.37 \pm 0.27_{a}$	$100.00 \pm 0.00_{a}$	$100.00 \pm 0.00_{a}$
2.0K + 1.5I	$3.97 \pm 0.26_{a}$	$100.00 \pm 0.00_{a}$	$100.00 \pm 0.00_{a}$
2.0K + 2.0I	$3.10 \pm 0.24_{bc}$	$100.00 \pm 0.00_{a}$	$100.00 \pm 0.00_{a}$

Data represents mean value ± standard error (SE) with 30 explants in each treatment. Means with different letters in the same column are significantly different at p=0.01according to Duncan's multiple range test (DMRT). K, KIN; I, IBA.

Table 3.7: Effects of different concentrations of single hormones on the mean height and diameter of shoots after 4 months.

MS + Hormone (mg/l)	Mean shoot height (cm)	Mean shoot diameter (cm)
control	$1.43 \pm 0.15_{ab}$	$0.75 \pm 0.05_{cd}$
0.5KIN	$0.15 \pm 0.13_{bc}$	$0.90 \pm 0.10_{bc}$
1.0KIN	$1.05 \pm 0.07_{bcd}$	$1.05 \pm 0.04_{b}$
1.5KIN	$1.37 \pm 0.09_{ab}$	$1.03 \pm 0.02_{b}$
2.0KIN	$1.70 \pm 0.10_{a}$	$1.30 \pm 0.05_{a}$
0.5IBA	$0.67 \pm 0.10_{de}$	$0.61 \pm 0.07_{de}$
1.0IBA	$0.82 \pm 0.12_{cde}$	$0.67 \pm 0.09_{cde}$
1.5IBA	$0.67 \pm 0.08_{de}$	$0.62 \pm 0.07_{de}$
2.0IBA	$0.45 \pm 0.07_{e}$	$0.46 \pm 0.07_{e}$

Data represents mean value ± standard error (SE) with 30 explants in each treatment. Means with different letters in the same column are significantly different at p=0.01according to Duncan's multiple range test (DMRT).

Table 3.8: Effects of different concentrations and combinations of hormones on the mean height and diameter of shoots after 4 months.

MS + Hormone (mg/l)	Mean shoot height (cm)	Mean shoot diameter (cm)
control	$1.43 \pm 0.15_{cde}$	$0.75 \pm 0.05_{ef}$
0.5K + 0.5I	$1.58 \pm 0.16_{bcd}$	$0.95 \pm 0.04_{bcd}$
0.5K + 1.0I	$0.93 \pm 0.11_{efgh}$	$0.73 \pm 0.05_{ef}$
0.5K + 1.5I	$0.41 \pm 0.09_{h}$	$0.38 \pm 0.08_{g}$
0.5K + 2.0I	$0.60 \pm 0.08_{gh}$	$0.49 \pm 0.03_{g}$
1.0K + 0.5I	$1.93 \pm 0.08_{be}$	$1.08 \pm 0.03_{ab}$
1.0K + 1.0I	$1.12 \pm 0.07_{defg}$	$1.08 \pm 0.03_{ab}$
1.0K + 1.5I	$1.35 \pm 0.11_{def}$	$1.07 \pm 0.03_{ab}$
1.0K + 2.0I	$0.73 \pm 0.08_{gh}$	$0.65 \pm 0.04_{f}$
1.5K + 0.5I	$2.00 \pm 0.11_{b}$	$1.02 \pm 0.02_{abc}$
1.5K + 1.0I	$0.87 \pm 0.07_{efgh}$	$0.83 \pm 0.04_{de}$
1.5K + 1.5I	$0.85 \pm 0.04_{efgh}$	$0.98 \pm 0.02a_{bcd}$
1.5K + 2.0I	$1.18 \pm 0.09_{defg}$	$0.85 \pm 0.05_{cde}$
2.0K + 0.5I	$2.72 \pm 0.43_{a}$	$1.12 \pm 0.04_{ab}$
2.0K + 1.0I	$1.68 \pm 0.08_{bcd}$	$1.08 \pm 0.03_{ab}$
2.0K + 1.5I	$1.38 \pm 0.09_{cdef}$	$1.13 \pm 0.04_{a}$
2.0K + 2.0I	$0.83 \pm 0.08_{fgh}$	$0.83 \pm 0.06_{de}$

Data represents mean value ± standard error (SE) with 30 explants in each treatment. Means with different letters in the same column are significantly different at p=0.01according to Duncan's multiple range test (DMRT). K, KIN; I, IBA.

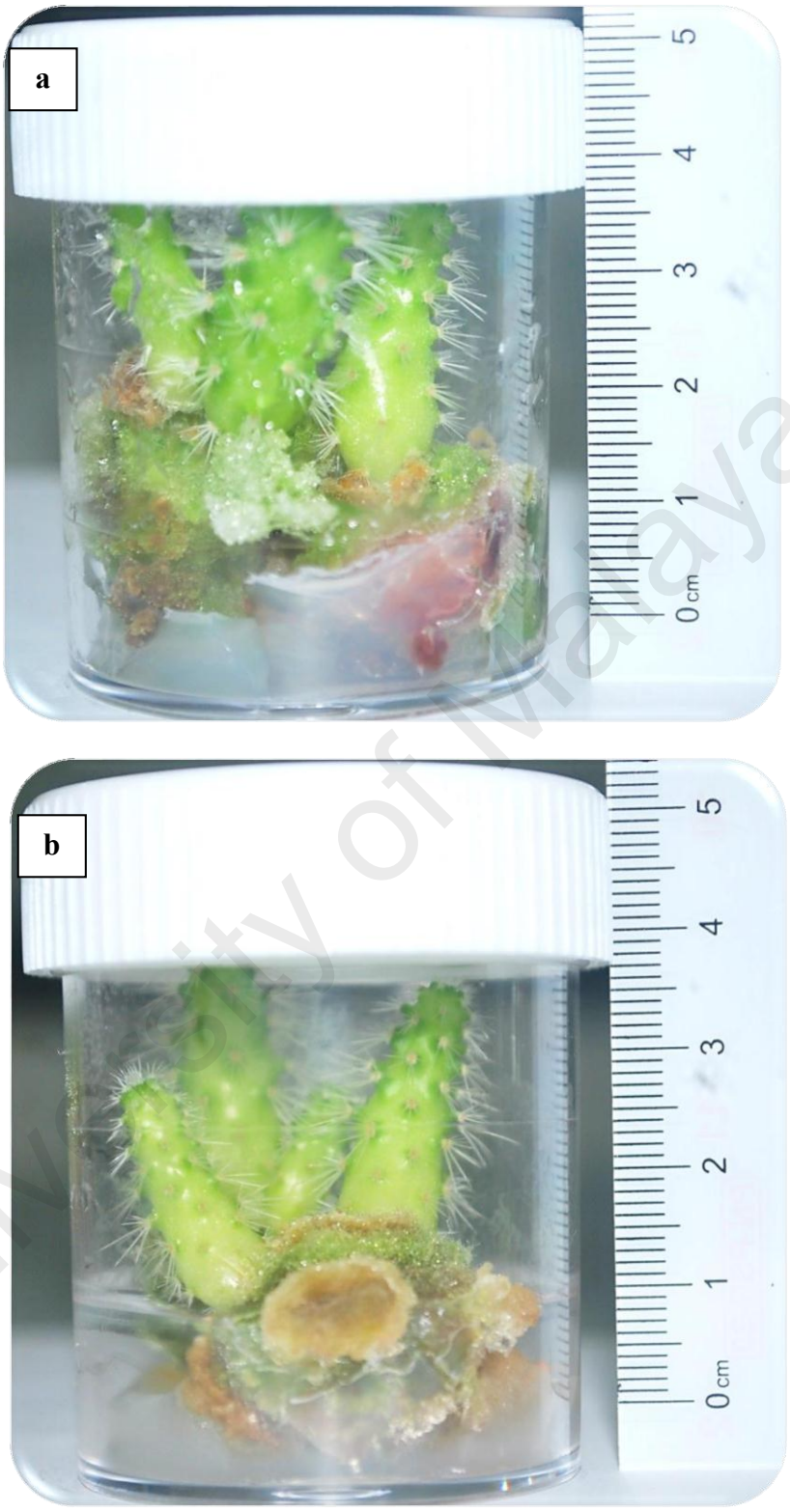


Figure 3.6: Production of (a) 3 shoots per explant, (b) 4 shoots per explant incorporated with callus after 4 months of culture on MS medium supplemented with 2.0 mg/l Kinetin + 1.0 mg/l IBA.

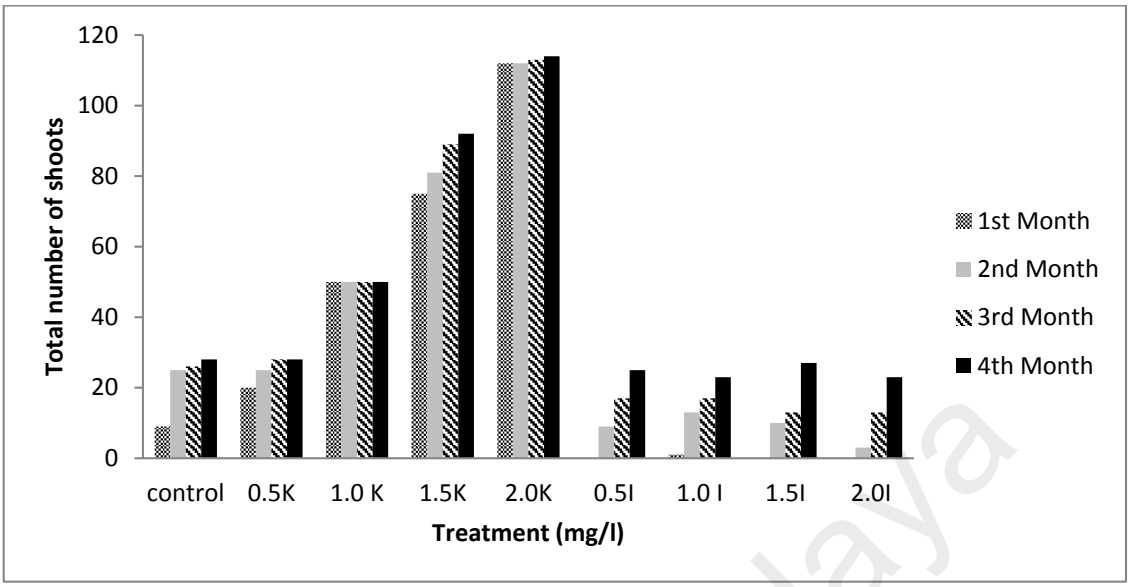


Figure 3.7: Total number of shoots produced in different concentrations of single hormones observed monthly. K, KIN; I, IBA.

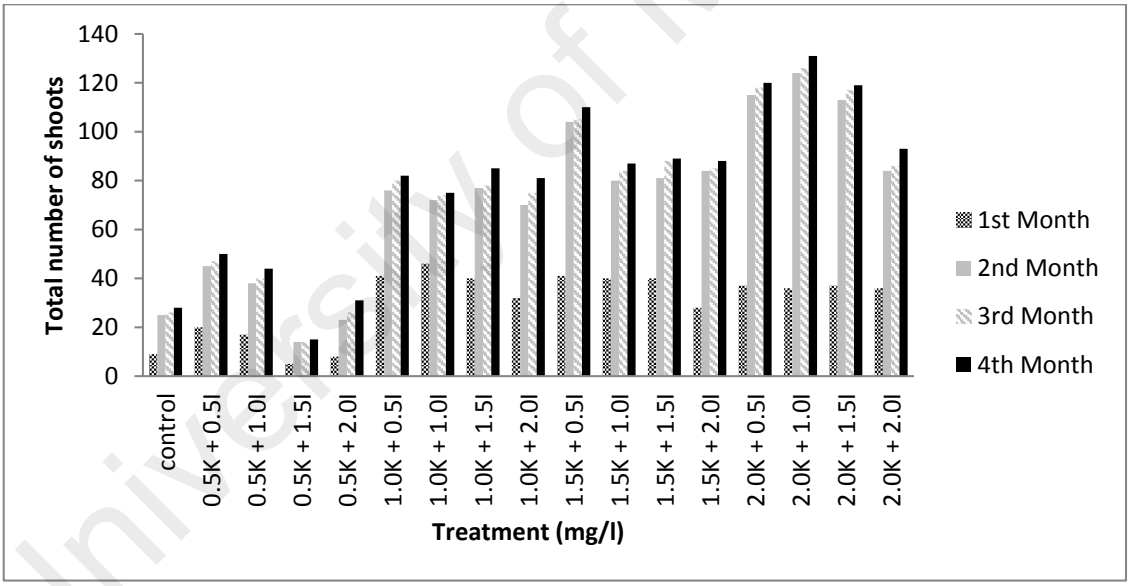


Figure 3.8: Total number of shoots produced in different concentrations and combinations of hormones observed monthly. K, KIN; I, IBA.

3.3.3 *In Vitro* Rooting

Trial experiments on rooting *in vitro* were done and plantlets cultured on MS basal medium (Figure 3.9) promoted a higher percentage of rooting than those on MS medium fortified with hormones, such as NAA or IBA (data not shown) applied singly. Plantlets rooted abundantly *in vitro* on MS basal medium within 2 to 3 months. These complete plantlets grown with well-developed roots (Figure 3.10) were harvested and selected for acclimatization.

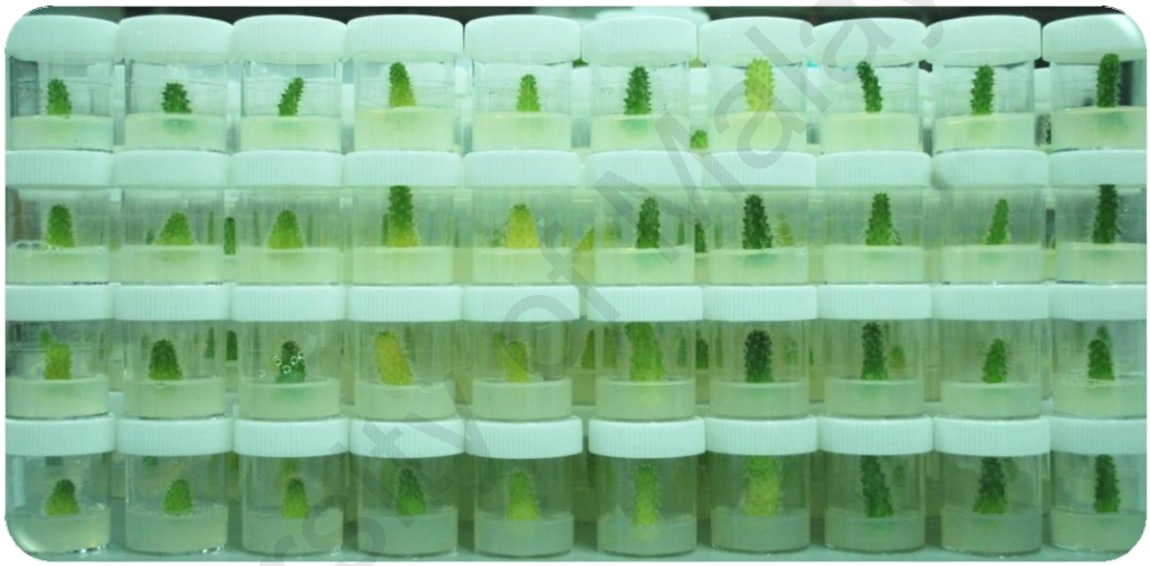


Figure 3.9: Selected shoots produced in proliferation medium transferred to MS basal medium for *in vitro* rooting.

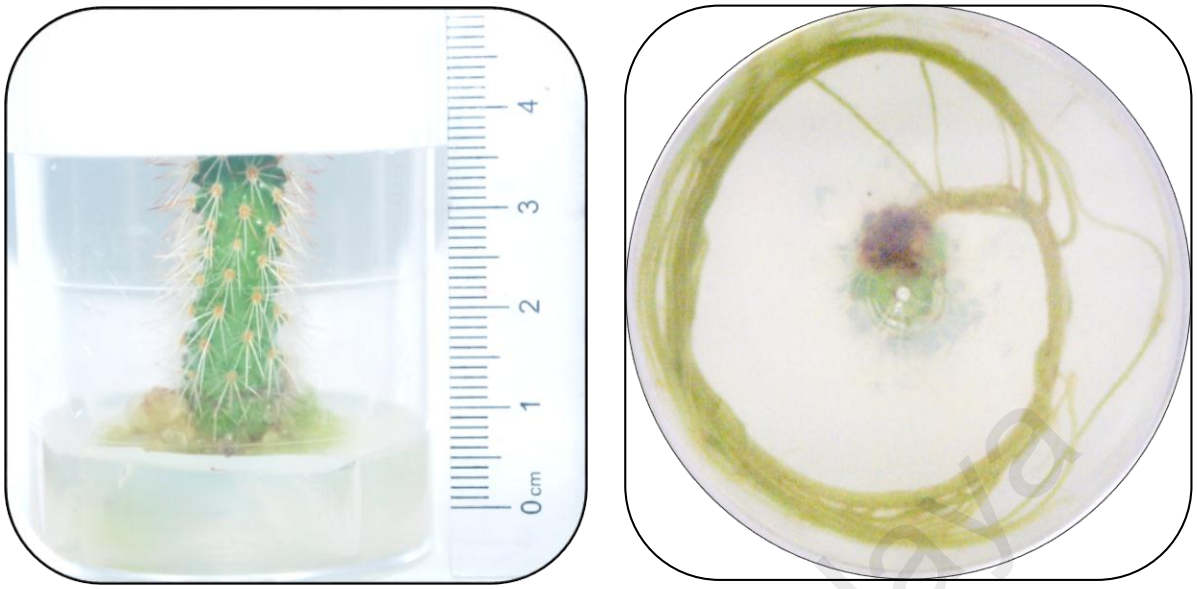


Figure 3.10: Plantlet become (a) a complete plantlet with (b) well-developed roots after 3 months in MS basal medium.

3.3.4 Microscopic Studies

Several comparisons were made on morphology and anatomy of *in vivo, in vitro* and *ex vitro* samples. Investigations reported similar structures of shoot apical, vascular bundles, starch grains and type of stomata for all of the samples tested. Analysis of shoot apical revealed interesting morphology and anatomy of the areoles, ribs and spines of *Echinocereus cinerascens* which observed under SEM (Figure: 3.11a) and light microscope (Figure: 3.11b,c,d). Areoles could be defined as a cushion like structures with hairs where branches, flowers, shoots and spines emerge. This unique structure of cactus species was found along the stems on the ribs or on the top of tubercles. Spines are present on cactus species as modified leaves that provide protection against predators. Besides, observations also reported the presence of starch grains in the mesophyll cells (Figure: 3.12) which normally function to store starch and a ring of vascular bundles including xylem and phloem (Figure: 3.13, 3.14) which are very important for transportation of water, minerals or nutrients in the plants.

Further studies in ultrastructural revealed the type of stomata of *Echinocereus cinerascens.* Analysis on epidermis surfaces viewed under SEM verified that the structure of stomata is anisocytic, as most of the samples depicted the stomata surrounded by three subsidiary cells of which one is distinctly smaller than the other two (Figure 3.15). Comparisons between epidermis surfaces of *in vivo* and *in vitro* samples viewed under SEM suggested that the mean size of stomata, length and width of stomata opening were similar (Figure 3.16, 3.17). However, the mean number of stomata per area (mm^2) was unable to be quantified as the epidermis surfaces of *in vitro* samples were wrinkled. Therefore, additional studies were performed using FESEM as an alternative method that offers better result in analysing epidermis surfaces of *Echinocereus cinerascens.* Nevertheless, analysis with FESEM was conducted only for *in vitro* and *ex vitro* samples since the observations showed that no significant

difference between *in vivo* and *in vitro* samples in previous studies (Figure 3.11, 3.12, 3.13, 3.14, 3.16, 3.17).

Observations recorded epidermis cells of *Echinocereus cinerascens* were sinuous and stomata dispersed widely on the surfaces (Figure 3.18). Mean number of stomata per area (1 mm^2) present on epidermis surfaces of *in vitro* samples was 14.77 μm whereas for *ex vitro* samples was 15.45 μm (Figure 3.18), which slightly higher than *in vitro* samples. However, statistical analysis confirmed that no significant difference between the mean number of stomata per area (mm^2), both *in vitro* and *ex vitro* samples (Table 3.9). Similarly, mean size of stomata, mean length and width of opening stomata for both *in vitro* (Table 3.10) and *ex vitro* samples (Table 3.11) were comparable with no different statistically. The mean size of stomata for *ex vitro* samples was 26.12 μm, which slightly higher than *in vitro* samples, 25.20 μm (Figure 3.18a). In contrast, mean length and width of stomata opening for *in vitro* samples which correspond to 17.87 μm of length, 2.94 μm of width was slightly higher than *ex vitro* samples with 14.96 μm of length, 2.41 μm of width (Figure 3.18b).

Table 3.9: Comparison of the mean number of stomata per area (mm^2) for *in vitro* and *ex vitro* samples viewed under FESEM.

	Mean no. of stomata per area (mm^2)	
Replicate	*In vitro*	*Ex vitro*
1	20.00	17.23
2	15.73	11.36
3	19.10	12.50
4	9.00	18.18
5	10.00	18.00
Mean ± SE	14.77 ± 2.27*	15.45 ± 1.46*

Data represents mean value ± standard error (SE) with 5 replicates correspond to 5 different areas captured on the epidermis surfaces. *Means are not significantly different at p=0.01 (Independent samples T-test).

Table 3.10: Analysis on the size of stomata, length and width of opening stomata for *in vitro* samples viewed under FESEM.

		Opening stomata (µm)	
Replicate	Size of stomata (µm)	Length	Width
1	26.67	18.94	2.53
2	22.00	16.74	2.18
3	30.00	18.65	3.58
4	27.33	22.61	2.69
5	20.00	12.39	3.72
*Mean ± SE	25.2 ± 1.83	17.87 ± 1.67	2.94 ± 0.30

Data represents mean value ± standard error (SE) with 5 replicates correspond to 5 different areas captured on the epidermis surfaces. *Means are not significantly different with *ex vitro* samples (Independent samples T-test, p=0.01).

Table 3.11: Analysis on the size of stomata, length and width of opening stomata for *ex vitro* samples viewed under FESEM.

		Opening stomata (µm)	
Replicate	Size of stomata (µm)	Length	Width
1	22.60	11.60	2.72
2	27.00	16.60	2.68
3	28.00	14.80	1.17
4	28.40	17.60	2.37
5	24.60	14.20	3.09
*Mean ± SE	26.12 ± 1.10	14.96 ± 1.04	2.41 ± 0.33

Data represents mean value ± standard error (SE) with 5 replicates correspond to 5 different areas captured on the epidermis surfaces.*Means are not significantly different with *in vitro* samples (Independent samples T-test, p=0.01).

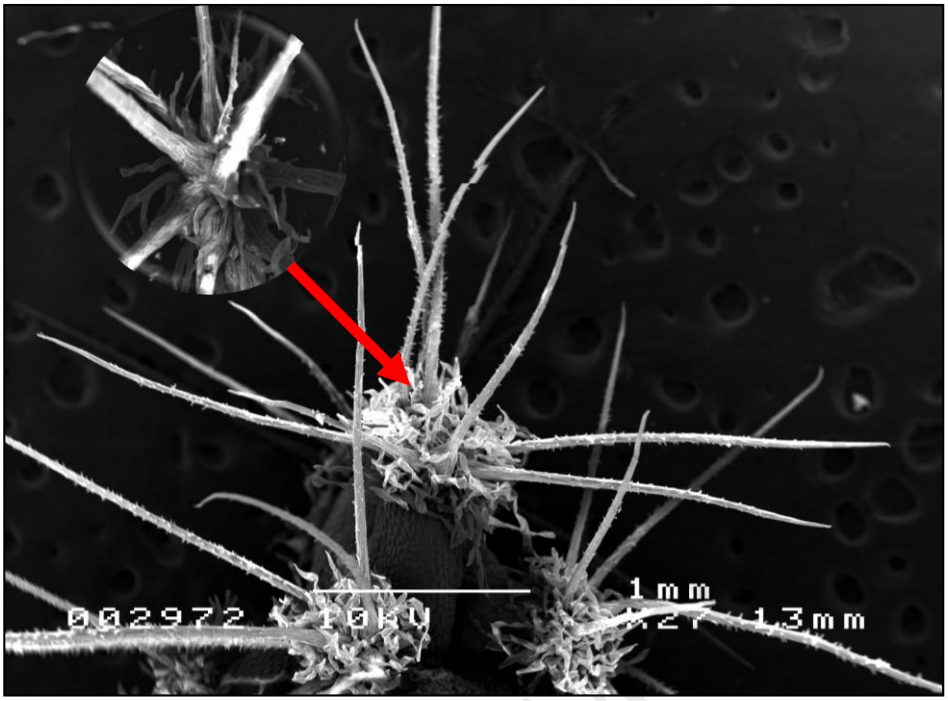


Figure 3.11(a): SEM micrograph (Magnification: X 27) illustrated the *in vivo* shoot apical of *Echinocereus cinerascens* with areoles, the unique structure of cactus where the spines, shoots and flowers will emerge.

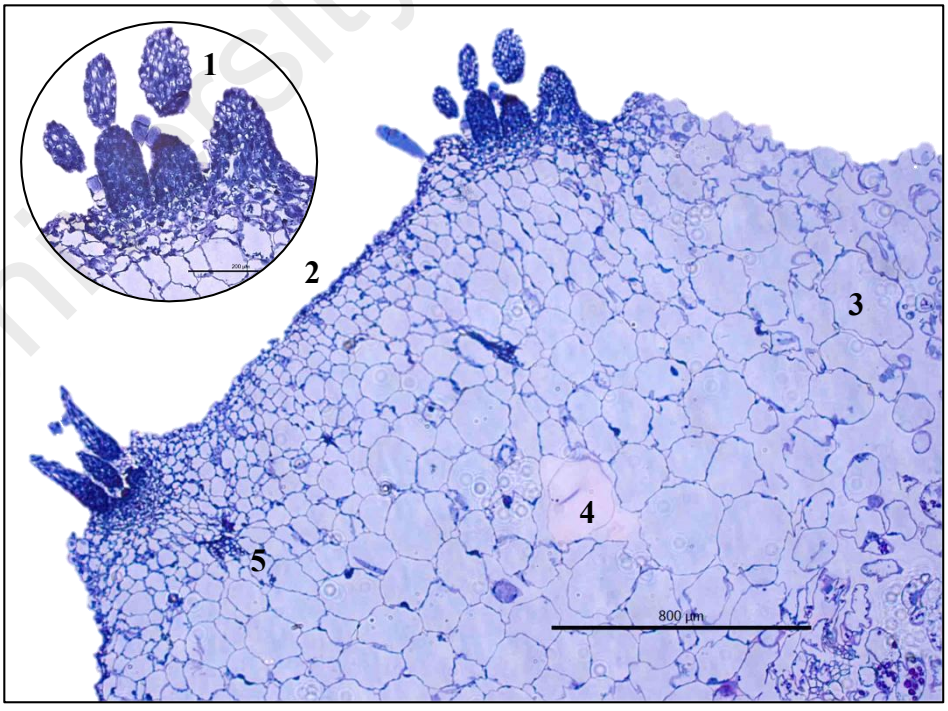


Figure 3.11(b): LS through the *in vitro* apical shoot and areoles of *Echinocereus cinerascens* (Magnification: X 5). 1 = areoles, 2 = epidermis, 3 = pith, 4 = mucilage cell, 5 = areoles trace.

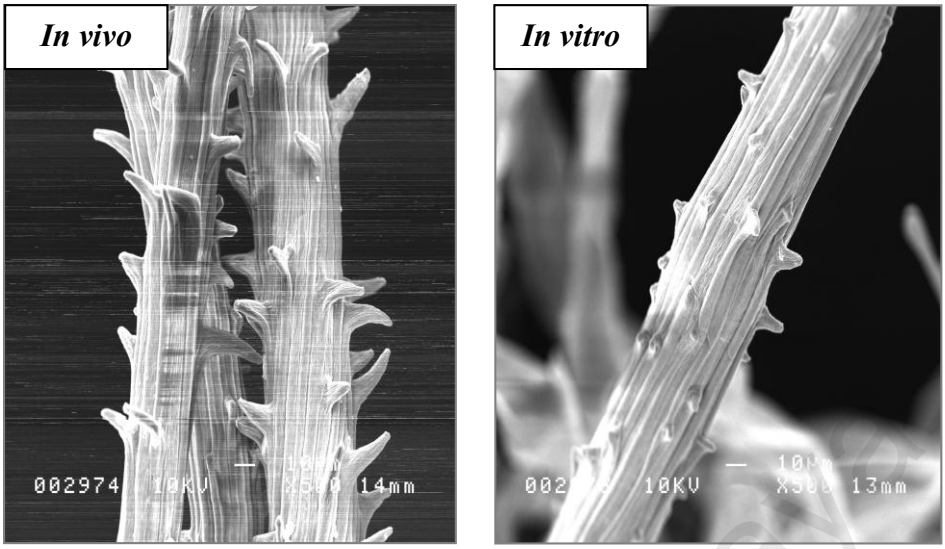


Figure 3.11(c): SEM micrograph (Magnification: X 500) illustrated the spine structure of *Echinocereus cinerascens* for *in vivo* and *in vitro* samples.

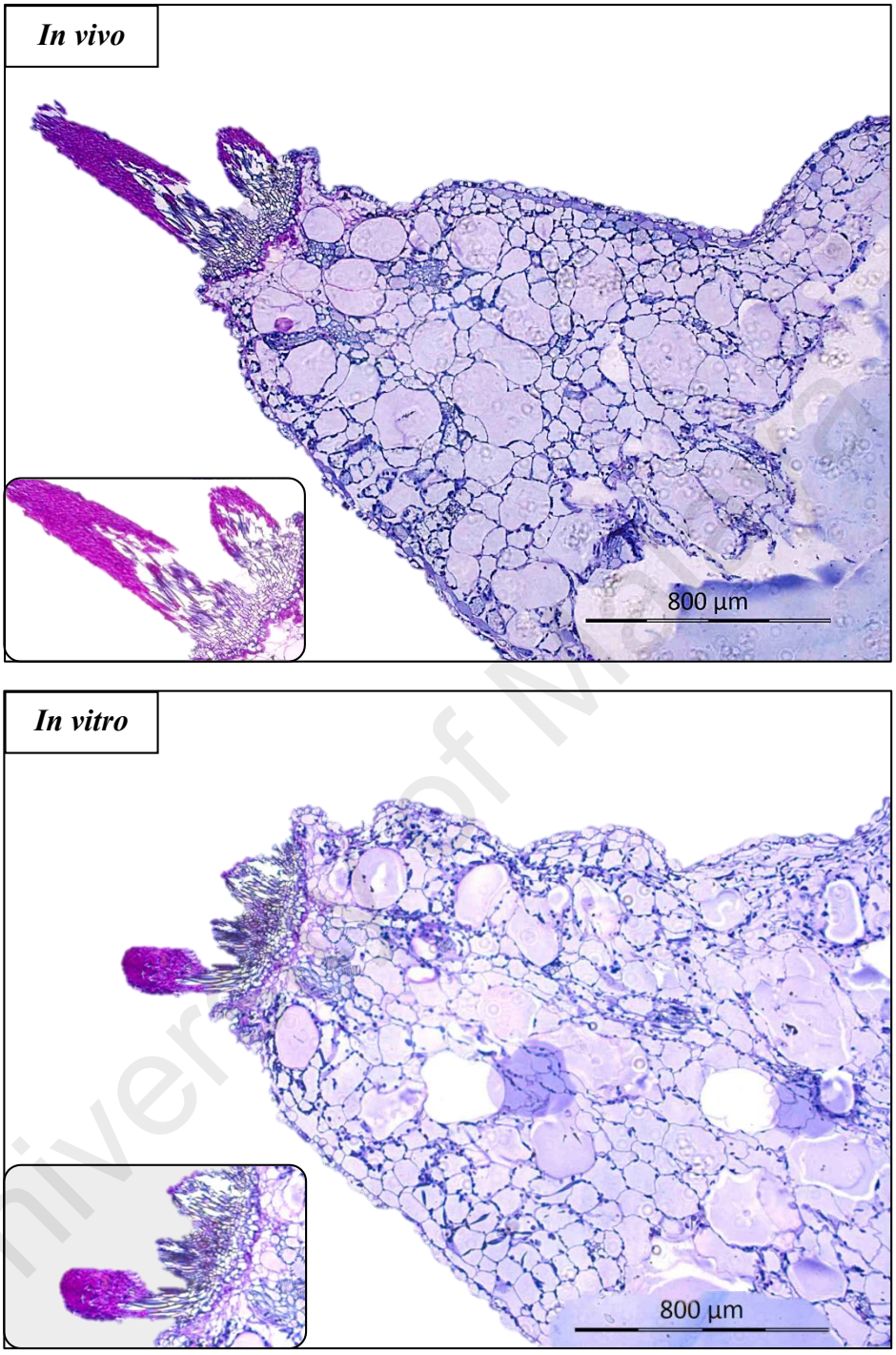


Figure 3.11(d): LS through the rib and spines of *Echinocereus cinerascens* for *in vivo* and *in vitro* samples (Magnification: X 5). 1 = epidermis, 2 = pith, 3 = mucilage cell, 4 = spines.

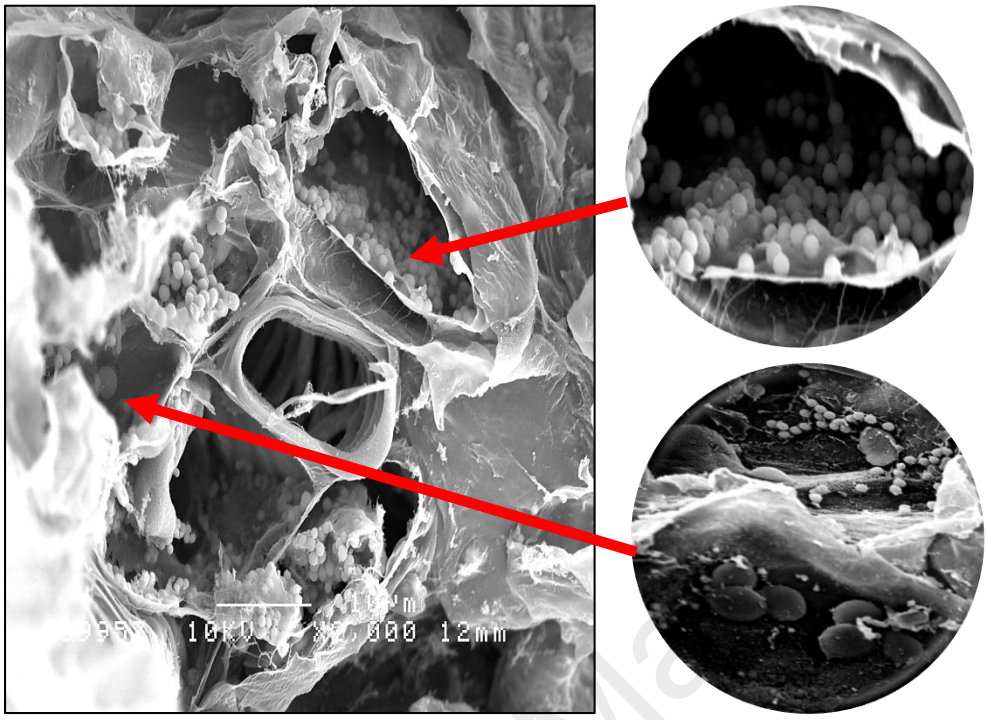


Figure 3.12(a): SEM micrograph (Magnification: X 2000) illustrated the starch grains of *Echinocereus cinerascens* with various size (in the range of 1 to 5 μm) in mesophyll cells for *in vivo* sample.

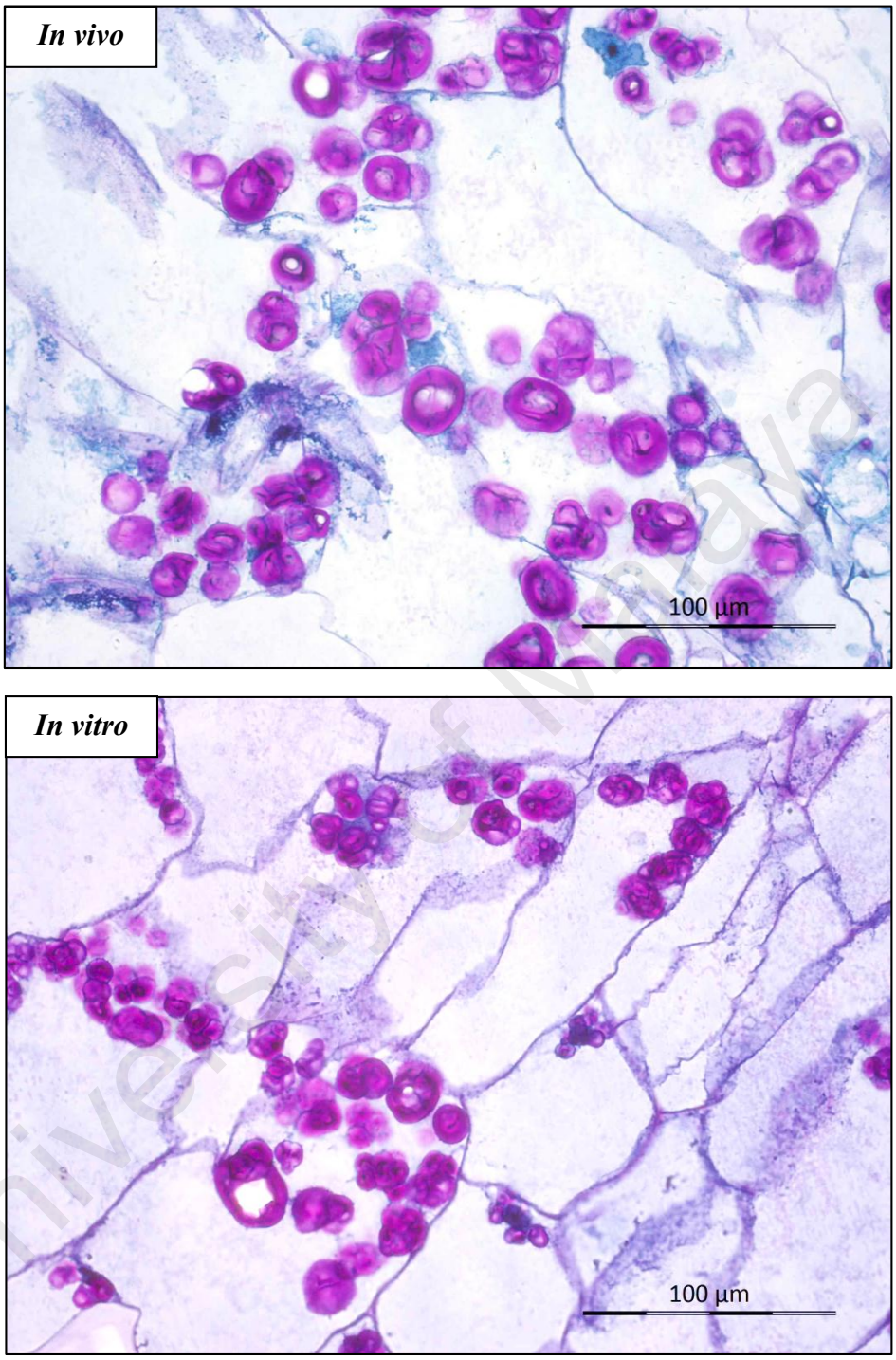


Figure 3.12(b): TS through the stem of Echinocereus cinerascens showed the parenchyma cortex packed with red-stained grains of starch (Magnification: X 40) for *in vivo* and *in vitro* samples.

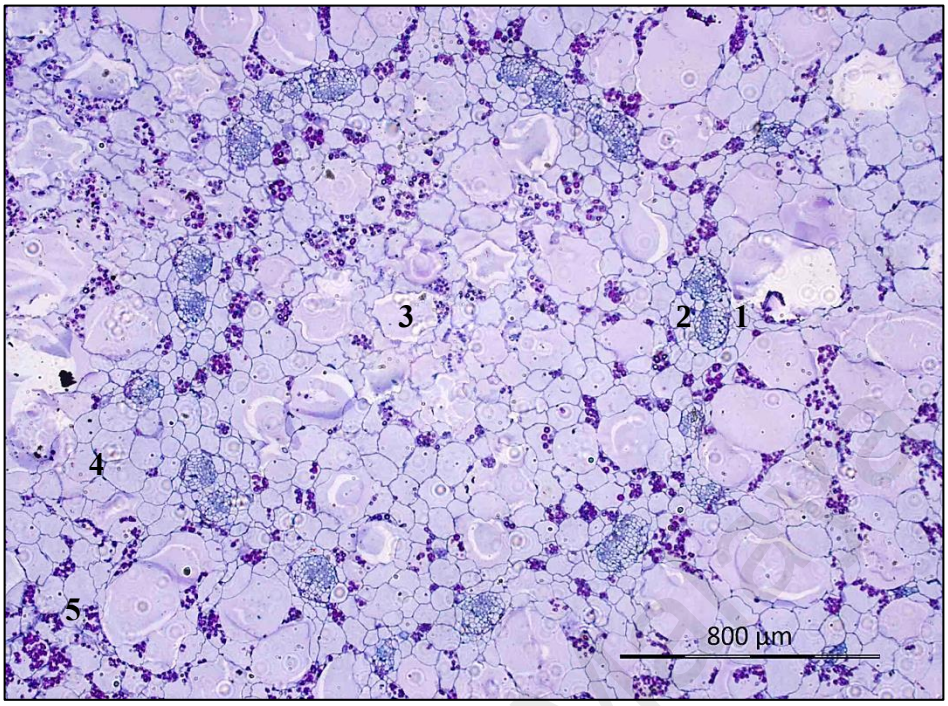


Figure 3.13(a): TS through the stem of *Echinocereus cinerascens* showed a ring of vascular bundles with xylem lying internally and phloem externally (Magnification: X 5) for *in vivo* sample. 1 = phloem, 2 = xylem, 3 = pith, 4 = cortex, 5 = starch grains.

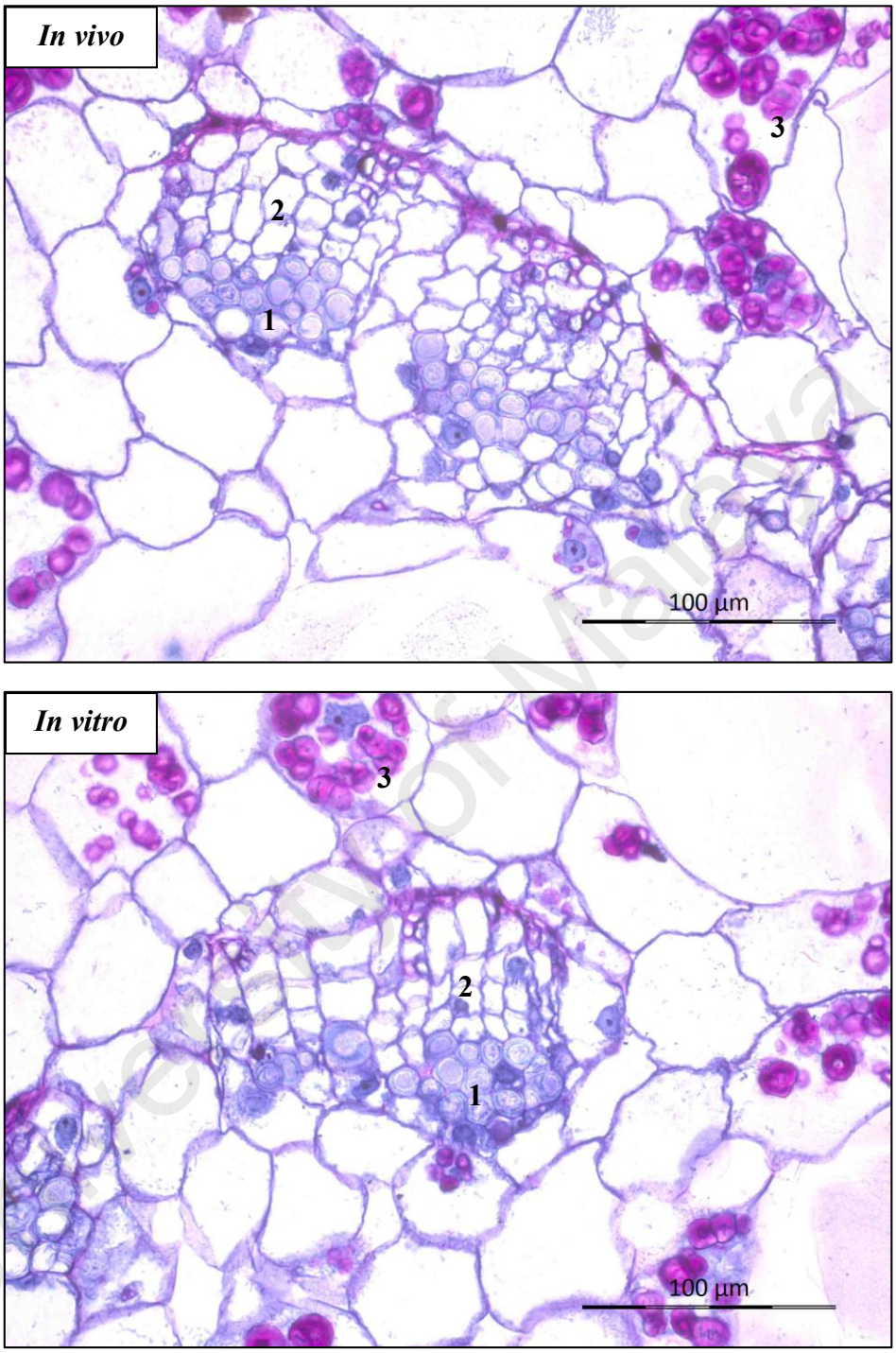


Figure 3.13 (b): TS through the vascular bundles (xylem, phloem) and starch grains of *Echinocereus cinerascens* (Magnification: X 40) for *in vivo* and *in vitro* samples. 1 = xylem, 2 = phloem, 3 = starch grains.

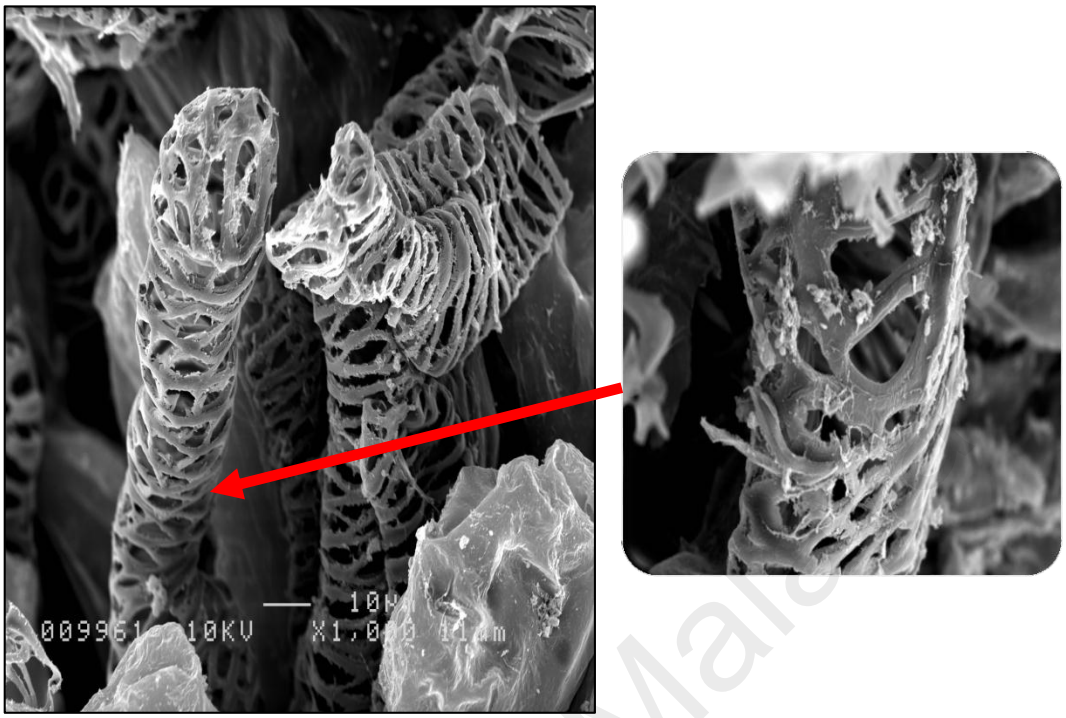


Figure 3.14 (a): SEM micrograph (Magnification: X 2000) illustrated the vascular bundles of *Echinocereus cinerascens*, about 20 µm in length for *in vivo* sample.

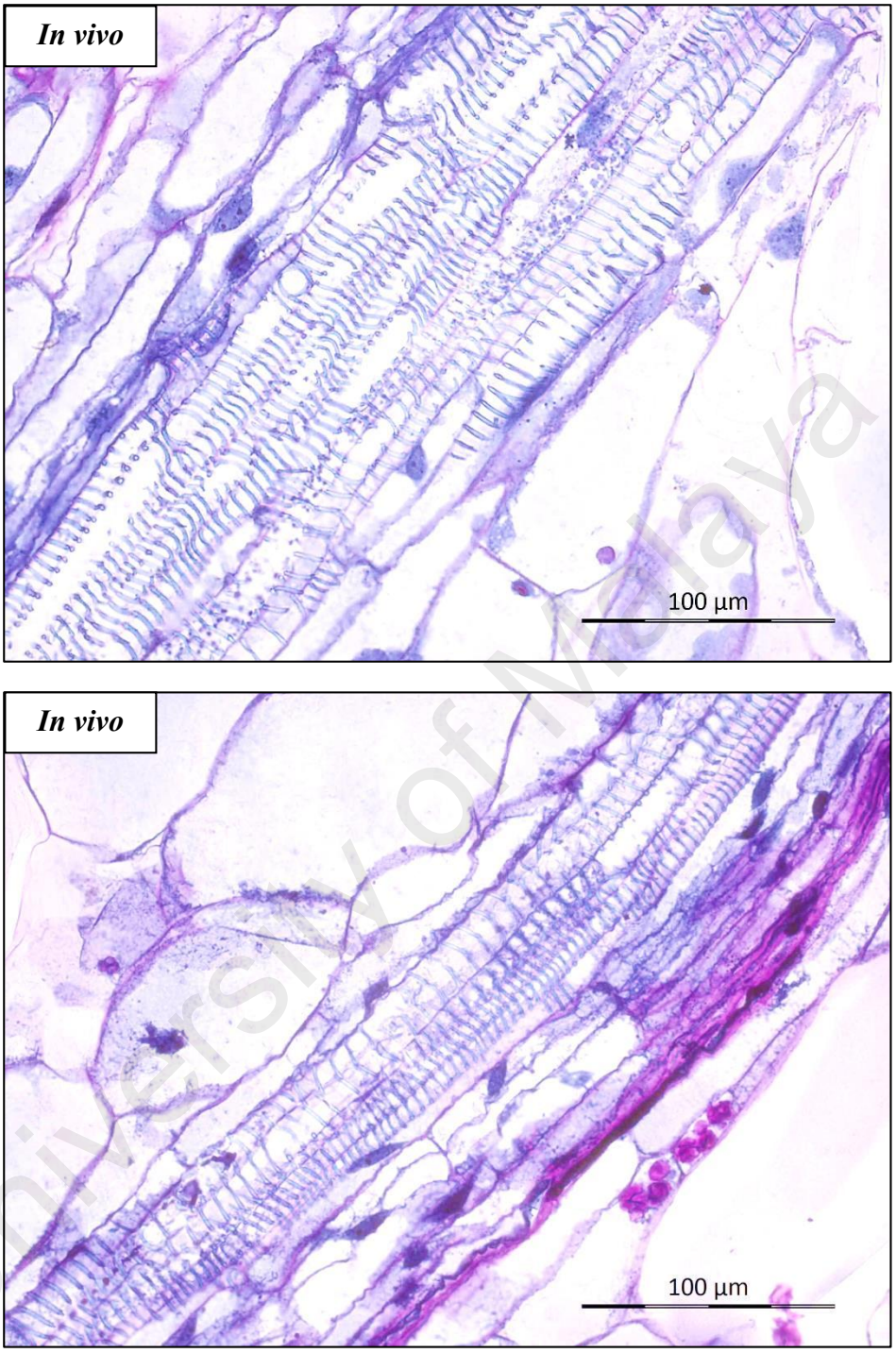


Figure 3.14 (b): LS through the vascular bundles of *Echinocereus cinerascens* showed annular or spiral pattern of xylem (Magnification: X 40) for *in vivo* and *in vitro* samples.

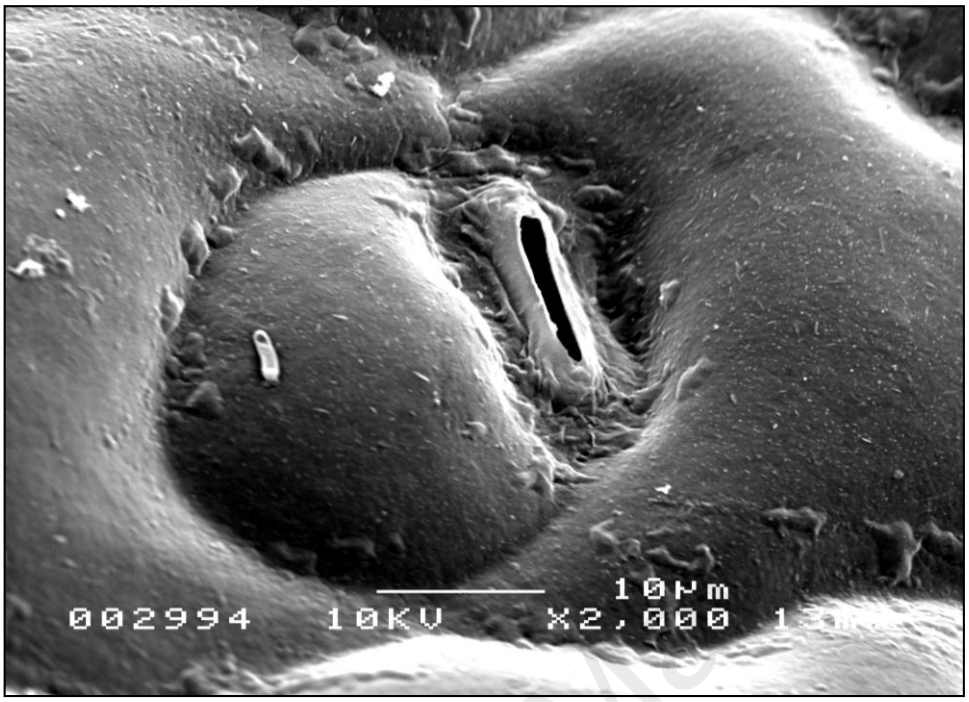


Figure 3.15: SEM micrograph (Magnification: X 2000) illustrated a stoma structure of *Echinocereus cinerascens*, anisocytic – the stoma surrounded by three subsidiary cells of which one is distinctly smaller than the other two for *in vivo* sample.

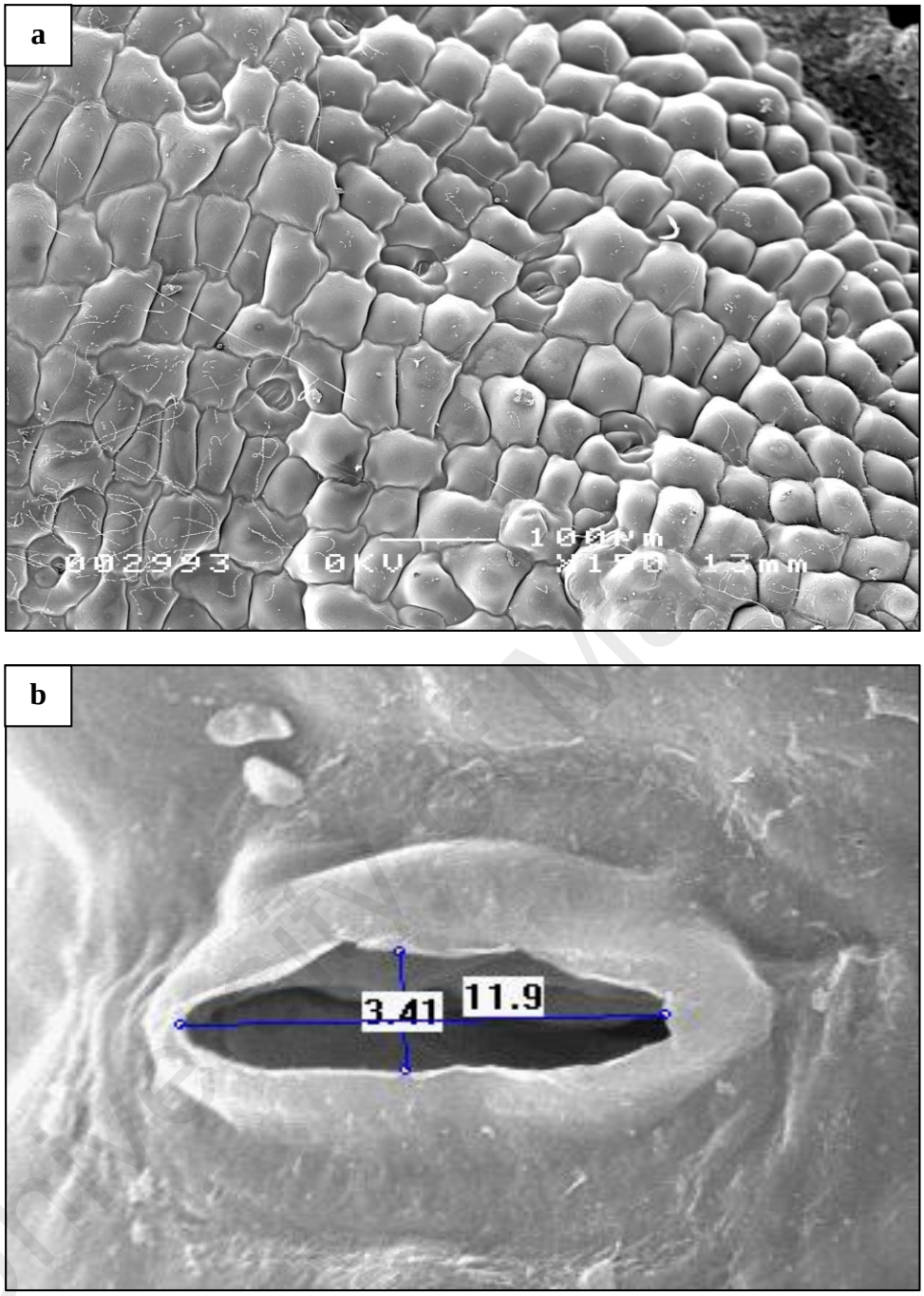


Figure 3.16: (a) SEM micrograph (Magnification: X 150) illustrated the stomata scattered on smooth epidermis surface of *Echinocereus cinerascens* (*in vivo* sample). (b) SEM micrograph showed the measurement of stoma opening (Length = 11.90 µm, Width = 3.41µm).

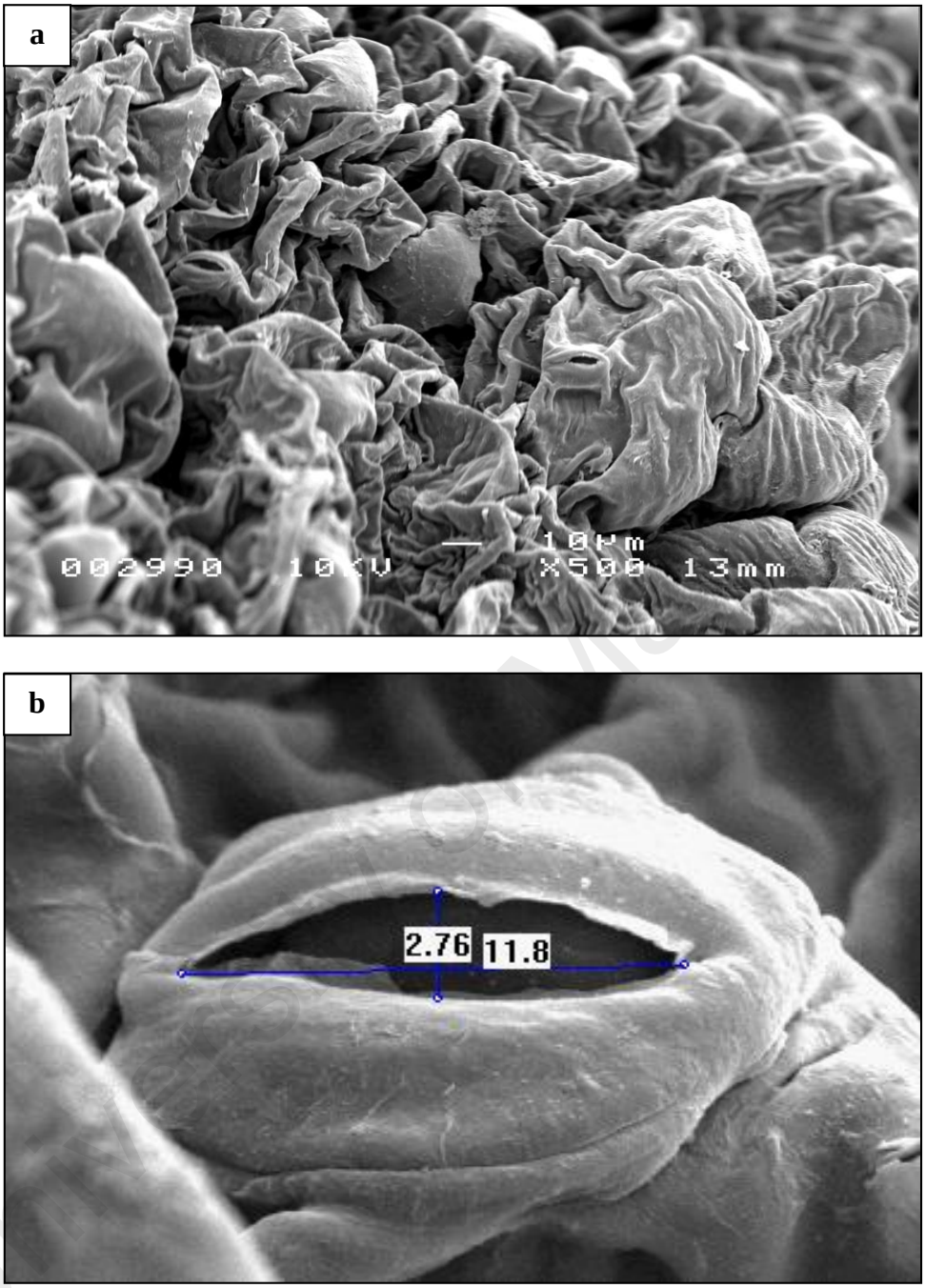


Figure 3.17: (a) SEM micrograph (Magnification: X 500) illustrated the stomata scattered on wrinkle epidermis surface of *Echinocereus cinerascens* (*in vitro* sample). (b) SEM micrograph showed the measurement of stoma opening (Length = 11.80 µm, Width = 2.76 µm).

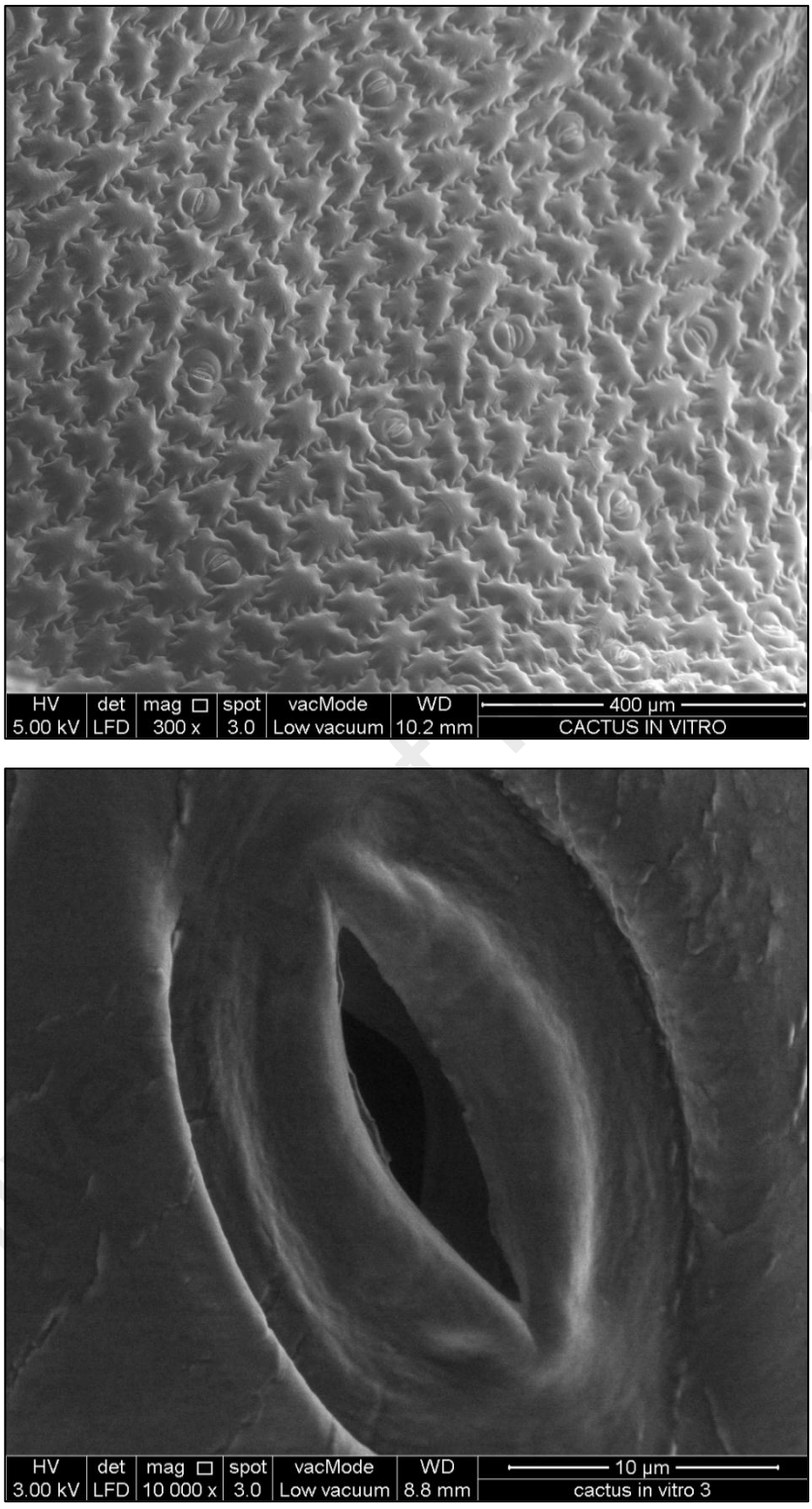
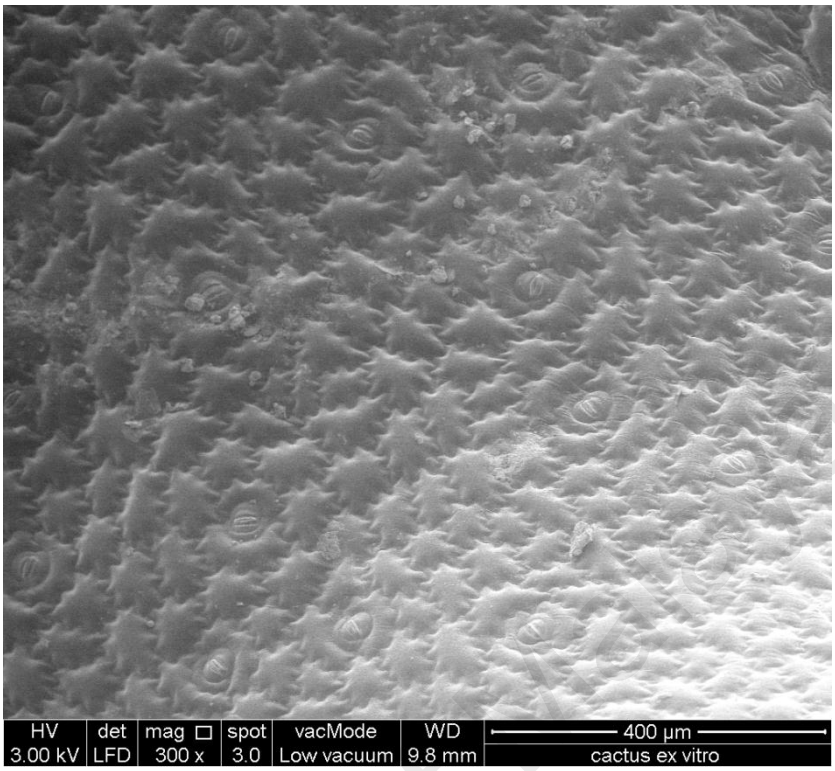


Figure 3.18 (a): FESEM micrograph (Magnification: X 300) illustrated the epidermis surface view of *in vitro* sample with the mean number of stomata per area (mm2) = 14.77, mean size of stomata = 25.20 µm, mean length and width of stomata opening = 17.87 µm, 2.94 µm.



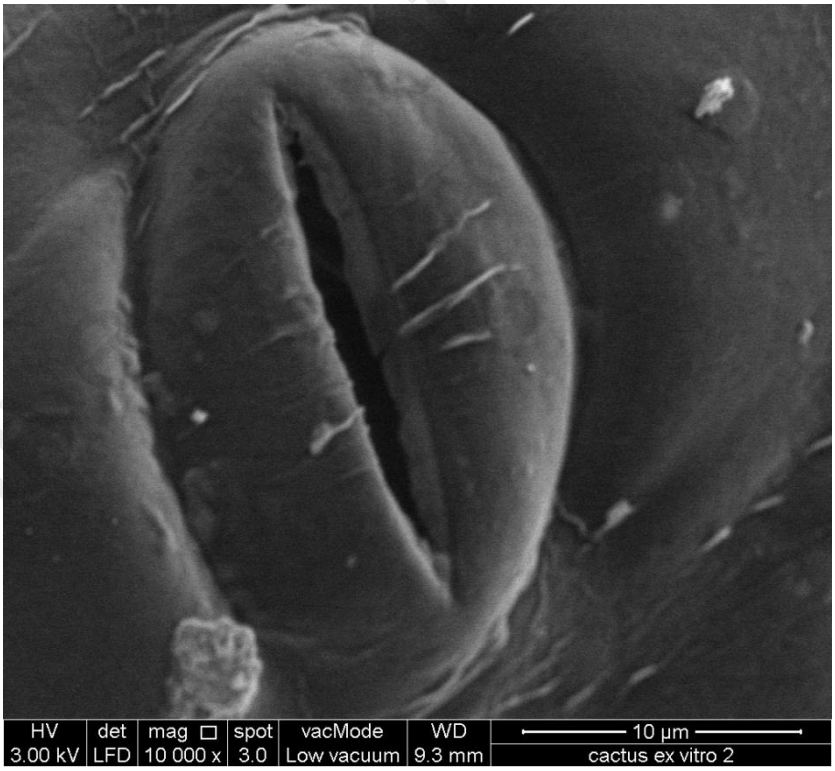


Figure 3.18 (b): FESEM micrograph (Magnification: X 300) illustrated the surface view of *ex vitro* sample with the mean number of stomata per area (mm2) = 15.45, mean size of stomata = 26.12 µm, mean length and width of stomata opening = 14.96 µm, 2.41 µm.

3.4 SUMMARY OF RESULTS

1. Direct *in vitro* regeneration of *Echinocereus cinerascens* was successfully achieved as the stem explants developed and produced shoots directly in most of the media tested.
2. MS medium supplemented with 2.0 mg/l Kinetin + 1.0 mg/l IBA was considered as the optimum medium for direct *in vitro* regeneration which produced the highest mean number of shoots (4.37 ± 0.27) that gave 131 shoots in total after 4 months.
3. Mean number of shoots produced in MS medium added with hormone combinations (Kinetin + IBA, NAA + BAP) was higher compared to MS medium treated with hormones applied singly (Kinetin, BAP, IBA, NAA).
4. The greatest shoot height (2.72 ± 0.43) was observed in MS medium supplemented with 2.0 mg/l Kinetin + 0.5 mg/l IBA whereas the greatest shoot diameter (1.30 ± 0.05) was observed in MS supplemented with 2.0 mg/l Kinetin.
5. Analysis on the production of shoots monthly indicated that media added with hormones applied singly such as BAP and Kinetin produced higher number of shoots after 1 month. In contrast, media added with hormone combinations, NAA + BAP and Kinetin + IBA produced higher number of shoots after 2 and the following months.
6. Ultrastructural analysis showed similar structure of apical shoot, vascular bundles, starch grains, areoles, ribs, spines and type of stomata for *in vivo, in vitro* and *ex vitro* samples examined.
7. Mean number of stomata per area observed in *ex vitro* samples (15.45 ± 1.46) was slightly higher than *in vitro* samples (14.77 ± 2.27).
8. Similarly, mean size of stomata observed in *ex vitro* samples (26.12 ± 1.10) was slightly higher compared to *in vitro* samples (25.20 ± 1.83).

9. In contrast, mean length and width of stomata opening for *in vitro* samples (length =17.87 ± 1.67, width = 2.94 ± 0.30) was slightly higher than *ex vitro* samples (length = 14.96 ± 1.04, width = 2.41 ± 0.33).

10. Even though the results promoted slightly different in mean number of stomata per area, mean size of stomata, length and width of stomata opening but it was proven that there was no significant difference statistically among all the samples tested.

CHAPTER 4

SOMATIC EMBRYOGENESIS OF *Echinocereus cinerascens*

4.1 EXPERIMENTAL AIMS

Somatic embryogenesis, also known as nonzygotic embryogenesis is a process in which the embryo that is genetically identical to the parent plant is produced without prior fertilization process. This somatic embryo which looks like zygotic embryo structure is generated from somatic or vegetative cell (Deo *et al*., 2011). Previous research indicated that the anatomy and physiological features of somatic embryos are similar to zygotic embryos either through direct or indirect somatic embryogenesis (Cheng and Raghavan, 1985; Gray, 1992; Zimmerman, 1993).

Somatic embryogenesis is considered as multi-step regeneration process that is initiated from the formation of proembryogenic masses, then followed by somatic embryo formation, maturation, desiccation and plant regeneration (Von Arnold *et al*., 2002). According to Zimmerman (1993), somatic embryos undergo several phases of development such as globular, heart, torpedo and cotyledonary stages prior to production of microshoots. The key stages of embryo development of somatic embryogenesis are similar to zygotic embryogenesis (Sharma and Millam, 2004).

Several factors that influence the induction of somatic embryogenesis and subsequent plant development include exogenous PGRs applied in the culture medium, types of explant used, culture medium, physical and environmental factors and others. Early studies on somatic embryogenesis reported that *Daucus carota* cell suspension cultures treated with coconut milk did not continue to multiply but differentiated into miniature embryo-like structure (Steward *et al*., 1958). In the meantime, Reinert (1959) independently discovered similar results in *Daucus carota* treated with high auxin concentrations in agar as the inducing material.

Several studies have been reported on the induction of somatic embryogenesis in cactus species mostly identified in solid medium including *Mediocactus coccineus* (Infante, 1992), *Ariocarpus retusus* (Stuppy and Nagl, 1992), *Turbinicarpus pseudomacrochele* (Torres-Muñoz and Rodríguez-Garay, 1996), *Mammillaria san-angelensis* (Marín-Hernández *et al*., 1998), *Opuntia ficus-indica* (Da Costa *et al*., 2001), *Ariocarpus kotschoubeyanus* (Moebius-Goldammer *et al*., 2003), *Copiapoa tenussima* (Lema-Rumińska *et al*., 2013) and some in liquid medium such as in *Aztekium ritteri* (Santacruz-Ruvalcaba *et al*., 1998) *Schlumbergera truncata* (Al-Ramamneh *et al*., 2006) which subsequently transferred to solid medium for somatic embryos germination or development.

In this chapter, somatic embryogenesis of *Echinocereus cinerascens* was induced in both solid and liquid medium using stem (for direct somatic embryogenesis) and callus (for indirect somatic embryogenesis) explants. The explants were cultured in media supplemented with various combinations and concentrations of hormones (BAP, 2,4-D) and vitamin (thiamine HCl) to establish the optimum medium for induction of somatic embryogenesis of *Echinocereus cinerascens*. In direct somatic embryogenesis, early detection of somatic embryos was observed by the formation of globular, heart, torpedo-shaped and cotyledonary stage. In contrast, early detection of indirect somatic embryogenesis was detected by the formation of embryogenic callus which could be distinguished through double staining technique (Gupta and Durzan, 1987). The embryogenic callus will further developed into globular, heart, torpedo-shaped and cotyledonary stage. Somatic embryos were then grow into plantlets as subjected into MS basal medium. In this study, parameters recorded including the mean production of somatic embryos (%) and also total mean production of somatic embryos monthly (%).

4.2 MATERIALS AND METHODS

4.2.1 Source of Explants

Explant sources used in the induction of somatic embryos studies were standardized. In solid culture, age (6-month-old plantlets) and size (6 cm height and 1 cm diameter) of explant sources used were similar to the *in vitro* regeneration studies with some modifications as the explants were peeled before sectioning into square size. This procedure was quite difficult since there would be slippery conditions with the sticky mucilage produced by the explants. Small pieces of explants were transferred cautiously to avoid injury as the explants became more friable and the explants were cultured on the media prepared in universal sterile containers. In liquid or suspension culture, embryogenic callus (6-month-old) was used as the explant sources and cultured in the liquid media prepared.

4.2.2 Preparation of Culture Media

Preparation of culture medium for the induction of somatic embryogenesis basically followed the procedures in *in vitro* regeneration studies. Generally, to prepare 1 liter medium, 800 ml of distilled water was filled into 1 liter conical flask. Then, 4.4 g/l of MS medium including vitamin (commercial powder) and 30 g/l of sucrose were added to the conical flask. The conical flask was placed on the hot plate and the medium solution was stirred constantly with a magnetic stirrer to allow the chemicals homogenized or dissolved in the solution. Next, the conical flask was refilled with distilled water up to 1 liter before the pH of the medium solution was adjusted to 5.7 with either 1.0 N sodium hydroxide (NaOH) or 1.0 N hydrochloride acid (HCl). For the preparation of liquid culture, the medium solution was autoclaved for 20 minutes at temperature of 121 °C, pressure of 104 kPa (15 psi) subsequently after synthetic hormones were added. For solid culture, the medium solution was solidified with 8 g/l agar technical (Agar No.3) and then added with selected synthetic hormones before

autoclaved. After autoclaved, sterilized medium was transferred to the laminar air flow cabinet and 20 ml were dispensed into each of 60 ml sterile universal container.

4.2.3 Culture Conditions

Culture conditions for the induction of somatic embryos completely similar to the procedures in *in vitro* regeneration studies. All the apparatus essential for culturing such as forceps, scalpels, jam jars, conical flask and etc. were autoclaved for 20 minutes to sterile the apparatus. Besides, UV light in the laminar flow cabinet was turned on for 15 minutes to provide aseptic culture conditions while the laminar flow cabinet surfaces were disinfected with 70% ethanol before culturing started. All explants cultured in the solid medium were allowed to develop and maintained in the culture room at 25 ± 2 °C under 16 hours light provided by cool daylight or fluorescent light (36W), PHILIPS. Meanwhile, the explants cultured in liquid medium were placed on a shaker (New Brunswick Scientific G-10 Gyrotory Shaker) adjusted to 100 rpm in the culture room with the same conditions.

4.2.4 Induction of Somatic Embryogenesis in Solid and Liquid Medium

The effects of 2,4-D, BAP and Thiamine HCl applied in combinations were studied to obtain the optimum medium in the induction of somatic embryos for indirect regeneration of the species *in vitro*. Stages of somatic embryos including globular-shaped, heart-shaped, torpedo-shaped and cotyledonary stage were observed both in the solid and liquid medium. Percentage of the production of proembryo masses (PEM), globular-shaped (G), heart-shaped (H), all stages (AS) recorded monthly. In this study, thirty explants were cultured and tested for each treatment.

4.2.4.1 Effects of 2,4-D Applied in Combinations of BAP or Thiamine HCl (solid medium)

All treatments or concentrations of 2,4-D applied in combinations of BAP or thiamine HCl tested throughout this study were prepared as listed below;

1. MS basal (medium without hormone/ control)
2. MS + 0.1 mg/l 2,4-D + 0.1 mg/l BAP
3. MS + 0.1 mg/l 2,4-D + 0.5 mg/l BAP
4. MS + 0.1 mg/l 2,4-D + 0.5 mg/l thiamine HCl
5. MS + 0.5 mg/l 2,4-D + 0.1 mg/l BAP
6. MS + 0.5 mg/l 2,4-D + 0.5 mg/l BAP
7. MS + 0.5 mg/l 2,4-D + 0.5 mg/l thiamine HCl
8. MS + 1.0 mg/l 2,4-D + 0.1 mg/l BAP
9. MS + 1.0 mg/l 2,4-D + 0.5 mg/l BAP
10. MS + 1.0 mg/l 2,4-D + 0.1 mg/l thiamine HCl
11. MS + 1.5 mg/l 2,4-D + 0.1 mg/l BAP
12. MS + 1.5 mg/l 2,4-D + 0.5 mg/l BAP
13. MS + 1.5 mg/l 2,4-D + 0.5 mg/l thiamine HCl

4.2.4.2 Effects of 2,4-D, BAP and Thiamine HCl Applied in Combinations (liquid medium)

All treatments or concentrations of 2,4-D and BAP applied in combinations with the addition of thiamine HCl tested throughout this study were prepared as listed below;

1. MS basal (medium without hormone/ control)
2. MS + 0.1 mg/l 2,4-D + 0.1 mg/l BAP + 0.1 mg/l thiamine HCl
3. MS + 0.1 mg/l 2,4-D + 0.1 mg/l BAP + 0.5 mg/l thiamine HCl
4. MS + 0.1 mg/l 2,4-D + 0.5 mg/l BAP + 0.1 mg/l thiamine HCl
5. MS + 0.1 mg/l 2,4-D + 0.5 mg/l BAP + 0.5 mg/l thiamine HCl

6. MS + 0.1 mg/l 2,4-D + 1.0 mg/l BAP + 0.1 mg/l thiamine HCl
7. MS + 0.1 mg/l 2,4-D + 1.0 mg/l BAP + 0.5 mg/l thiamine HCl
8. MS + 0.5 mg/l 2,4-D + 0.1 mg/l BAP + 0.1 mg/l thiamine HCl
9. MS + 0.5 mg/l 2,4-D + 0.5 mg/l BAP + 0.1 mg/l thiamine HCl
10. MS + 0.5 mg/l 2,4-D + 1.0 mg/l BAP + 0.1 mg/l thiamine HCl
11. MS + 0.5 mg/l 2,4-D + 0.1 mg/l BAP + 0.5 mg/l thiamine HCl
12. MS + 0.5 mg/l 2,4-D + 0.5 mg/l BAP + 0.5 mg/l thiamine HCl
13. MS + 0.5 mg/l 2,4-D + 1.0 mg/l BAP + 0.5 mg/l thiamine HCl
14. MS + 1.0 mg/l 2,4-D + 0.1 mg/l BAP + 0.1 mg/l thiamine HCl
15. MS + 1.0 mg/l 2,4-D + 0.5 mg/l BAP + 0.1 mg/l thiamine HCl
16. MS + 1.0 mg/l 2,4-D + 1.0 mg/l BAP + 0.1 mg/l thiamine HCl
17. MS + 1.0 mg/l 2,4-D + 0.1 mg/l BAP + 0.5 mg/l thiamine HCl
18. MS + 1.0 mg/l 2,4-D + 0.5 mg/l BAP + 0.5 mg/l thiamine HCl
19. MS + 1.0 mg/l 2,4-D + 1.0 mg/l BAP + 0.5 mg/l thiamine HCl

4.2.5 Identification of Embryogenic Callus

Embryogenic callus could be easily distinguished and differentiated from non embryogenic callus through double staining technique (Gupta and Durzan, 1987). Initially, a small piece of callus (0.2 – 0.5 cm) was placed on a clean glass slide. After that, 2% acetocarmine solution (2-3 drops) was dropped onto the callus. The callus was gently divided into very small pieces using needles and subsequently heated over a low flame for a few seconds. Heating for too long may destroy the cells. Next, the callus were carefully washed with distilled water (2-3 times) and then the slide was rinsed to remove all liquid before 0.5% Evan's blue solution (2-3 drops) was dropped onto acetocarmine stained cells. After 30 seconds, the callus and slide were rinsed again with distilled water (2-3 times) to remove all excess liquid. Lastly, a few drops of glycerol

(1-2 drops) were added to the stained cells (essential to prevent the cells from drying). Cover slip was applied before the slide was observed under a light microscope.

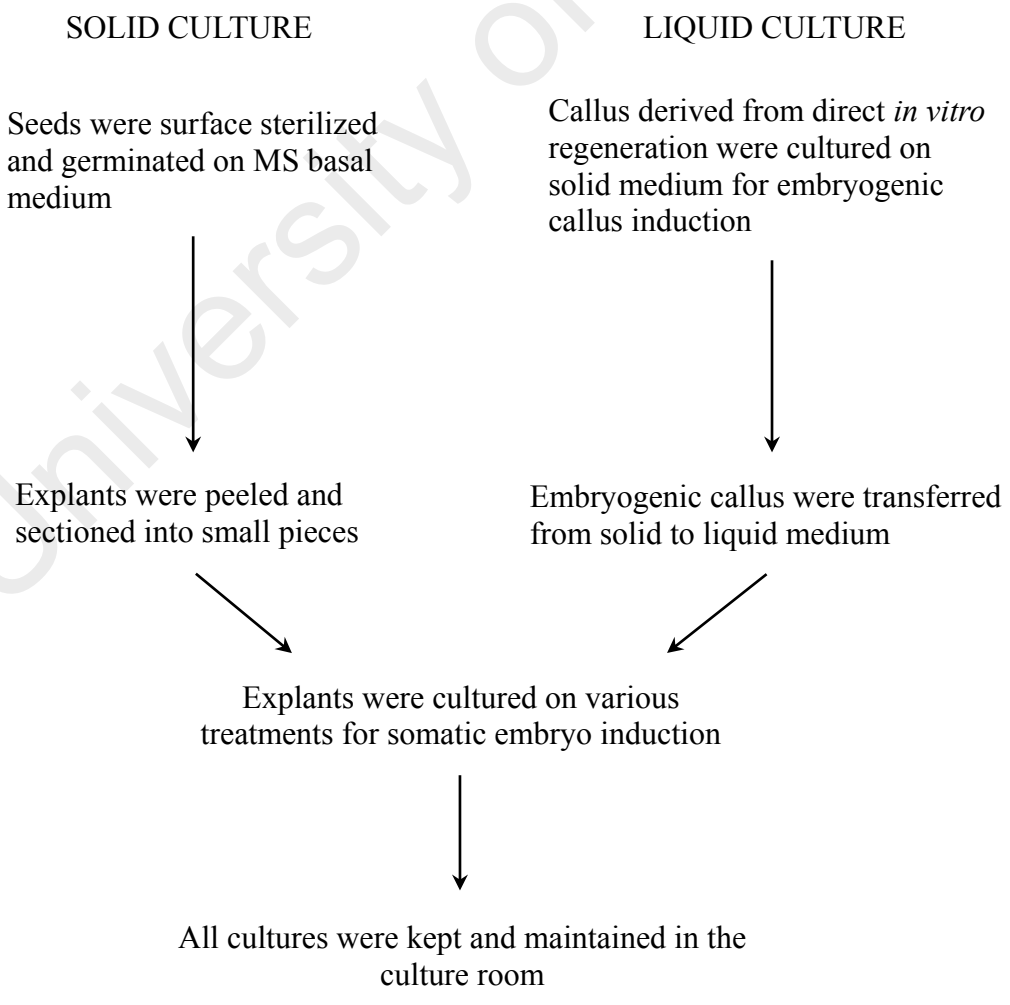
4.2.6 Development of Plantlets from Somatic Embryos

Somatic embryos produced (liquid culture) were subcultured in MS basal medium (solid culture) to further development into plantlets. Only somatic embryos from liquid culture were selected and subcultured in MS basal medium as the induction of somatic embryos in the solid medium was inefficient.

4.2.7 Data Analysis

Data obtained were statistically analysed using Duncan's Multiple Range Test (DMRT). Means with different letters in the same column differ significantly at p=0.01.

4.2.8 Experimental Outline



4.3 RESULTS

Somatic embryogenesis could be induced direct or indirectly through solid or liquid/ suspension cultures. In the induction of somatic embryogenesis, stem explants which cultured on solid medium produced somatic embryos directly. Meanwhile, when callus explants were cultured on liquid medium, indirect somatic embryogenesis was observed. Somatic embryos produced indirectly were subjected to MS basal medium for further development into plantlets.

4.3.1 Effects of 2,4-D Applied in Combinations of BAP or Thiamine HCl (solid medium)

Direct somatic embryogenesis could be observed after 1 month of culture as several globular-shaped (Figure 4.1a) were produced directly from the explants cultured in a few treatments tested. Treatments which showed the production of globular after 1 month include MS medium supplemented with 0.5 mg/l 2,4-D + 0.5 mg/l thiamine HCl, MS medium supplemented with 1.0 mg/l 2,4-D + 0.1 mg/l thiamine HCl and MS medium supplemented with 1.0 mg/l 2,4-D + 0.1 mg/l BAP that produced 33%, 27% and 13% of globular, respectively (Table 4.1).

Somatic embryogenesis increased drastically after 2 months where the observations reported that almost half of the treatments tested showed the production of globular and heart-shaped (Figure 4.1b). Production of globular (Figure 4.2a) and heart-shaped (Figure 4.2b) could be seen clearly in media added with combination of (0.5, 1.0, 1.5 mg/l) 2,4-D and (0.1, 0.5 mg/l) BAP or thiamine HCl (Table 4.1). Even though early detection after 1 month showed that the highest production of globular was 33%, however after 2 months the production of globular and heart-shaped recorded in the same treatment was 93%. The highest production of globular and heart-shaped after 2 months was 100% observed in MS medium supplemented with 1.0 mg/l 2,4-D + 0.1

mg/l thiamine HCl which showed no significant difference with 93% production of globular in MS medium supplemented with 0.5 mg/l 2,4-D + 0.5 mg/l thiamine HCl.

Total mean production of somatic embryos recorded after 1 month was in the range of 13 – 33% while 13 – 100% was obtained after 2 months (Figure 4.3). Nevertheless, further observations discovered unsatisfactory results as slow growth performance of globular and heart-shaped were observed after 4 months of culture.

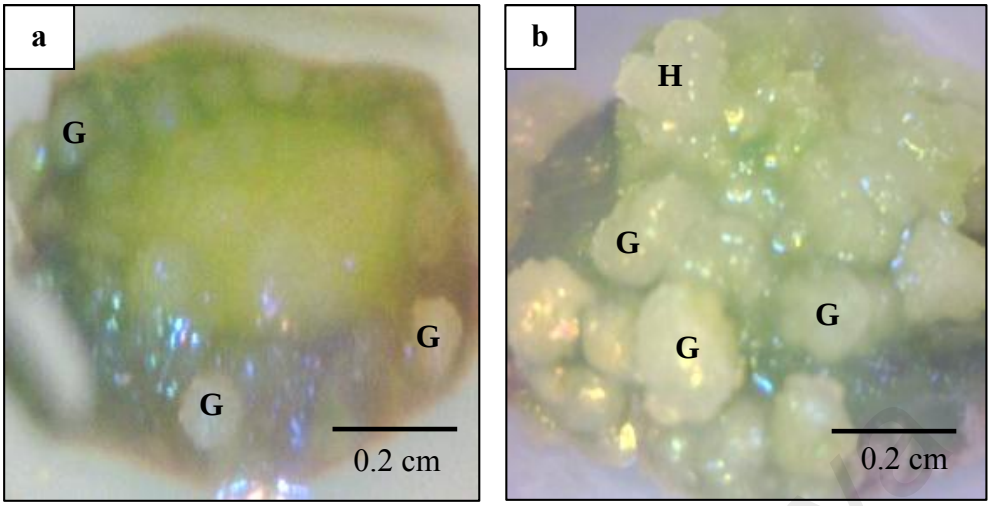


Figure 4.1: (a) Globular-shaped, G formed in solid medium after 1 month of culture. (b) Two stages (Globular, G and heart-shaped, H) of somatic embryos formed in solid medium after 2 months of culture.

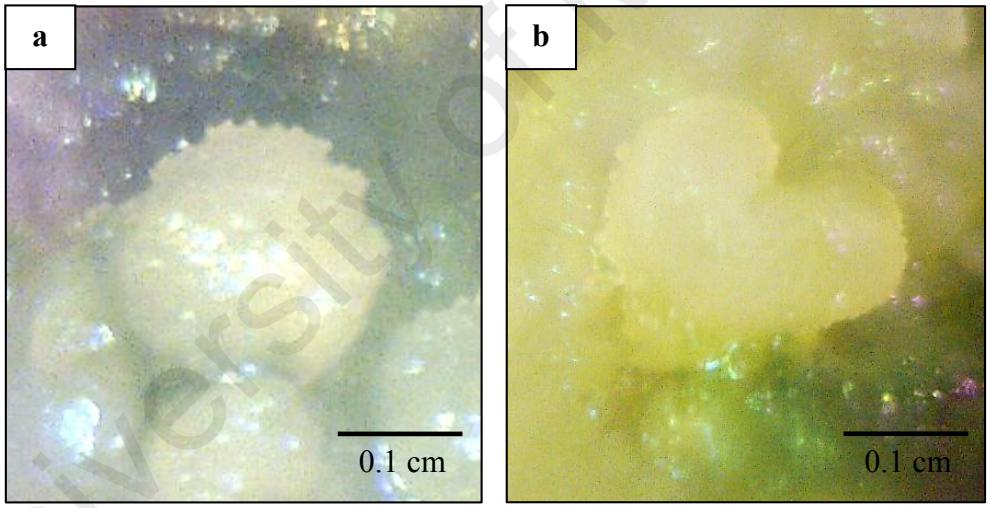


Figure 4.2: (a) Globular and (b) heart-shaped observed on MS medium + 0.5 mg/l 2,4-D + 0.5 mg/l thiamine HCl after 2 months of culture.

Table 4.1: Induction of somatic embryogenesis in solid medium (after 1 and 2 months of culture).

MS + Hormones (mg/l)	1 Month	2 Months
	Mean (% G) ± SE	Mean (% G + H) ± SE
control	0 ± 0.00 c	0 ± 0.00 c
0.1 2,4-D + 0.1 BAP	0 ± 0.00 c	0 ± 0.00 c
0.1 2,4-D + 0.5 BAP	0 ± 0.00 c	0 ± 0.00 c
0.1 2,4-D + 0.5 thiamine HCl	0 ± 0.00 c	0 ± 0.00 c
0.5 2,4-D + 0.1 BAP	0 ± 0.00 c	23 ± 0.08 b
0.5 2,4-D + 0.5 BAP	0 ± 0.00 c	0 ± 0.00 c
0.5 2,4-D + 0.5 thiamine HCl	33 ± 0.09 a	93 ± 0.05 a
1.0 2,4-D + 0.1 BAP	13 ± 0.06 bc	13 ± 0.06 bc
1.0 2,4-D + 0.5 BAP	0 ± 0.00 c	0 ± 0.00 c
1.0 2,4-D + 0.1 thiamine HCl	27 ± 0.08 ab	100 ± 0.00 a
1.5 2,4-D + 0.1 BAP	0 ± 0.00 c	23 ± 0.08 b
1.5 2,4-D + 0.5 BAP	0 ± 0.00 c	0 ± 0.00 c
1.5 2,4-D + 0.5 thiamine HCl	0 ± 0.00 c	13 ± 0.06 bc

Data represents mean value ± standard error (SE) with 30 explants in each treatment. Means with different letters in the same column are significantly different at p=0.01 according to Duncan's multiple range test (DMRT). G, Globular-shaped; H, Heart-shaped.

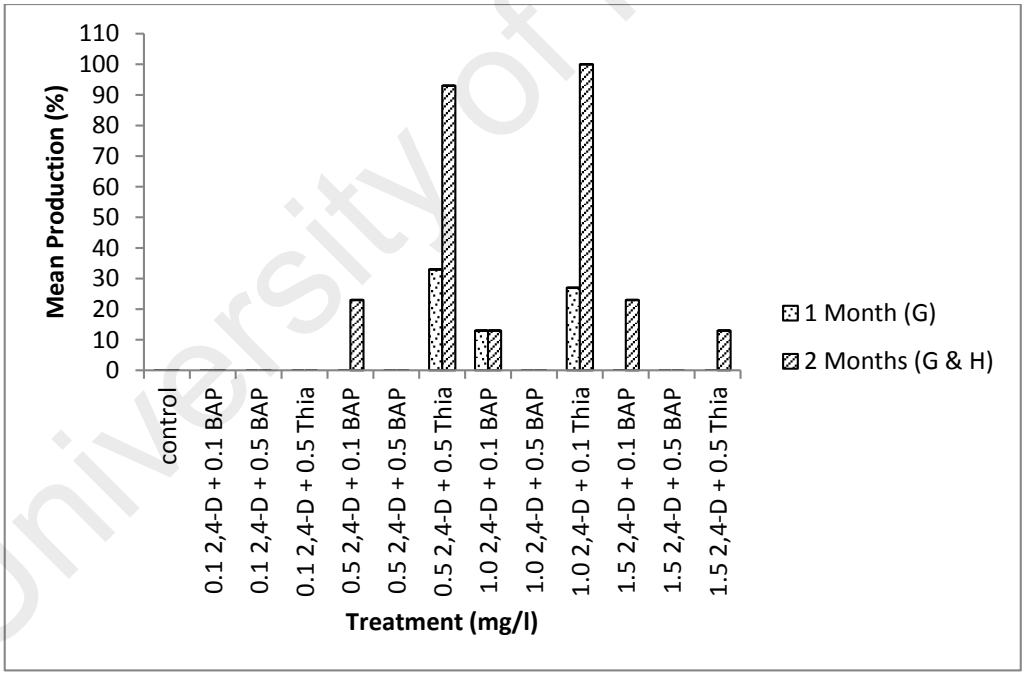


Figure 4.3: Total mean production of somatic embryos (globular and heart-shaped) in solid medium (after 1 and 2 months of culture).

4.3.2 Effects of 2,4-D, BAP and Thiamine HCl Applied in Combinations (liquid medium)

Non embryogenic callus derived from *in vitro* regeneration studies were subcultured on MS basal for a few months before subjected to the induction medium of somatic embryogenesis. This intermediate step is essential to neutralize effects of plant growth regulators that were applied previously. Non embryogenic callus that undergoes cell division and proliferation in the induction media for 6 months were tested using double staining method, the simplest technique to distinguish the presence of embryogenic callus. Observations under light microscope verified that the green and friable structure of callus developed in the induction medium of somatic embryogenesis were embryogenic callus. Embryogenic cells could be detected by an intense bright red stained (acetocarmine) of large nuclei and dense cytoplasm (Figure 4.4) whereas non embryogenic cells could be identified by characteristics such as very small nuclei and the whole cells stained blue (Evan's blue).

The embryogenic callus (Figure 4.5a) was transferred to liquid culture in the same induction medium to further growth. After a few months, the embryogenic callus proliferated and developed into more advanced structures (Figure 4.5b). Then, the callus was subsequently distributed and cultured on various media tested (liquid medium). Among all media tested, MS medium supplemented with 0.1, 0.5, 1.0 mg/l 2,4-D + 0.1, 0.5, 1.0 mg/l BAP + 0.1, 0.5 mg/l thiamine HCl gave positive response which showed the production of proembryo masses in liquid medium after 1 month (Table 4.2). The production of proembryo masses is an indicator for early stage production of globular-shaped. Total mean production of proembryo masses after 1 month was in the range of 33 – 67% (Figure 4.6).

Production of indirect somatic embryos increased after 2 months. However, it was found that only treatments that gave positive response after 1 month showed the production of proembryo masses, globular and all stages of somatic embryos. All stages of somatic embryos include the production of globular, heart, torpedo-shaped and cotyledonary stage (Figure 4.7). After 2 months, the treatments produced about 27 – 47% of proembryo masses, 3 – 20% of globulr-shaped and 20 – 23% of somatic embryos in all stages (Table 4.3). Though proembryo masses and globular-shaped could be observed in these treatments, however all stages of somatic embryos (Figure 4.8) could only be seen clearly in two treatments such as MS medium supplemented with 0.5 mg/l 2,4-D + 0.1 mg/l BAP + 0.5 mg/l thiamine HCl and MS medium supplemented with 0.5 mg/l 2,4-D + 0.5 mg/l BAP + 0.5 mg/l thiamine HCl that gave higher total mean production of somatic embryos, 90% and 73% respectively (Figure 4.9).

Indirect somatic embryogenesis that observed after 2 and 3 months were quite similar with slightly different in the production of somatic embryos. After 3 months, somatic embryos further proliferation and showed the production of proembryo masses in the range of 30 – 40%, while globular-shaped with 7 – 20% and all stages of somatic embryos about 33 – 37% (Table 4.4). Besides, higher total mean production of somatic embryos after 3 months, 91% and 83% (Figure 4.10) were detected on the same treatments. Analysis total mean production of somatic embryos verified that somatic embryogenesis was slightly increased after 3 months (Figure 4.10).

Indirect somatic embryogenesis gradually increased after 4 months and almost all treatments gave positive response with nearly 50% production of proembryo masses as well as the production of all stages of somatic embryos (Figure 4.11). Additionally, a few treatments showed approximately 20% production of globular. Besides, results after 4 months promoted the production of proembryo masses in the range of 33 – 50% whereas 40% and 47% were observed in the production of all stages of somatic embryos

(Table 4.5). Meanwhile, the production of globular was quite low which in the range of 7 – 20% (Table 4.5). Nevertheless, total mean production of somatic embryos was 100% after 4 months that obtained in both treatments such as MS medium supplemented with 0.5 mg/l 2,4-D + 0.1 mg/l BAP + 0.5 mg/l thiamine HCl and MS medium supplemented with 0.5 mg/l 2,4-D + 0.5 mg/l BAP + 0.5 mg/l thiamine HCl, which indicated the highest total mean production of somatic embryos (Figure 4.12). On the other hand, other treatments showed that more than 50% production of somatic embryos (Figure 4.12).

4.3.3 Development of Plantlets from Somatic Embryos

Early stage of globular could be observed with the formation of proembryo masses on the callus surface that cultured in several liquid media tested. The proembryo masses further growth and cell division to form globular-shaped which subsequently developed into heart, torpedo-shaped and finally cotyledonary stage. Further development into plantlets (Figure 4.13) was achieved once somatic embryos were transferred into MS basal medium.

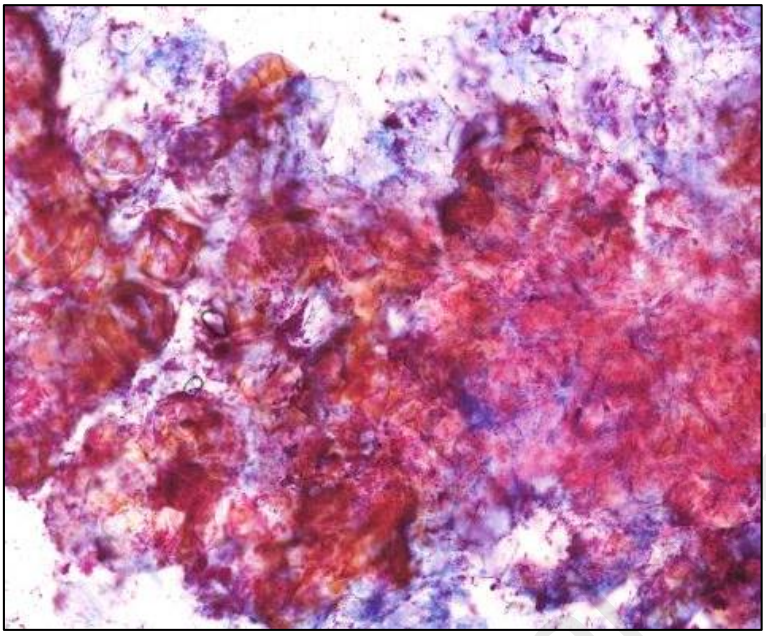


Figure 4.4: Embryogenic cells promoted bright red stained of large nuclei as tested with double staining technique observed under a light microscope (Magnification: X 50).

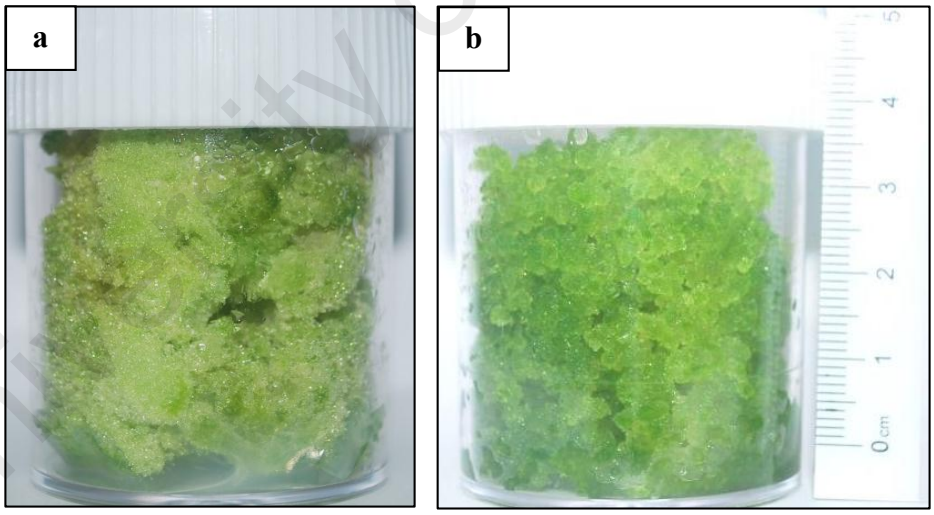


Figure 4.5: (a) Embryogenic callus with friable structure induced in solid medium (b) developed into more advanced structures in liquid medium (MS medium + 0.5 mg/l 2,4-D + 0.5 mg/l thiamine HCl).

Table 4.2: Induction of somatic embryogenesis in liquid medium (after 1 month of culture).

MS + Hormones (mg/l)	1 Month
	Mean (% PEM) ± SE
Control	0 ± 0.00 c
0.1 2,4-D + 0.1 BAP + 0.1 thiamine HCl	40 ± 0.09 b
0.1 2,4-D + 0.1 BAP + 0.5 thiamine HCl	0 ± 0.00 c
0.1 2,4-D + 0.5 BAP + 0.1 thiamine HCl	0 ± 0.00 c
0.1 2,4-D + 0.5 BAP + 0.5 thiamine HCl	0 ± 0.00 c
0.1 2,4-D + 1.0 BAP + 0.1 thiamine HCl	33 ± 0.09 b
0.1 2,4-D + 1.0 BAP + 0.5 thiamine HCl	0 ± 0.00 c
0.5 2,4-D + 0.1 BAP + 0.1 thiamine HCl	0 ± 0.00 c
0.5 2,4-D + 0.5 BAP + 0.1 thiamine HCl	0 ± 0.00 c
0.5 2,4-D + 1.0 BAP + 0.1 thiamine HCl	0 ± 0.00 c
0.5 2,4-D + 0.1 BAP + 0.5 thiamine HCl	67 ± 0.09 a
0.5 2,4-D + 0.5 BAP + 0.5 thiamine HCl	63 ± 0.09 a
0.5 2,4-D + 1.0 BAP + 0.5 thiamine HCl	0 ± 0.00 c
1.0 2,4-D + 0.1 BAP + 0.1 thiamine HCl	0 ± 0.00 c
1.0 2,4-D + 0.5 BAP + 0.1 thiamine HCl	37 ± 0.09 b
1.0 2,4-D + 1.0 BAP + 0.1 thiamine HCl	0 ± 0.00 c
1.0 2,4-D + 0.1 BAP + 0.5 thiamine HCl	33 ± 0.09 b
1.0 2,4-D + 0.5 BAP + 0.5 thiamine HCl	0 ± 0.00 c
1.0 2,4-D + 1.0 BAP + 0.5 thiamine HCl	0 ± 0.00 c

Data represents mean value ± standard error (SE) with 30 explants in each treatment. Means with different letters in the same column are significantly different at p=0.01 according to Duncan's multiple range test (DMRT). PEM, Proembryo masses.

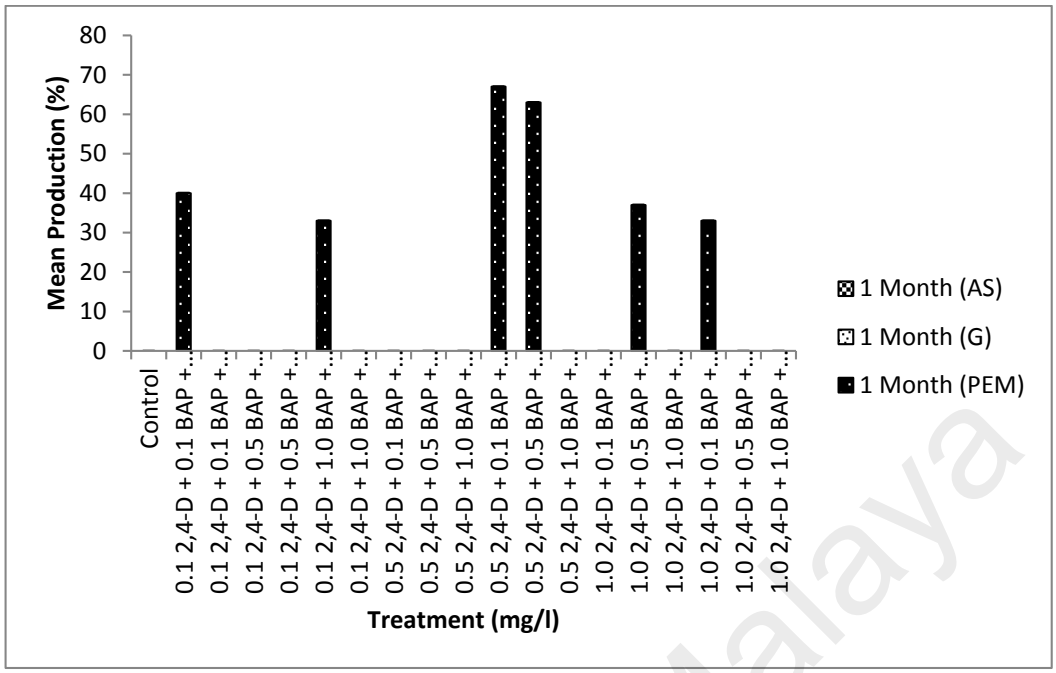


Figure 4.6: Total mean production of somatic embryos (proembryo masses) in liquid medium (after 1 month of culture).

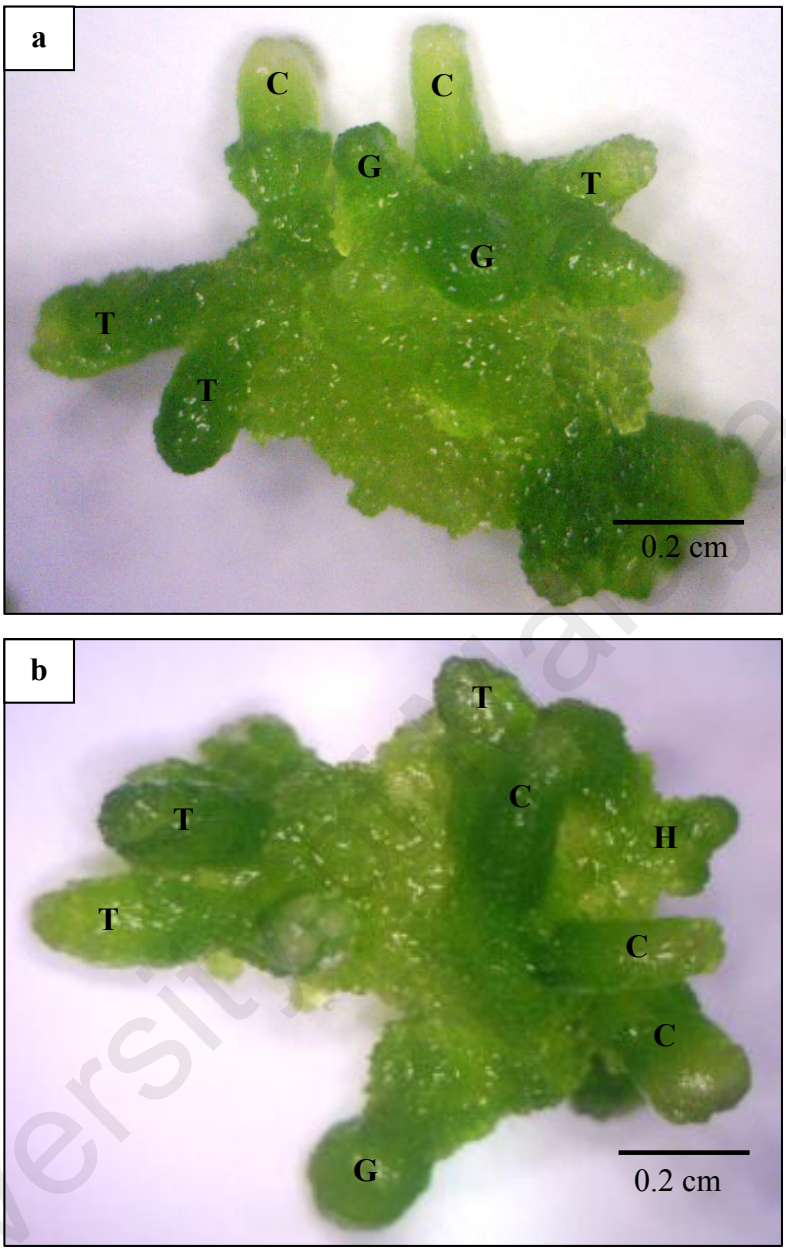


Figure 4.7: (a) and (b) All stages (globular, G; heart, H; torpedo-shaped, T and cotyledonary stage, C) of somatic embryos formed in liquid medium after 2 months of culture.

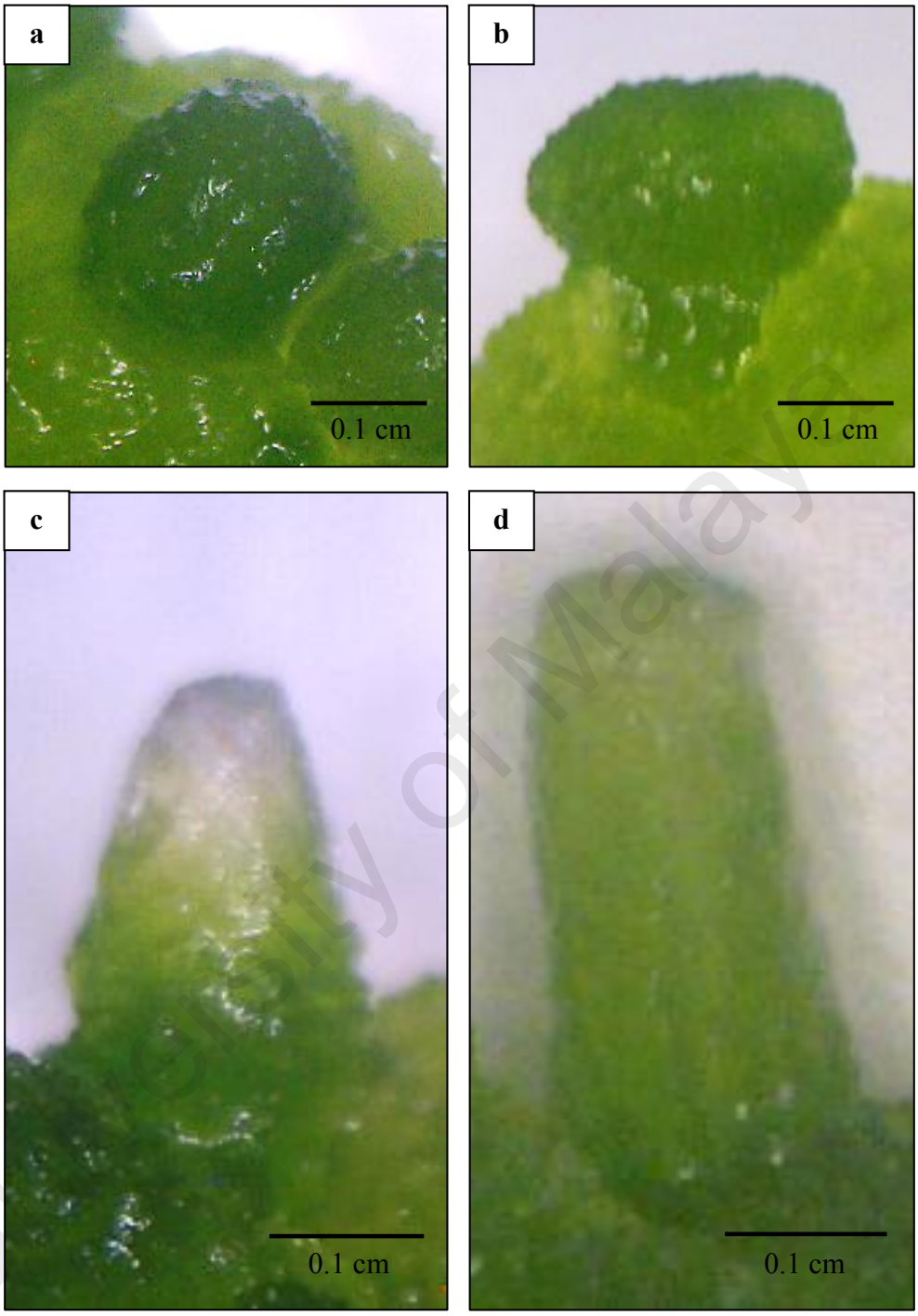


Figure 4.8: (a) Globular (b) heart (c) torpedo-shaped and (d) cotyledonary stage observed on MS medium + 0.5 mg/l 2,4-D + 0.1 mg/l BAP + 0.5 mg/l thiamine HCl after 2 months of culture.

Table 4.3: Induction of somatic embryogenesis in liquid medium (after 2 months of culture).

	2 Months		
MS + Hormones (mg/l)	Mean (% PEM) ± SE	Mean (% G) ± SE	Mean (% AS) ± SE
Control	0 ± 0.00 c	0 ± 0.00 b	0 ± 0.00 b
0.1 2,4-D + 0.1 BAP + 0.1 thia	33 ± 0.09 ab	13 ± 0.06 ab	0 ± 0.00 b
0.1 2,4-D + 0.1 BAP + 0.5 thia	0 ± 0.00 c	0 ± 0.00 b	0 ± 0.00 b
0.1 2,4-D + 0.5 BAP + 0.1 thia	0 ± 0.00 c	0 ± 0.00 b	0 ± 0.00 b
0.1 2,4-D + 0.5 BAP + 0.5 thia	0 ± 0.00 c	0 ± 0.00 b	0 ± 0.00 b
0.1 2,4-D + 1.0 BAP + 0.1 thia	27 ± 0.09 b	7 ± 0.05 b	0 ± 0.00 b
0.1 2,4-D + 1.0 BAP + 0.5 thia	0 ± 0.00 c	0 ± 0.00 b	0 ± 0.00 b
0.5 2,4-D + 0.1 BAP + 0.1 thia	0 ± 0.00 c	0 ± 0.00 b	0 ± 0.00 b
0.5 2,4-D + 0.5 BAP + 0.1 thia	0 ± 0.00 c	0 ± 0.00 b	0 ± 0.00 b
0.5 2,4-D + 1.0 BAP + 0.1 thia	0 ± 0.00 c	0 ± 0.00 b	0 ± 0.00 b
0.5 2,4-D + 0.1 BAP + 0.5 thia	47 ± 0.09 a	20 ± 0.07 a	23 ± 0.08 a
0.5 2,4-D + 0.5 BAP + 0.5 thia	33 ± 0.09 ab	20 ± 0.07 a	20 ± 0.07 a
0.5 2,4-D + 1.0 BAP + 0.5 thia	0 ± 0.00 c	0 ± 0.00 b	0 ± 0.00 b
1.0 2,4-D + 0.1 BAP + 0.1 thia	0 ± 0.00 c	0 ± 0.00 b	0 ± 0.00 b
1.0 2,4-D + 0.5 BAP + 0.1 thia	30 ± 0.09 ab	7 ± 0.05 b	0 ± 0.00 b
1.0 2,4-D + 1.0 BAP + 0.1 thia	0 ± 0.00 c	0 ± 0.00 b	0 ± 0.00 b
1.0 2,4-D + 0.1 BAP + 0.5 thia	30 ± 0.09 ab	3 ± 0.03 b	0 ± 0.00 b
1.0 2,4-D + 0.5 BAP + 0.5 thia	0 ± 0.00 c	0 ± 0.00 b	0 ± 0.00 b
1.0 2,4-D + 1.0 BAP + 0.5 thia	0 ± 0.00 c	0 ± 0.00 b	0 ± 0.00 b

Data represents mean value ± standard error (SE) with 30 explants in each treatment. Means with different letters in the same column are significantly different at p=0.01 according to Duncan's multiple range test (DMRT). thia, thiamine HCl; PEM, Proembryo masses; G, Globular; AS, All stages.

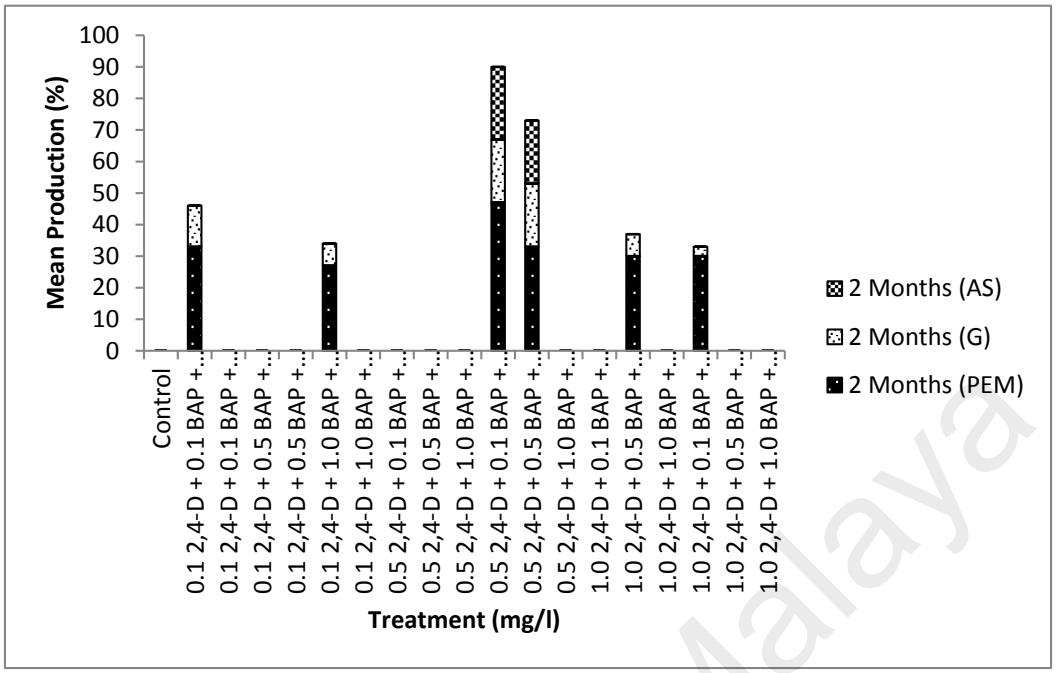


Figure 4.9: Total mean production of somatic embryos (proembryo masses, globular and all stages) in liquid medium (after 2 months of culture).

Table 4.4: Induction of somatic embryogenesis in liquid medium (after 3 months of culture).

	3 Months		
MS + Hormones (mg/l)	Mean (% PEM) ± SE	Mean (% G) ± SE	Mean (% AS) ± SE
Control	0 ± 0.00 b	0 ± 0.00 b	0 ± 0.00 b
0.1 2,4-D + 0.1 BAP + 0.1 thia	40 ± 0.09 a	13 ± 0.06 ab	0 ± 0.00 b
0.1 2,4-D + 0.1 BAP + 0.5 thia	0 ± 0.00 b	0 ± 0.00 b	0 ± 0.00 b
0.1 2,4-D + 0.5 BAP + 0.1 thia	0 ± 0.00 b	0 ± 0.00 b	0 ± 0.00 b
0.1 2,4-D + 0.5 BAP + 0.5 thia	0 ± 0.00 b	0 ± 0.00 b	0 ± 0.00 b
0.1 2,4-D + 1.0 BAP + 0.1 thia	30 ± 0.09 a	7 ± 0.05 ab	0 ± 0.00 b
0.1 2,4-D + 1.0 BAP + 0.5 thia	0 ± 0.00 b	0 ± 0.00 b	0 ± 0.00 b
0.5 2,4-D + 0.1 BAP + 0.1 thia	0 ± 0.00 b	0 ± 0.00 b	0 ± 0.00 b
0.5 2,4-D + 0.5 BAP + 0.1 thia	0 ± 0.00 b	0 ± 0.00 b	0 ± 0.00 b
0.5 2,4-D + 1.0 BAP + 0.1 thia	0 ± 0.00 b	0 ± 0.00 b	0 ± 0.00 b
0.5 2,4-D + 0.1 BAP + 0.5 thia	37 ± 0.09 a	17 ± 0.07 a	37 ± 0.09 a
0.5 2,4-D + 0.5 BAP + 0.5 thia	30 ± 0.09 a	20 ± 0.07 a	33 ± 0.09 a
0.5 2,4-D + 1.0 BAP + 0.5 thia	0 ± 0.00 b	0 ± 0.00 b	0 ± 0.00 b
1.0 2,4-D + 0.1 BAP + 0.1 thia	0 ± 0.00 b	0 ± 0.00 b	0 ± 0.00 b
1.0 2,4-D + 0.5 BAP + 0.1 thia	33 ± 0.09 a	7 ± 0.05 ab	0 ± 0.00 b
1.0 2,4-D + 1.0 BAP + 0.1 thia	0 ± 0.00 b	0 ± 0.00 b	0 ± 0.00 b
1.0 2,4-D + 0.1 BAP + 0.5 thia	33 ± 0.09 a	7 ± 0.05 ab	0 ± 0.00 b
1.0 2,4-D + 0.5 BAP + 0.5 thia	0 ± 0.00 b	0 ± 0.00 b	0 ± 0.00 b
1.0 2,4-D + 1.0 BAP + 0.5 thia	0 ± 0.00 b	0 ± 0.00 b	0 ± 0.00 b

Data represents mean value ± standard error (SE) with 30 explants in each treatment. Means with different letters in the same column are significantly different at p=0.01 according to Duncan's multiple range test (DMRT). thia, thiamine HCl; PEM, Proembryo masses; G, Globular; AS, All stages.

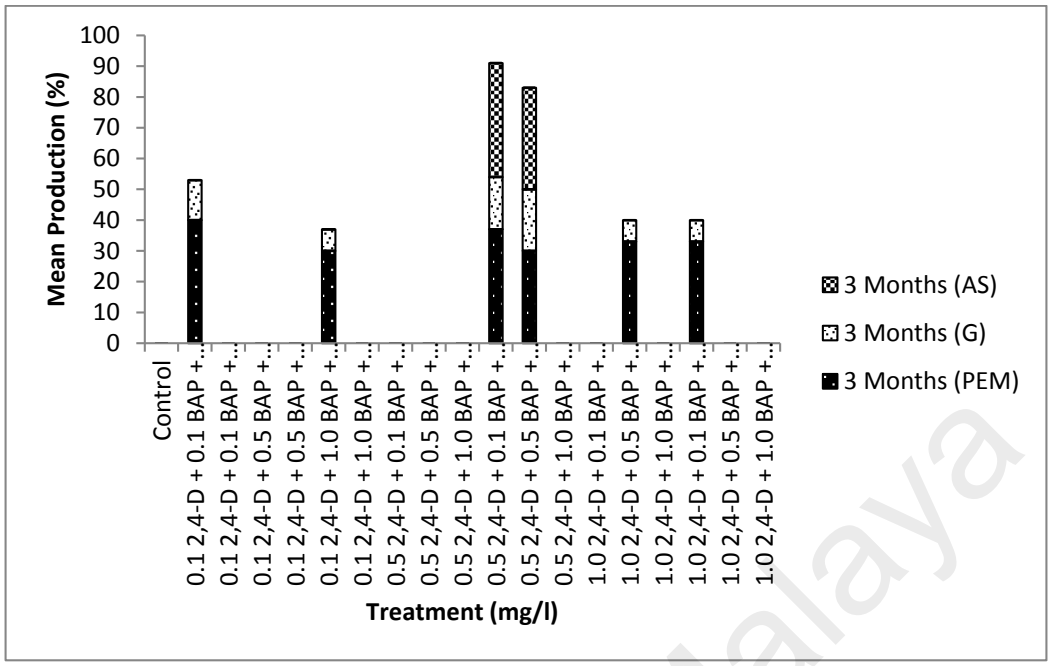


Figure 4.10: Total mean production of somatic embryos (proembryo masses, globular and all stages) in liquid medium (after 3 months of culture).

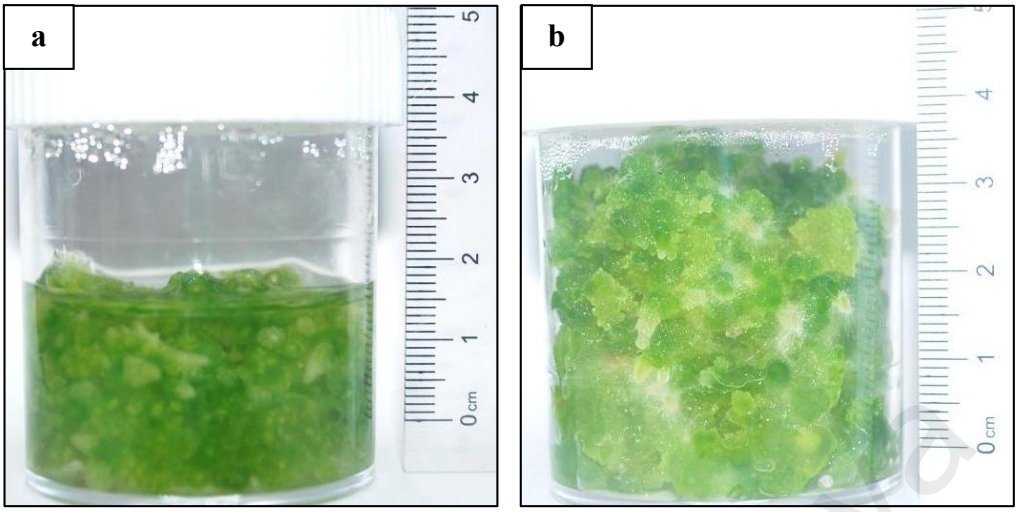


Figure 4.11: Somatic embryos (proembryo masses, globular, all stages) (a) after 4 months and (b) 6 months of culture in liquid medium (MS medium + 0.5 mg/l 2,4-D + 0.1 mg/l BAP + 0.5 mg/l thiamine HCl).

Table 4.5: Induction of somatic embryogenesis in liquid medium (after 4 months of culture).

	4 Months		
MS + Hormones (mg/l)	Mean (% PEM) ± SE	Mean (% G) ± SE	Mean (% AS) ± SE
Control	0 ± 0.00 b	0 ± 0.00 b	0 ± 0.00 b
0.1 2,4-D + 0.1 BAP + 0.1 thia	47 ± 0.09 a	17 ± 0.07 a	0 ± 0.00 b
0.1 2,4-D + 0.1 BAP + 0.5 thia	0 ± 0.00 b	0 ± 0.00 b	0 ± 0.00 b
0.1 2,4-D + 0.5 BAP + 0.1 thia	0 ± 0.00 b	0 ± 0.00 b	0 ± 0.00 b
0.1 2,4-D + 0.5 BAP + 0.5 thia	0 ± 0.00 b	0 ± 0.00 b	0 ± 0.00 b
0.1 2,4-D + 1.0 BAP + 0.1 thia	40 ± 0.09 a	10 ± 0.06 ab	0 ± 0.00 b
0.1 2,4-D + 1.0 BAP + 0.5 thia	0 ± 0.00 b	0 ± 0.00 b	0 ± 0.00 b
0.5 2,4-D + 0.1 BAP + 0.1 thia	0 ± 0.00 b	0 ± 0.00 b	0 ± 0.00 b
0.5 2,4-D + 0.5 BAP + 0.1 thia	0 ± 0.00 b	0 ± 0.00 b	0 ± 0.00 b
0.5 2,4-D + 1.0 BAP + 0.1 thia	0 ± 0.00 b	0 ± 0.00 b	0 ± 0.00 b
0.5 2,4-D + 0.1 BAP + 0.5 thia	33 ± 0.09 a	20 ± 0.07 a	47 ± 0.09 a
0.5 2,4-D + 0.5 BAP + 0.5 thia	43 ± 0.09 a	17 ± 0.07 a	40 ± 0.09 a
0.5 2,4-D + 1.0 BAP + 0.5 thia	0 ± 0.00 b	0 ± 0.00 b	0 ± 0.00 b
1.0 2,4-D + 0.1 BAP + 0.1 thia	0 ± 0.00 b	0 ± 0.00 b	0 ± 0.00 b
1.0 2,4-D + 0.5 BAP + 0.1 thia	50 ± 0.09 a	10 ± 0.06 ab	0 ± 0.00 b
1.0 2,4-D + 1.0 BAP + 0.1 thia	0 ± 0.00 b	0 ± 0.00 b	0 ± 0.00 b
1.0 2,4-D + 0.1 BAP + 0.5 thia	47 ± 0.09 a	7 ± 0.05 ab	0 ± 0.00 b
1.0 2,4-D + 0.5 BAP + 0.5 thia	0 ± 0.00 b	0 ± 0.00 b	0 ± 0.00 b
1.0 2,4-D + 1.0 BAP + 0.5 thia	0 ± 0.00 b	0 ± 0.00 b	0 ± 0.00 b

Data represents mean value ± standard error (SE) with 30 explants in each treatment. Means with different letters in the same column are significantly different at p=0.01 according to Duncan's multiple range test (DMRT). thia, thiamine HCl; PEM, Proembryo masses; G, Globular; AS, All stages.

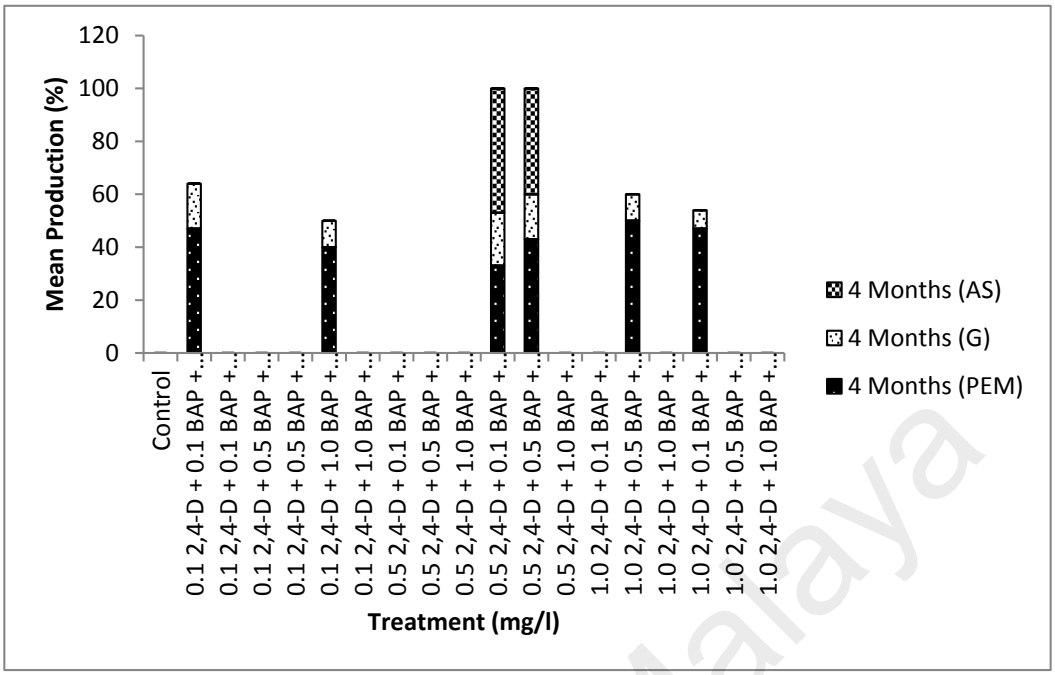


Figure 4.12: Total mean production of somatic embryos (proembryo masses, globular and all stages) in liquid medium (after 4 months of culture).

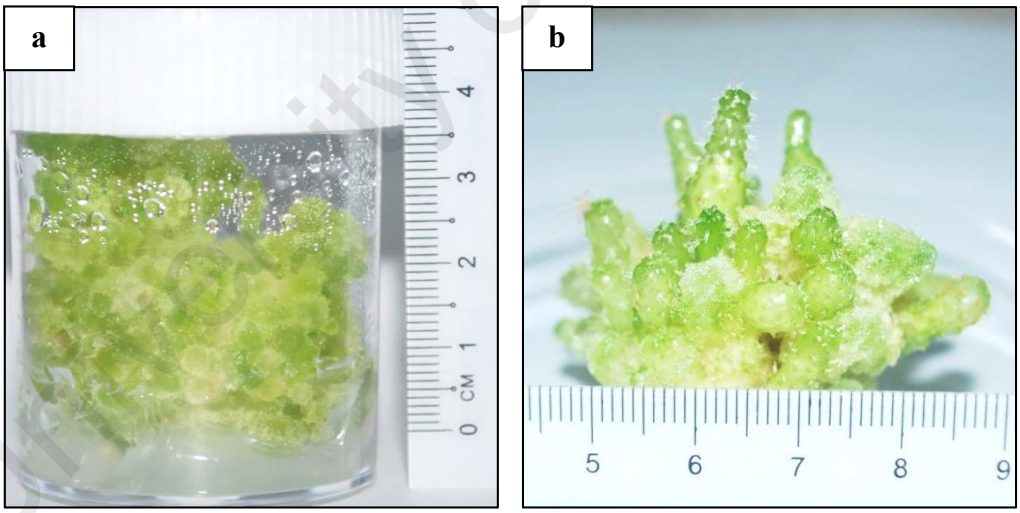


Figure 4.13: (a) Development of somatic embryos and (b) production of multiple shoots from somatic embryos cultured on MS basal medium (solid medium).

4.4 SUMMARY OF RESULTS

1. Direct somatic embryogenesis of *Echinocereus cinerascens* was obtained in solid culture as the stem explants produced somatic embryos directly without callus phase in several solid media tested. Somatic embryos could be detected with the formation of globular-shaped after 1 month while after 2 months, both globular and heart-shaped were observed. However, the globular and heart-shaped were retained with no further development after 3 and 4 months.
2. The highest total mean production of somatic embryos (globular and heart-shaped only) in solid culture was 100% observed after 2 months in MS medium supplemented with 1.0 mg/l 2,4-D + 0.1 mg/l thiamine HCl.
3. Indirect somatic embryogenesis was observed in liquid culture as the callus explants produced somatic embryos in several liquid media tested. Somatic embryos could be detected with the formation of proembryo masses after 1 month which further development to all stages of somatic embryos (globular, heart, torpedo-shaped and cotyledonary stage) after 2 months.
4. Total mean production of somatic embryos (all stages) in liquid culture after 2 months was 90% observed in MS supplemented with 0.5 mg/l 2,4-D + 0.1 mg/l BAP + 0.5 mg/l thiamine HCl.
5. Somatic embryogenesis in liquid medium gradually increased and showed 100% total mean production of somatic embryos after 4 months in two treatments such as MS supplemented with 0.5 mg/l 2,4-D + 0.1 mg/l BAP + 0.5 mg/l thiamine HCl and MS supplemented with 0.5 mg/l 2,4-D + 0.5 mg/l BAP + 0.5 mg/l thiamine HCl. Therefore, both treatments were considered as the optimum medium for the induction of somatic embryogenesis of *Echinocereus cinerascens*.

6. Somatic embryos produced in liquid medium developed into plantlets when transferred to MS basal medium (solid medium).

CHAPTER 5

SYNTHETIC SEED PRODUCTION OF *Echinocereus cinerascens*

5.1 EXPERIMENTAL AIMS

Synthetic seed technology is an advancement of *in vitro* plant regeneration (Naik and Chand, 2006) and has become popular nowadays which accelerates a new vista in the agriculture industry that largely contributes in mass propagation of commercial plants production. Synthetic seed production offers an alternative procedure to produce unlimited desirable planting materials throughout the year, hence is applicable for *in vitro* conservation of endangered species (West *et al.*, 2006). Other significant application of synthetic seeds include propagation of plants with non-viable seeds that are difficult to propagate by other means (Daud *et al.*, 2008). Additionally, it is also known as a channel for various plants produced through advanced biotechnology technique to be delivered directly to the greenhouse or field. Thus, allowing production and propagation of elite plant varieties. Synthetic seed production overcomes many seed germination problems in conventional propagation such as unbreakable and hard seed coat, thick and stiff endosperm, dormant seeds etc.

Somatic embryos or other non-embryogenic *in vitro*-derived explants (shoot tips, micro shoots, axillary shoots and others) can be utilized as propagules and encapsulated in artificial or synthetic endosperm to produce synthetic seeds. Somatic embryos are typically used in the production of synthetic seeds but recent studies indicated that non-embryogenic *in vitro*-derived explants are preferred for some specific applications (Muruyama *et al.*, 1997; Standardi and Piccioni, 1998; Soneji *et al*., 2002; Danso and Ford-Lloyd, 2003; Chand and Singh, 2004; Tsvetkov and Hausman, 2005). Furthermore, non-embryogenic artificial seeds are comparatively inexpensive and easy to handle, transport and grow (Danso and Ford-Lloyd, 2003).

Recently, the production of synthetic seeds has been studied in various plant species using different types of explants as propagules. Studies reported that there are several factors which influenced the production and germination of synthetic seeds including the concentration of encapsulation matrix (normally sodium alginate), nutrients and carbohydrate source in the encapsulation matrix, types and culture conditions during germination, the quality of the encapsulated propagules etc.

In this study, synthetic seeds were produced according to the hydrogel encapsulation method developed by Redenbaugh *et al*. (1987). In the production of synthetic seeds of *Echinocereus cinerascens*, different types of explants were tested to investigate the most responsive explant as propagule. The selected propagules were encapsulated in several treatments. Observations on the effects of different encapsulation matrix and types of media tested on germination of synthetic seeds produced were recorded. The viability of synthetic seeds produced was examined as the synthetic seeds were stored for certain periods.

Table 5.1: Plant species and the type of explant used for production of synthetic seeds.

Plant species	Type of explant (Propagule)	Reference
Acacia mangia Willd. x *A. auriculaformis* Cunn. Ex Benth	Shoots and axillary buds	Asmah *et al*., 2013
Begonia x hiemalis Forch	Somatic embryos	Awal *et al*., 2007
Brassica oleracea var botrytis	Micro shoots	Rihan *et al*.,2011
Cassia angustifolia Vahl.	Nodal segments	Bukhari *et al*., 2014
Catharantus roseus (L.) G. Don	Somatic embryos	Maqsood *et al*., 2012
Cucumis sativus L.	Somatic embryos	Tabassum *et al*., 2010
Dalbergia sissoo Roxb.	Somatic embryos	Singh and Chand, 2010
Flickingeria nodosa (Dalz.) Seidenf.	Protocorm like bodies	Nagananda *et al*., 2011
Gerbera jamesonii Bolus ex Hook. f	Micro shoots and somatic embryos	Taha *et al*., 2009a
Helianthus annuus L.	Shoot tips	Katouzi *et al*., 2011
Musa balbisiana 'Kluai Hin' (BBB group)	Micro shoots	Kanchanapoom and Promsorn, 2012
Musa paradisiaca L.	Shoot tips	Hassanein *et al*., 2011
Olea europaea L.	*In vitro* derived shoots	Ikhiaq *et al*., 2010
Parkia speciosa Hassk.	Embryos	Ummi *et al*., 2011
Picrorhiza kurroa Royle. ex. Benth	Micro shoots	Mishra *et al*., 2011
Pseudostellaria heterophylla (Miquel) Pax.	Micro tubers	Ma *et al*., 2011
Rauvolfia serpentina (L.) Benth. ex Kurz.	Micro shoots	Faisal *et al*., 2012
Rauvolfia tetraphylla L.	Micro shoots	Alatar and Faisal, 2012
Ruta graviolens L.	Roots	Vdovitchenko and Kuzovkina, 2011
Saintpaulia ionantha Wendl.	Somatic embryos	Taha *et al*., 2009b
Saintpaulia ionantha Wendl.	Micro shoots	Daud *et al*., 2008
Salvia officinalis L.	Shoot tips	Grzegorczyk and Wysokińska, 2011
Scutellaria baicalensis Georgi	Roots	Vdovitchenko and Kuzovkina, 2011
Solanum nigrum L.	Shoot tips	Verma *et al*., 2010
Stevia rebaudiana Bertoni	Shoot tips	Andlib *et al*., 2011
Stevia rebaudiana Bertoni	Shoot tips and axillary buds	Ali *et al*., 2012
Trichosanthes dioica Roxb.	Shoot tips	Malek, 2009
Tylophora indica (Burm. F.) Merrill	Somatic embryos	Devendra *et al*., 2011
Vanda coerulea Grifft.	Protocorm like bodies	Sarmah *et al*., 2010
Vitex negundo L.	Nodal segments	Ahmad and Anis, 2010
Zingiber officinale Rosc.	Micro shoots	Sundararaj *et al*., 2010

5.2 MATERIALS AND METHODS

5.2.1 Source of Explants

Micro shoot and stem explants were preferred for the production of synthetic seeds of *Echinocereus cinerascens* instead of somatic embryos after considering several factors. Even though the production of plantlets from indirect regeneration (induction of somatic embryos) was significantly higher than plantlets produced through direct regeneration, however, production of somatic embryos indirectly was time consuming. Therefore, micro shoot and stem explants were selected properly, whereby the size of explant to be encapsulated was standardized to produce uniform synthetic seeds. Micro shoots (2-month-old) and stems of 6-month-old plantlets were excised approximately 0.5 cm in size before encapsulated in the encapsulation matrix.

5.2.2 Preparation of Solutions for Encapsulation

Production of synthetic seeds involved the encapsulation of explants (propagules) in encapsulation matrix consisting of nutrients and plant growth regulators. The encapsulation matrix serves as synthetic endosperm for the encapsulated explants aimed to increase the efficiency of viability and germination of synthetic seeds. In this study, synthetic seeds were produced using the hydrogel encapsulation method which requires the preparation of sodium alginate and calcium chloride dehydrate solutions. The concentrations of both solutions were standardized, 3% sodium alginate solution and 100 mM of calcium chloride dehydrate solution were prepared and applied throughout this study since both are considered as the optimum concentration for the production of ideal synthetic seeds of *Echinocereus cinerascens*.

5.2.2.1 Preparation of Modified MS Medium

The basic composition and formulation of MS medium (commercial MS powder) is given in Appendix III. In this study, the basic composition of MS medium is modified for preparation of synthetic seeds. Modified MS medium (Table 5.2) comprised of macronutrients without calcium chloride dehydrate ($CaCl_2.2H_2O$), micronutrients, vitamins and irons which were prepared separately in the Schott bottles and kept in the refrigerator at 4 ± 1 ºC. Modified MS medium is essential for the preparation of sodium alginate solution (encapsulation matrix of synthetic seeds).

Table 5.2: Modified MS medium for preparation of synthetic seeds.

Components	Concentrations (mg/l)		To prepare 1 liter MS medium	To prepare 100 ml MS medium
Macronutrients without CaCl2.2H2O		**X 10**		
$MgSO_4.7H_2O$	370.00	3700.0		
KH_2PO_4	170.00	1700.0	**100 ml was used**	**10 ml was used**
KNO_3	1900.00	19000.0		
NH_4NO_3	1650.00	16500.0		
Micronutrients		**X 100**		
H_3BO_3	6.200	620.0		
$MnSO_4.H_2O$	15.600	1560.0		
$ZnSO_4.7H_2O$	8.600	860.0		
$NaMoO_4.2H_2O$	0.250	25.0	**10 ml was used**	**1 ml was used**
$CuSO_4.5H_2O$	0.025	2.5		
$CoCl_2.6H_2O$	0.025	2.5		
KI	0.830	8.3		
Irons		**X 100**		
$FeSO_4.7H_2O$	27.850	2785.0	**10 ml was used**	**1 ml was used**
Na_2EDTA	37.250	3725.0		
Vitamins		**X 100**		
Thiamine HCl	0.100	10.0		
Pyridoxine HCl	0.500	50.0	**10 ml was used**	**1 ml was used**
Nicotinic acid	0.500	50.0		

5.2.2.2 Preparation of (3%) Sodium Alginate Solution ($NaC_6H_7O_6$)

To prepare 3% sodium alginate as an encapsulation matrix, 50 ml of distilled water was dispensed into a conical flask. Subsequently, modified MS medium (10 ml of macronutrients without calcium chloride dehydrate + 1 ml of micronutrients + 1 ml of irons + 1 ml of vitamins), 3.0 g of sucrose and 0.1 g of myo-inositol were added into the conical flask. The solution was placed on a hot plate and mixed using magnetic stirrer. Once the solution was homogenized, the heater was turned on and 3.0 g of sodium alginate powder was added slowly to the solution to allow the powder to dissolve completely. The solution was heated according to the method by Fabre and Dereuddre (1990). Distilled water was then filled in the solution up to 100 ml. The heater was turned off once the powder was well dissolved and cooled at room temperature. After that, the pH was adjusted to 5.7 and the selected plant growth regulator was added before the solution was autoclaved at 121 ºC for 20 minutes.

5.2.2.3 Preparation of (100 mM) Calcium Chloride Dehydrate ($CaCl_2.2H_2O$) Solution

Calcium chloride dehydrate solution plays an important role for the hardening of encapsulation matrix. The hardness of the synthetic seed beads produced depend on the concentration of calcium chloride dehydrate solution. In this study, 100 mM of calcium chloride dehydrate solution was chosen since trial experiment proved that it was the best concentration for the production of the ideal bead of synthetic seeds. To prepare 100 mM of calcium chloride dehydrate solution, 3.676 g of calcium chloride dehydrate powder was dissolved in 250 ml distilled water and subsequently autoclaved at 121 ºC for 20 minutes.

5.2.3 Encapsulation of Micro shoot and Stem Explants

In this study, synthetic seeds were produced through encapsulation technique which was introduced by Lynch (2002). The encapsulation technique requires micropipette with approximately 0.5 mm diameter of pipette tip as an important apparatus while sodium alginate and calcium chloride dehydrate solutions as the main constituents in the production of synthetic seeds. The explants (micro shoots and stems) were submerged in various types of encapsulation matrix tested. Then, both of the explant and the encapsulation matrix were drawn up using micropipette and subsequently dropped into calcium chloride dehydrate solution. Round shaped bead formed as the droplet comprised of explant was dropped into the calcium chloride dehydrate. The occurrence of round shaped bead was clarified by the reaction of ion exchange between Na^+ (in sodium alginate solution) and Ca^{2+} (in calcium chloride dehydrate solution) leads to the establishment of insoluble calcium alginate. During this process, the calcium chloride dehydrate solution was slowly agitated to prevent them from blending together as a lot of beads were formed. The beads remained in the calcium chloride dehydrate solution for 30 minutes to be hardened (the explants were encapsulated or synthetic seeds were formed). After that, the synthetic seeds were transferred into a clean container and rinsed three times with sterile distilled water for a few minutes to wash out excess solution of calcium chloride dehydrate. Finally, the synthetic seeds were dried on sterile tissue paper before being cultured in appropriate media or stored at 4 ± 1 ºC in the refrigerator.

5.2.4 Germination of Synthetic Seeds

Synthetic seeds produced (stored for 0 and 30 days) were cultured in MS basal medium as a control and also in MS medium supplemented with 2.0 mg/l Kinetin + 0.5 mg/l IBA which was the optimum medium for direct *in vitro* regeneration to observe the viability of synthetic seeds, as well as to investigate the mean (%) germination of

synthetic seeds and their response toward media tested. All cultures were maintained in the culture room at 25 ± 2 °C under 16 hours light provided by cool daylight or fluorescent light (36W), PHILIPS. Observations were recorded after 4 months of culture. Analysis on the results determined the most responsive propagules, the optimum medium and encapsulation matrix for synthetic seeds germination of *Echinocereus cinerascens*.

5.2.4.1 Effects of Different Types of Media and Encapsulation Matrix on Germination of Synthetic Seeds

All treatments or types of media and encapsulation matrix applied throughout this study were prepared as listed below.

Type of medium tested including;

1. MS basal medium (control)
2. MS medium + 2.0 mg/l Kinetin + 0.5 mg/l IBA (optimum medium for direct *in vitro* regeneration)

Encapsulation matrix tested including;

1. Sodium alginate
2. Sodium alginate + 2.0 mg/l Kinetin + 0.5 mg/l IBA
3. Sodium alginate without sucrose

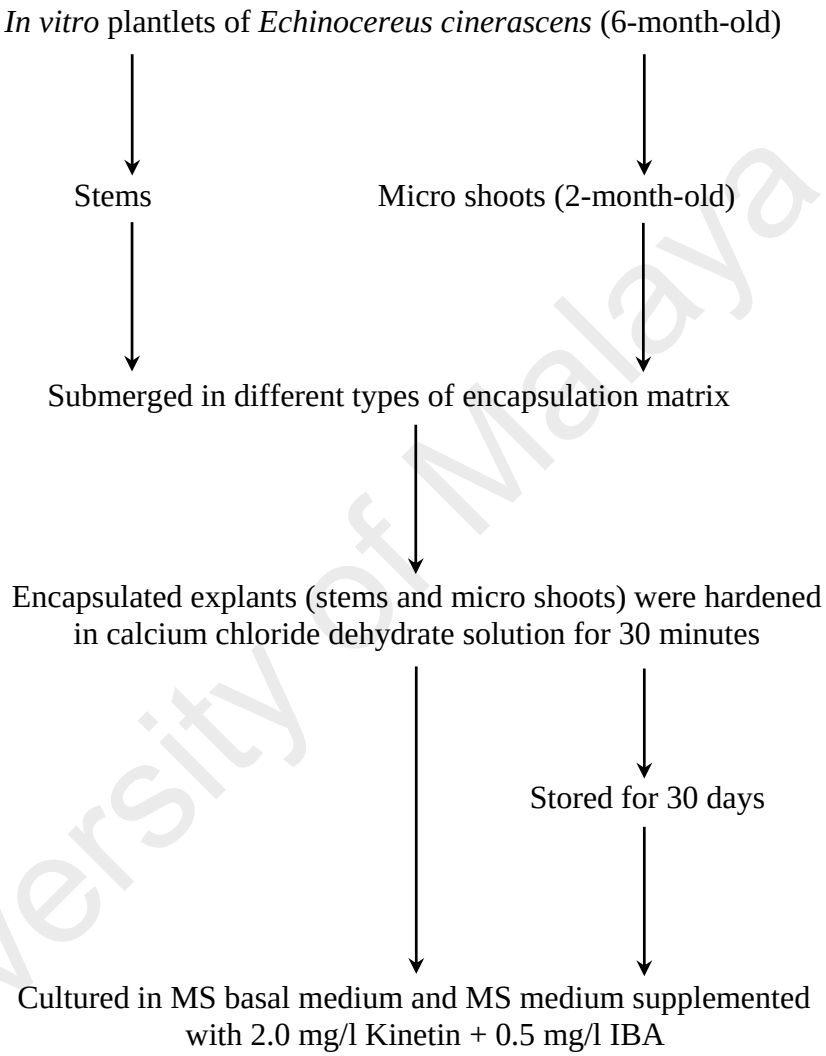
5.2.5 Low Temperature Storage

Synthetic seeds produced were kept in the dark, in a container wrapped with aluminium foil and preserved at 4 ± 1 ºC in the refrigerator for a month. After storage period, the synthetic seeds were subjected to media tested and maintained in the culture room at 25 ± 2 °C under 16 hours light provided by cool daylight or fluorescent light (36W), PHILIPS. No germination of synthetic seed was observed in all treatments tested after storage for a month.

5.2.6 Data Analysis

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

5.2.7 Experimental Outline



5.3 RESULTS

In the production of synthetic seeds, selected propagules including micro shoots and stems (Figure 5.1) were encapsulated in different types of encapsulation matrix (Figure 5.2) as synthetic endosperms. The propagules were encapsulated in 3% sodium alginate and subsequently hardened in 100 mM of calcium chloride dehydrate for 30 minutes to produce ideal synthetic seeds with characteristics of firm and round shaped beads. The synthetic seeds produced (stored for 0 and 30 days) were then germinated on MS basal medium and MS medium supplemented with 2.0 mg/l Kinetin + 0.5 mg/l IBA. Both selected propagules were able to germinate and develop into plantlets in all the treatments tested.

5.3.1 Effects of Different Types of Media and Encapsulation Matrix on Germination of Synthetic Seeds (micro shoots as propagules)

Synthetic seeds comprised of micro shoots as propagules were germinated after a month of culture and further developed into more advanced shoots after 4 months of culture. Encouraging results were obtained after 4 months of culture as 100% of synthetic seeds germinated in all treatments tested (Table 5.3). This indicated that micro shoot was responsive for synthetic seeds production which encapsulated in synthetic endosperm with sufficient nutrients for germination in the media tested.

In MS basal medium, micro shoots encapsulated in sodium alginate (synthetic endosperm) as a control (Figure 5.3) showed single and multiple shoots formation with germination rate of 70% and 30%, respectively. Similarly, micro shoots encapsulated in sodium alginate without sucrose (Figure 5.4) also gave comparable result which showed single and multiple shoots formation. However, the germination rate of single shoot formation was 73% and multiple shoots formation was 27% which were slightly different. Statistical analysis proved that there was no significant difference for the results in both treatments tested. Both treatments showed germination of synthetic seeds

with higher formation of single shoot compared to multiple shoots. In contrast, micro shoots encapsulated in sodium alginate added with 2.0 mg/l Kinetin + 0.5 mg/l IBA (Figure 5.5) showed equal (50%) germination of synthetic seeds of single shoot and multiple shoots. These results suggest that plant growth regulators supplemented in encapsulation matrix were suitable for induction of multiple shoots.

Further study was done where synthetic seeds produced with micro shoots encapsulated in sodium alginate were germinated on MS medium supplemented with 2.0 mg/l Kinetin + 0.5 mg/l IBA. Remarkable result was observed as 100% of synthetic seeds showed multiple shoots formation which were significantly different with control. Formation of multiple shoots was higher as the plant growth regulators were supplemented in the medium for germination of synthetic seeds. It was found that the highest formation of single shoot (Figure 5.6) could be obtained by encapsulating micro shoots in sodium alginate and sodium alginate without sucrose germinated in MS basal medium. Nevertheless, the highest formation of multiple shoots (Figure 5.7) could be obtained by encapsulating micro shoot in sodium alginate germinated in MS medium supplemented with 2.0 mg/l Kinetin + 0.5 mg/l IBA. Furthermore, analysis of the mean height and diameter reported that shoot with the highest height was observed in synthetic seeds (micro shoot encapsulated in sodium alginate) germinated in MS supplemented with 2.0 mg/l Kinetin + 0.5 mg/l IBA, while shoot with the greatest diameter was observed as synthetic seeds (micro shoot encapsulated in sodium alginate) germinated in MS basal medium.

5.3.2 Effects of Different Types of Media and Encapsulation Matrix on Germination of Synthetic Seeds (stems as propagules)

For further production of synthetic seeds, stems were selected as propagules instead of micro shoots which were tested in the same treatments. However, observations indicated that the stems were less responsive than micro shoots.

Germination of synthetic seeds with stems encapsulated in encapsulation matrix could obviously be seen after 2 months of culture. In fact, most of the synthetic seeds showed less than 50% germination rate after 4 months of culture (Table 5.4) and it was found that the synthetic seeds germinated in all treatments produced shoots incorporated with callus.

In MS basal medium, synthetic seeds produced by encapsulating stems in sodium alginate (control) and sodium alginate without sucrose (Figure 5.8, Figure 5.9) presented similar germination rate. Both treatments showed 23% germination rate of synthetic seeds. In addition, all the synthetic seeds germinated on MS basal medium in these treatments produced single shoot. Higher germination rate of synthetic seeds, 43% was observed in treatment where stems encapsulated in sodium alginate added with 2.0 mg/l Kinetin + 0.5 mg/l IBA (Figure 5.10). In this treatment, 30% of synthetic seeds germinated in MS basal medium produced single shoot while 13% produced multiple shoots. These results supported the hypothesis that plant growth regulators were necessary to induce the formation of multiple shoots in germination of synthetic seeds.

Further study involved the production of synthetic seeds germinated in MS medium supplemented with 2.0 mg/l Kinetin + 0.5 mg/l IBA. In this treatment, the stems encapsulated in sodium alginate showed the highest germination rate (50%) and the result was significantly different from control. The formation of single shoot was 17% while the formation of multiple shoots was 33%. Observations indicated that the highest formation of single shoot in synthetic seeds germinated (stems encapsulated in sodium alginate added with 2.0 mg/l Kinetin + 0.5 mg/l IBA) in MS basal medium while the highest formation of multiple shoots was detected in MS medium supplemented with 2.0 mg/l Kinetin + 0.5 mg/l IBA. Moreover, the highest height of shoot, 1.55 cm was obtained in synthetic seeds (stems encapsulated with sodium alginate) germinated on MS medium supplemented with 2.0 mg/l Kinetin + 0.5 mg/l

IBA as well. Nevertheless, the greatest diameter of shoots, 0.65 cm was observed in synthetic seeds (stems encapsulated with sodium alginate added with 2.0 mg/l Kinetin + 0.5 mg/l IBA) germinated in MS basal medium.

Table 5.3: Germination of synthetic seeds (micro shoot explants) on different types of media and encapsulation matrix.

Medium	MS basal			MS + 2K + 0.5I
Encapsulation Matrix	sodium alginate	sodium alginate + 2K + 0.5I	sodium alginate - sucrose	sodium alginate
Germination (%)	100.00 ± 0.00a	100.00 ± 0.00a	100.00 ± 0.00a	100.00 ± 0.00a
Single shoot (%)	70.00 ± 0.09ab	50.00 ± 0.09b	73.00 ± 0.08a	0.00 ± 0.00c
Multiple shoots (%)	30.00 ± 0.09bc	50.00 ± 0.09b	27.00 ± 0.08c	100.00 ± 0.00a
Height (cm)	1.33 ± 0.01b	1.18 ± 0.01c	0.94 ± 0.01d	2.24 ± 0.04a
Diameter (cm)	1.21 ± 0.06a	1.14 ± 0.02a	0.85 ± 0.03b	1.18 ± 0.02a

Data represents mean value ± standard error (SE) with 30 explants in each treatment. Means with different letters in the same row are significantly different at p=0.05according to Duncan's multiple range test (DMRT). 2K, 2.0 mg/l Kinetin; 0.5I, 0.5 mg/l IBA.

Table 5.4: Germination of synthetic seeds (stem explants) on different types of media and encapsulation matrix.

Medium	MS basal			MS + 2K + 0.5I
Encapsulation Matrix	sodium alginate	sodium alginate + 2K + 0.5I	sodium alginate - sucrose	sodium alginate
Germination (%)	23.00 ± 0.08b	43.00 ± 0.09ab	23.00 ± 0.08b	50.00 ± 0.09a
Single shoot (%)	23.00 ± 0.08a	30.00 ± 0.09a	23.00 ± 0.08a	17.00 ± 0.07a
Multiple shoots (%)	0.00 ± 0.00b	13.00 ± 0.06b	0.00 ± 0.00b	33.00 ± 0.09a
Height (cm)	0.92 ± 0.01c	1.01 ± 0.02b	0.74 ± 0.01d	1.55 ± 0.03a
Diameter (cm)	0.50 ± 0.01b	0.65 ± 0.02a	0.62 ± 0.01a	0.49 ± 0.05b

Data represents mean value ± standard error (SE) with 30 explants in each treatment. Means with different letters in the same row are significantly different at p=0.05according to Duncan's multiple range test (DMRT). 2K, 2.0 mg/l Kinetin; 0.5I, 0.5 mg/l IBA.

5.3.3 Effects of Storage Period on Germination of Synthetic Seeds

Synthetic seeds (micro shoots and stems encapsulated in sodium alginate) preserved at 4 ± 1 ºC for 30 days cultured in MS basal medium and MS medium supplemented with 2.0 mg/l Kinetin + 0.5 mg/l IBA showed unsatisfactory results. After storage, some morphological changes were detected on the propagules (micro shoots and stems) that have been encapsulated. Initially, the propagules were green colour but eventually turned reddish and brown (Figure 5.11) after storage. Unfortunately, no germination of synthetic seed was observed and this indicated that the synthetic seeds were dormant or perhaps some injuries on the propagules might cause the cells dead after being stored at 4 ± 1 ºC for 30 days.

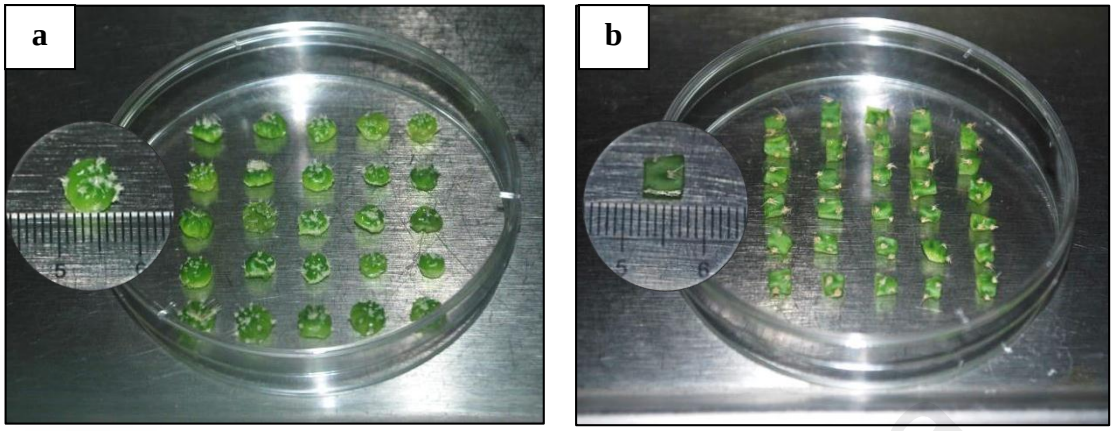


Figure 5.1: (a) Micro shoots and (b) stems were selected (approximately 0.5 cm in size) for the production of synthetic seeds.

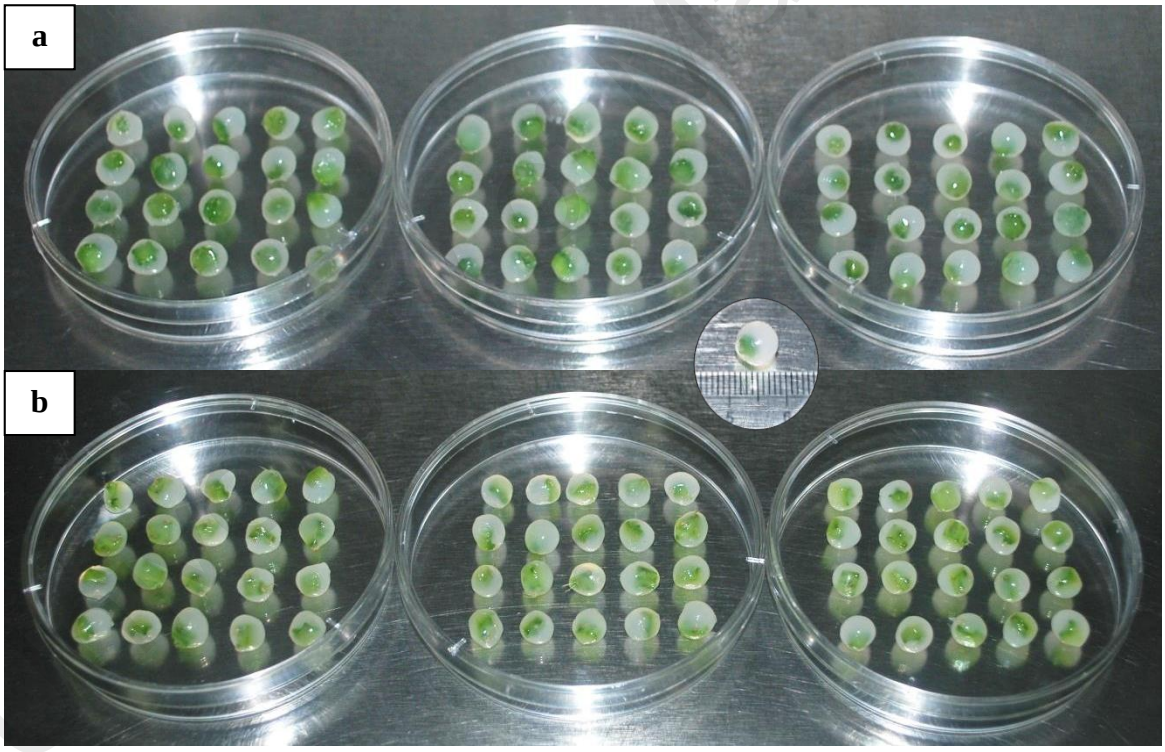


Figure 5.2: Encapsulated (a) micro shoots and (b) stems in various encapsulation matrix (sodium alginate, sodium alginate added with 2.0 mg/l Kinetin + 0.5 mg/l IBA, sodium alginate without sucrose).

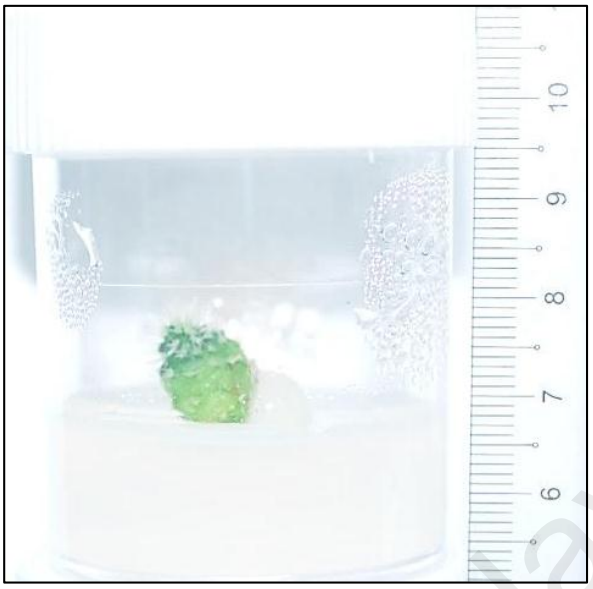


Figure 5.3: Synthetic seed (encapsulated micro shoot in sodium alginate) germinated and produced shoot in MS basal medium after 2 months of culture.

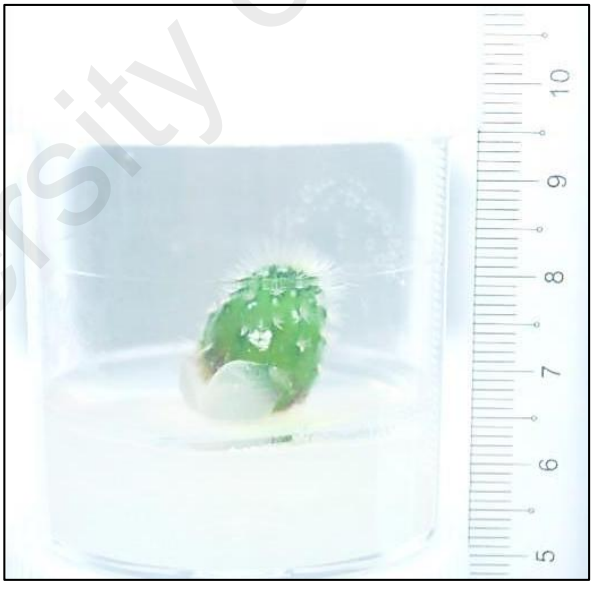


Figure 5.4: Synthetic seed (encapsulated micro shoot in sodium alginate without sucrose) germinated and produced shoot in MS basal medium after 2 months of culture.

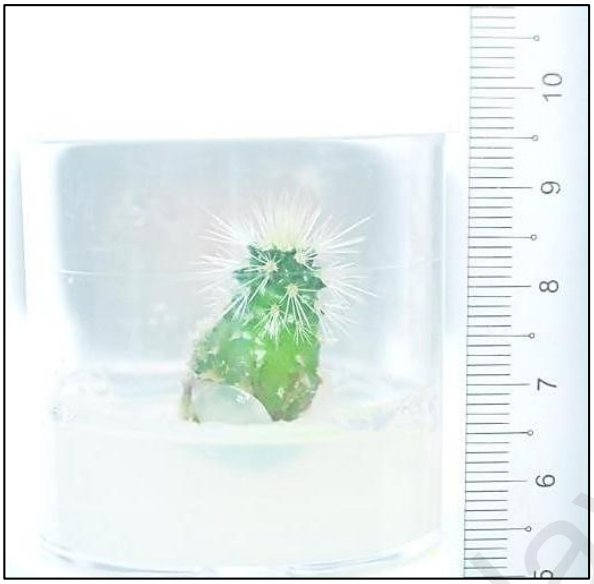


Figure 5.5: Synthetic seed (encapsulated micro shoot in sodium alginate added with 2.0 mg/l Kinetin + 0.5 mg/l IBA) germinated and produced shoot in MS basal medium after 2 months of culture.

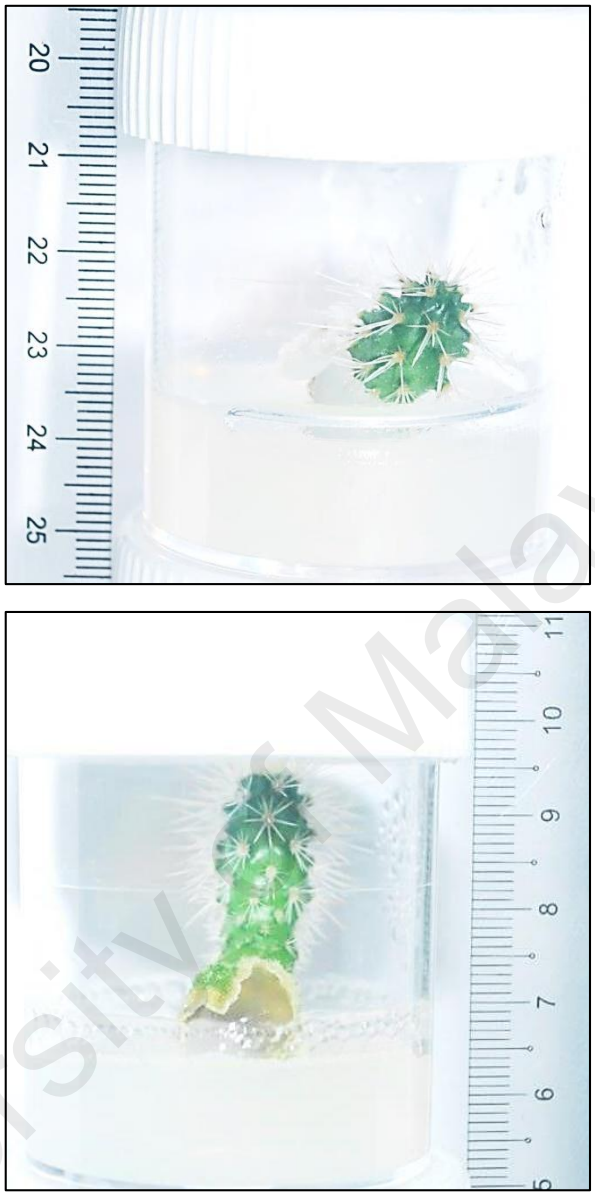


Figure 5.6: Synthetic seeds (encapsulated micro shoots in sodium alginate) germinated and produced single shoot after 4 months of culture in MS basal medium.

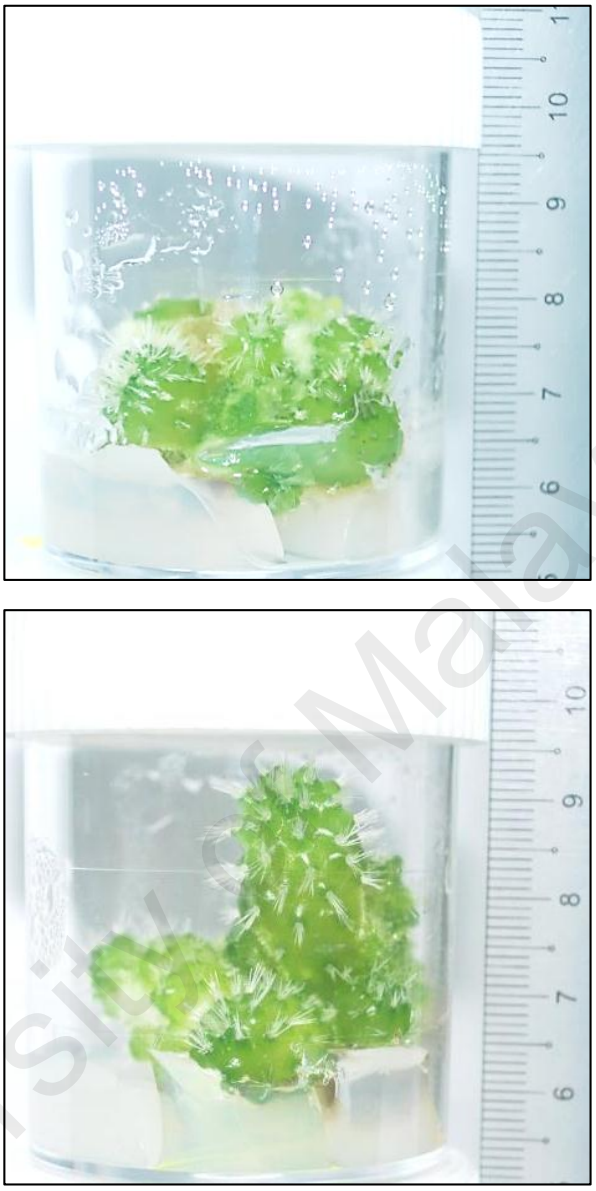


Figure 5.7: Synthetic seeds (encapsulated micro shoots in sodium alginate) germinated and produced multiple shoots in MS medium supplemented with 2.0 mg/l Kinetin + 0.5 mg/l IBA after 4 months of culture.

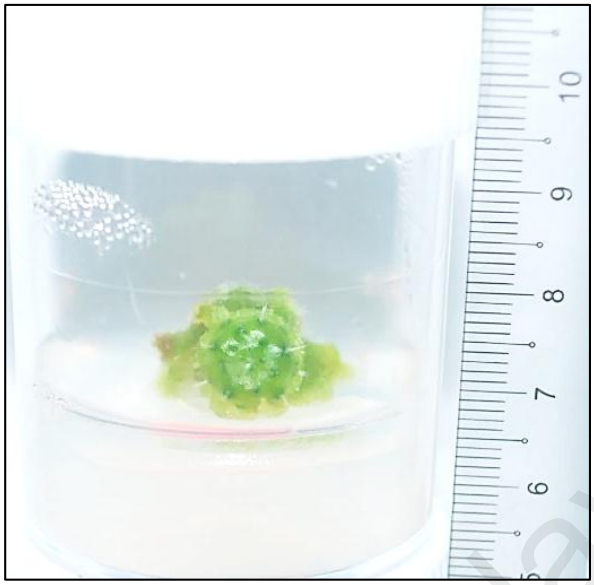


Figure 5.8: Synthetic seed (encapsulated stem in sodium alginate) germinated in MS basal medium after 2 months of culture.

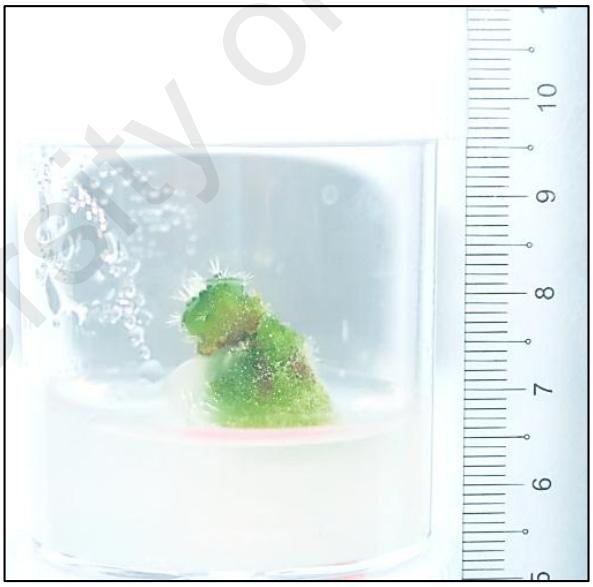


Figure 5.9: Synthetic seed (encapsulated stem in sodium alginate without sucrose) germinated in MS basal medium after 2 months of culture.

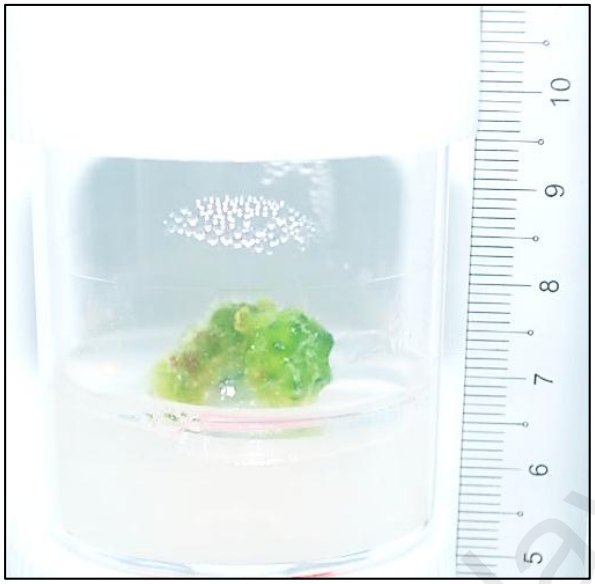


Figure 5.10: Synthetic seed (encapsulated stem in sodium alginate supplemented with 2.0 mg/l Kinetin + 0.5 mg/l IBA) germinated in MS basal medium after 2 months of culture.

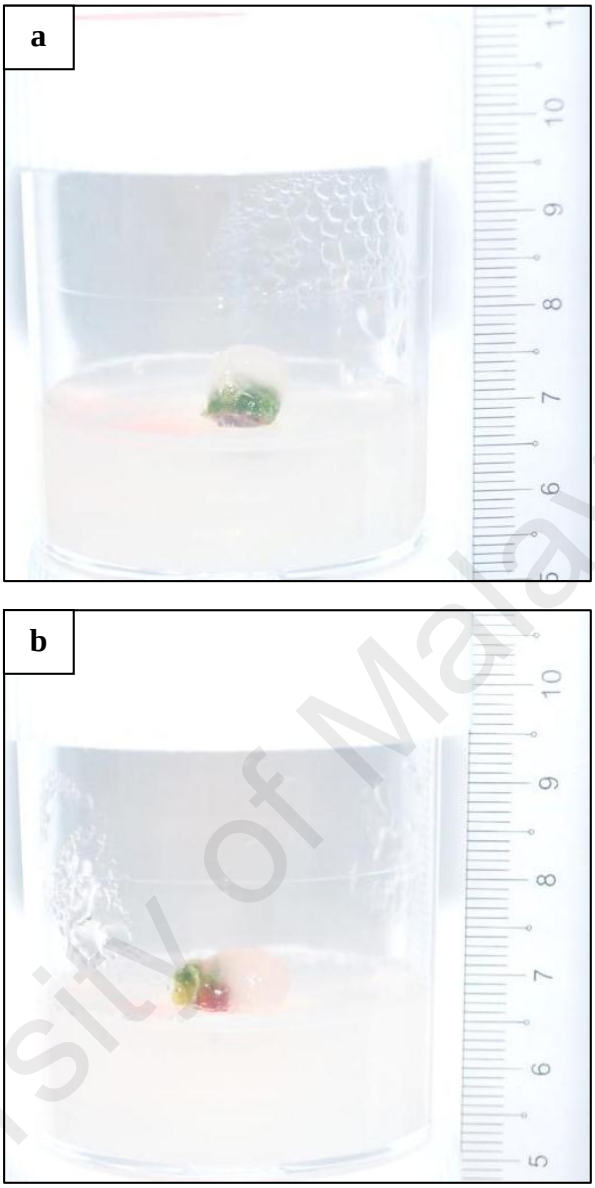


Figure 5.11: Synthetic seeds produced by encapsulating (a) micro shoot and (b) stem in synthetic endosperm were dormant or dead and the propagules became reddish and brown after being stored for 30 days at 4 ± 1 ºC in the refrigerator (dark condition). Stem showed more reddish and brown colour compared to micro shoot.

5.4 SUMMARY OF RESULTS

1. Production of ideal synthetic seed of *Echinocereus cinerascens* with firm, isodiametric and round shape was established by encapsulating propagules in 3% sodium alginate which serves as synthetic endosperm and then, hardened in 100 mM of calcium chloride dehydrate for 30 minutes.
2. Among the propagules tested, micro shoot was found to be more responsive than stem since synthetic seeds with micro shoots as propagules gave the highest (100%) germination rate after 4 months of culture while synthetic seeds with stems as propagules showed only 50% germination rate after 4 months of culture.
3. In MS basal medium, germination of synthetic seeds with synthetic endosperm contained sodium alginate added with 2.0 mg/l Kinetin + 0.5 mg/l IBA showed better result compared to the others (synthetic endosperm contained sodium alginate (control) and sodium alginate without sucrose).
4. Synthetic seeds produced by encapsulating micro shoots in sodium alginate without sucrose showed the highest production of single shoot (73%) which was not significantly different with control (70%). Meanwhile, the highest production of multiple shoots (50%) was observed as synthetic seeds produced by encapsulating micro shoots in sodium alginate added with 2.0 mg/l Kinetin + 0.5 mg/l IBA.
5. Synthetic seeds produced by encapsulating stems in sodium alginate added with 2.0 mg/l Kinetin + 0.5 mg/l IBA showed the highest production of single and multiple shoots with only 30% and 13%, respectively.

6. In MS medium supplemented with 2.0 mg/l Kinetin + 0.5 mg/l IBA (optimum medium for direct *in vitro* regeneration of *Echinocereus cinerascens*), both synthetic seeds produced by encapsulating micro shoots and stems in sodium alginate showed higher production of multiple shoots compared to single shoot.
7. Synthetic seeds produced by encapsulating micro shoots in sodium alginate showed the highest production of multiple shoots, 100% while synthetic seeds produced by encapsulating stems in sodium alginate gave the highest production of multiple shoots with only 33%.
8. Several factors influenced germination of synthetic seeds of *Echinocereus cinerascens* including types of propagules, encapsulation matrix or synthetic endosperm and selection of medium.

CHAPTER 6

ACCLIMATIZATION OF *Echinocereus cinerascens*

6.1 EXPERIMENTAL AIMS

An adaptation process of complete *in vitro* plantlets to grow in the natural environment (greenhouse or field) is termed acclimatization. According to Brainerd and Fuchigami (1981), acclimatization is a process of adaptation of an organism to an environmental change. Acclimatization entails the human interception in the adjustment process which differs from acclimation that implies the adaptation of an organism on its own to an environment change (Conover and Poole, 1984).

Acclimatization process is very important to ensure high survival rate and vigorous growth of *in vitro* plantlets when transferred to the natural environment. *In vitro* plantlets which were transferred to *ex vitro* condition were exposed to changes in temperature, light intensity and water stress conditions which require acclimatization for successful establishment and survival of plantlets (Chandra *et al*., 2010). *In vitro* (aseptic) condition is significantly different from the natural environment, with the controlled growth conditions (*in vitro*) leading to the development of different physiological and morphological characteristics of plants. Yokota *et al*. (2007) indicated that the heterotrophic condition (aseptic condition) plays important roles in the induction of plant physiological and structural modifications which leads to the differences of *in vitro* and *ex vitro* plants.

Difficulties in successfully transplanting *in vitro* plantlets to soil are well documented (Timmis and Richie, 1984) as they share certain characteristics that are inconsistent with development under greenhouse or field conditions. Therefore, the adaptation process is essential for modification of physiological and morphological of *in vitro* plantlets to survive in the natural environment. Previous studies suggested after *ex vitro* transplantation, plantlets need a few weeks of acclimatization with gradual

lowering in air humidity (Preece and Sutter, 1991; Kadleček, 1997; Bolar *et al*., 1998). Concerning this issue, acclimatization units have been developed with temperature, humidity, irradiance, CO_2 concentration and air flow rate controlled by a computer (Hayashi *et al*., 1988).

Plantlets which are successfully acclimatized display almost similar characteristics as intact plants (Mohamed and Vidaver, 1990). It was found that morphological changes of plantlets that grow *in vitro* can be clearly seen as the plantlets are transferred and grown vigorously *ex vitro*. Several morphological changes occur in a small percentage of plantlets and are termed as the somaclonal variation (Larkin and Scowcroft, 1981; Evans, 1989). Besides, stress during acclimatization may also cause variation in most plants. The evidence was supported by Swarts (1991) that indicated the acclimatization of *in vitro* plantlets could not promise that the plant produced is true-to-type to the mother plant in genotype aspect.

The present research aims to study the effects of different planting substrates during acclimatization of plantlets grown *in vitro*. In this study, planting substrates selected were soils and sand. A soil is comprised of materials such as organic and inorganic components, water with dissolved minerals, oxygen and carbon dioxide which were important for plant growth. The composition of materials varies depending on the type of soil. In contrast, sand is a heavy rooting medium, consists of small rock particles and virtually has no mineral nutrients. The mineral composition depends on the type of rocks for example quartz sand consists of a silica complex that is mostly used for plant propagation. Analysis on the planting substrates identified major element composition that plays an important role for the growth of acclimatized plantlets of *Echinocereus cinerascens*.

6.2 MATERIALS AND METHODS

6.2.1 Source of Explants

Shoots produced from direct *in vitro* regeneration were selected as a source of explants in this study. The shoots propagated from axillary buds grew into plantlets within 4 months. Subsequently, plantlets were separated and subcultured in MS basal medium for rooting. This would enable plantlets to undergo elongation process to become complete plantlets. Complete plantlets which fulfilled the criteria, such as 6 cm in height and possess healthy fibrous roots, were selected for acclimatization.

6.2.2 Acclimatization of *In* V*itro* Plantlets

The *in vitro* plantlets that have been developed into complete plantlets were selected and removed carefully from the sterile containers. Then, the plantlets were gently washed and dried on tissue paper to avoid any contamination or infection that may cause fatality before transferring to planting substrates. In this experiment, 50 plantlets were grown on different types of planting materials in jam jars which were covered with transparent plastic bags for 2 to 3 weeks. During this time, the plantlets were maintained in the culture room. Watering was done once a week because excess water resulted in damping off. After 3 months, the plantlets were transferred to round plastic pots and ready to be exposed to the natural environmental condition (Outdoors at 30 ± 1 °C, in shade). Plant survival rates were observed after 9 months.

6.2.2.1 Effects of Different Planting or Growing Substrates on Acclimatization of Complete Plantlets

Complete plantlets that have been successfully acclimatized on several types of planting substrates in jam jars were transferred to round plastic pots to be exposed to the natural environment. After 9 months grown in the natural environment, the survival rate of the plantlets was recorded. The most suitable planting substrate for acclimatization of *in vitro* plantlets of *Echinocereus cinreascens* was identified. In this study, three

different types of planting substrates applied which affected the growth and development of plantlets were as listed below:

1. Red soil
2. Black/ garden soil
3. Sand

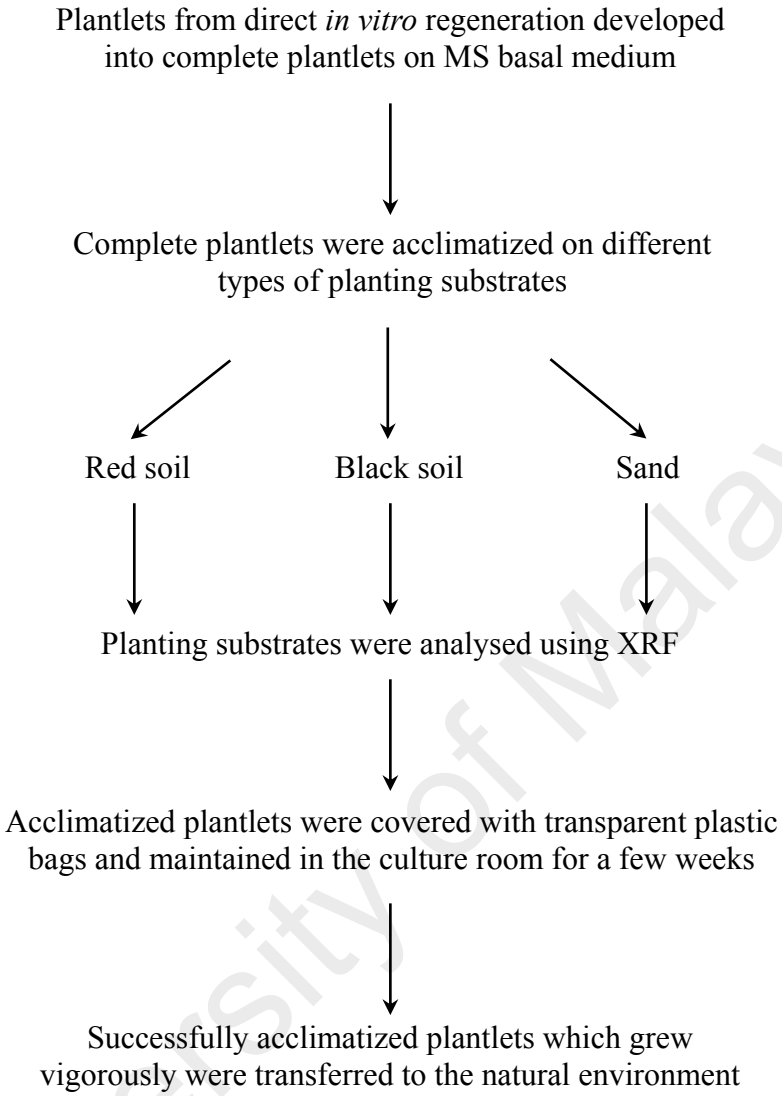
6.2.3 Analysis of Planting or Growing Substrates

Planting substrates such as red soil, black/ garden soil and sand were dried, ground manually using mortar and pestle into powder form. The powder was sieved and then compressed into a tablet before analysed using the XRF (X-Ray Fluorescence). The XRF method was used to analysis an element's total composition (%) in the planting substrate (Chappell *et al*., 1995).

6.2.4 Data Analysis

Data obtained were statistically analysed using Duncan's Multiple Range Test (DMRT). Means with different letters in the same column differ significantly at p=0.01.

6.2.5 Experimental Outline



6.3 RESULTS

Trial experiments on rooting *in vitro* were done and plantlets cultured on MS basal medium had a higher percentage of rooting than those on MS medium fortified with hormones, such as NAA or IBA (data not shown) applied singly. Plantlets rooted abundantly *in vitro* on MS basal medium within 2 to 3 months. These complete plantlets grown with well-developed roots were harvested and selected for acclimatization (Figure 6.1).

6.3.1 Effects of Different Planting or Growing Substrates on Acclimatization of Complete Plantlets

Initially, complete plantlets were acclimatized on different types of planting substrates (red soil, black/ garden soil, sand) in jam jars (Figure 6.2) covered with the transparent plastic bags as an adaptation stage between the humidity of the *in vitro* environment and the natural environment. After a few weeks, plantlets which survived were transferred to round plastic pots and exposed to the natural environment for further elongation and development (Figure 6.3).

Observation after 9 months (Tables 6.1) showed that plantlets propagated *in vitro* could grow as healthily and vigorously as intact plants that exist in nature. It was found that more than 50% survival rate of plantlets was observed in all planting substrates tested. Red soil showed 84% survival rate of plantlets which is not significantly different from the survival rate of plantlets recorded in black/ garden soil and sand. Black/ garden soil exhibited the lowest survival rate of plantlets, 66% while sand became the most suitable planting substrate which gave the highest survival rate of plantlets, 90%.

Table 6.1: Effects of different types of planting substrates on the survival rate (%) of complete plantlets after being acclimatized.

Planting substrate	Survival Rate (%)	Observations (after 9 months being acclimatized)
Red soil	84 ± 0.05 ab	Most of the plantlets survived and showed healthy growth after transferring to the natural environment.
Black/ Garden soil	66 ± 0.07 b	The plantlets survived and showed healthy growth after transferring to the natural environment.
Sand	90 ± 0.04 a	Most of the plantlets survived and showed healthy growth after transferring to the natural environment.

Data represents mean value ± standard error (SE) with 50 explants in each treatment. Means with different letters in the same column are significantly different at p=0.01according to Duncan's multiple range test (DMRT).

Figure 6.1: Vigorous plantlets with well-developed roots (complete plantlets) on MS basal medium were selected and prepared for the acclimatization process.

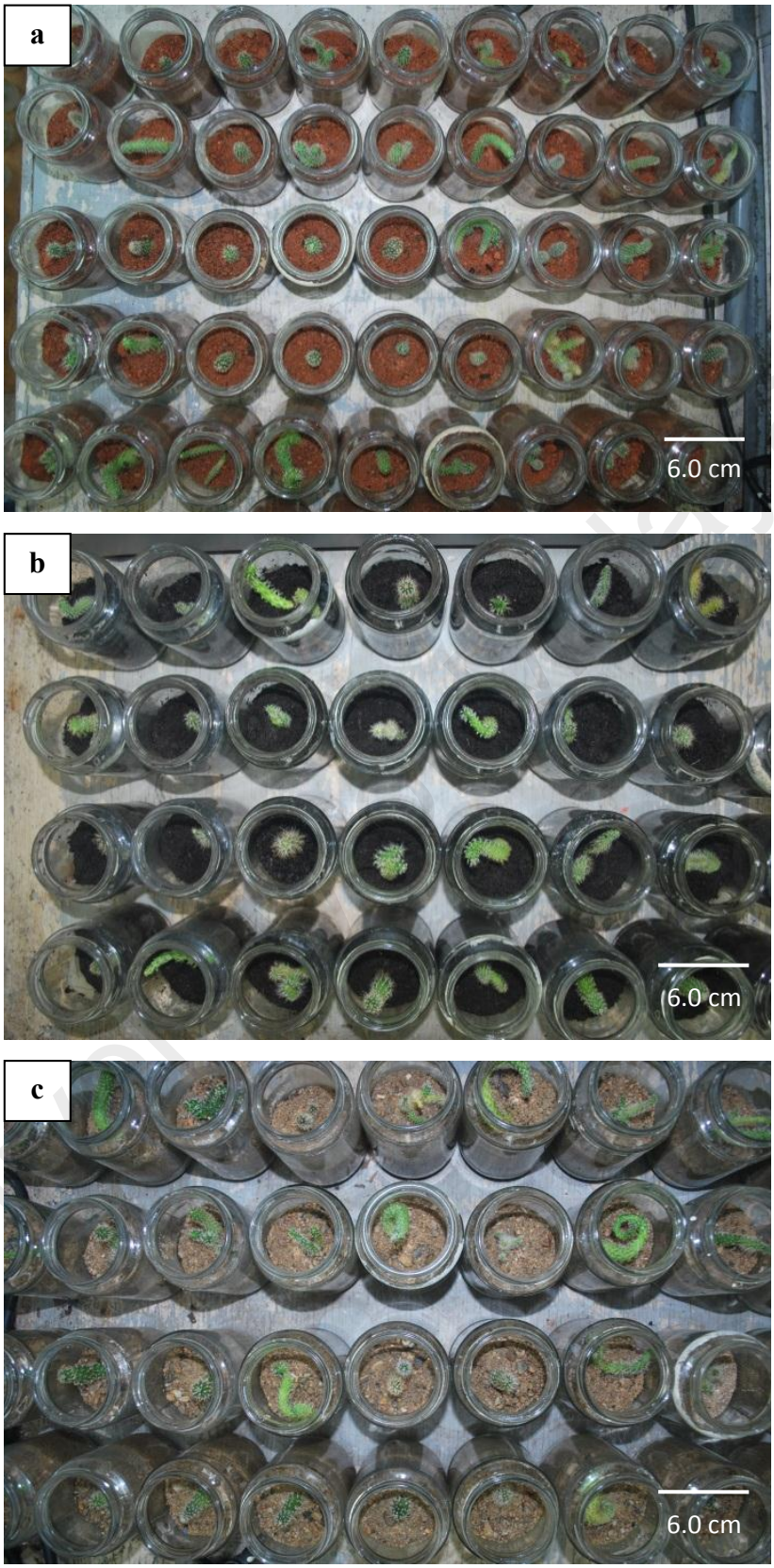


Figure 6.2: Complete plantlets acclimatized in different planting substrates such as (a) red soil, (b) black/ garden soil and (c) sand in jam jars for a few weeks before being transferred to round plastic pots

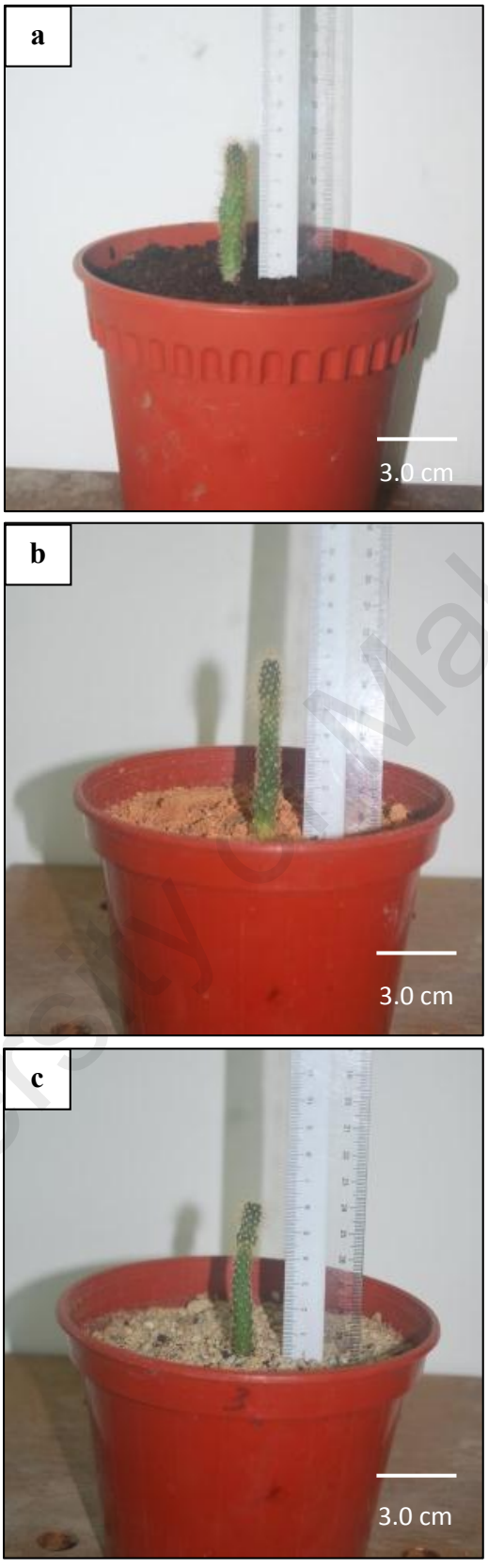


Figure 6.3: The *ex vitro* plants managed to survive and grow vigorously in the natural environment, which showed about 1.0 cm of height increment after 9 months.

6.3.2 Analysis of Planting or Growing Substrates

Different types of plants require different planting substrates for growth. Planting substrates possess various major and trace elements which play important role to support plant growth and development. An adequate supply of nutrients essential to ensure optimum growing condition for plants while good soil structures provide an adequate porosity to support root growth. In this study, analysis using XRF (X-ray fluorescence) showed the percentage (%) of elements distribution in various planting substrates (Table 6.2).

Overall, a few major elements commonly found in the planting substrates including Al_2O_3, SiO_2, SO_3, CaO and Fe_2O_3 whereas only two trace elements were detected such as Cl and Br. Observations on red soil showed the highest element identified was SiO_2 with 49%, then followed by Al_2O_3 with 42% and Fe_2O_3 with 7% whilst the rest gave less than 1%. In contrast, the highest element discovered in black/ garden soil was CaO with 72%. Other elements obtained such as Fe_2O_3 and SO_3 with 6% each, SiO_2 w ith 5%, K_2O with 3%, and the rest showed less than 3%. Nevertheless, higher elements detected in sand were similar to red soil such as Al_2O_3 and SiO_2. The highest element obtained in sand was SiO_2 with 80% which almost 2 times higher than in red soil. However, Al_2O_3 detected in sand was 13% which roughly 3 times lower than in red soil. In addition, the percentage of K_2O in sand was similar to black soil, 3% and the rest of elements detected in sand were less than 2%. Therefore, the two major elements identified which plays an important role for the growth of *Echinocereus cinerascens* were Al_2O_3 and SiO_2.

Table 6.2: Percentage (%) of various major and trace elements detected in different types of planting substrates.

Types of Planting Substrates	Red soil	Black soil	Sand
Elements		Value (%)	
Sodium oxide (Na_2O)	0.053	0.330	0.250
Magnesium oxide (MgO)	0.000	1.983	0.301
Aluminium oxide (Al_2O_3)	41.921	2.212	13.124
Silicon oxide (SiO_2)	48.687	5.371	80.425
Phosphorus pentoxide (P_2O_5)	0.156	1.384	0.090
Sulfur trioxide (SO_3)	0.221	5.709	0.067
Potassium oxide (K_2O)	0.362	2.596	3.043
Calcium oxide (CaO)	0.518	71.590	0.104
Titanium dioxide (TiO_2)	0.781	0.540	0.548
Manganese (II) oxide (MnO)	0.018	0.211	0.053
Iron (III) oxide (Fe_2O_3)	7.028	6.451	1.839
Zinc oxide (ZnO)	0.018	0.128	0.009
Strontium oxide (SrO)	0.005	0.128	0.000
Zirconium dioxide (ZrO_2)	0.060	0.045	0.031
Barium oxide (BaO)	0.000	0.167	0.018
Lead (IV) oxide (PbO)	0.008	0.120	0.006
Chlorine (Cl)	0.032	0.801	0.041
Bromine (Br)	0.000	0.234	0.000
Niobium pentoxide (Nb_2O_5)	0.011	0.000	0.020
Rubidium oxide (Rb_2O)	0.004	0.000	0.028
Yttrium (III) oxide (Y_2O_3)	0.008	0.000	0.003
Tin (IV) oxide (SnO_2)	0.020	0.000	0.000
Thallium (III) oxide (Tl_2O_3)	0.002	0.000	0.000
Thorium dioxide (ThO_2)	0.016	0.000	0.000
Gallium (III) oxide (Ga_2O_3)	0.058	0.000	0.000
Arsenic (III) oxide (As_2O_3)	0.013	0.000	0.000

6.4 SUMMARY OF RESULTS

1. MS medium was suggested to be the suitable medium for rooting *in vitro* of *Echinocereus cinerascens* as a high percentage of plantlets exhibited healthy growth with well-developed roots (complete plantlets).
2. Complete plantlets of *Echinocereus cinerascens* were successfully acclimatized in red soil, black/ garden soil and sand with different survival rates which showed healthily and vigorously growth after 4 months exposed to the natural environment.
3. After 9 months being acclimatized, the highest survival rate of plantlets recorded was 90%, observed in sand, then followed by red soil, 84% and black/ garden soil, 66%. Therefore, sand was identified as the most suitable planting substrate for acclimatization of *Echinocereus cinerascens.*
4. The survival rate was influenced by types of planting substrates applied which varies in nutrients supplies and very important for the establishment of plant growth.
5. Major and trace elements in red soil, black/ garden soil and sand were detected by XRF analysis. The XRF was used to analyse the element's total compositions in each of the planting substrates.
6. The highest major elements detected in sand and red soil were similar, SiO_2 which showed 80% and 49% respectively. Then, followed by Al_2O_3 with 13% in sand while 42% in red soil. In contrast, the highest major elements obtained in black soil was CaO with 72%, whereas for SiO_2 and Al_2O_3 corresponded to 5% and 2%.

CHAPTER 7

COLOURED CALLUS INDUCTION OF *Echinocereus cinerascens*

7.1 EXPERIMENTAL AIMS

Callus is the undifferentiated cell that usually occurs at the cut surfaces and is thought to be a protective response by the plant to seal off damaged tissues. In plant tissue culture, callus is induced by placing the explants on solid culture media. Callus formed from proliferating cells at the cut surfaces of the explant cultured on appropriate growth medium. Callus formation *in vitro* could be enhanced by the presence of exogenous PGRs in the medium that promote cell division and elongation. Skoog and Miller (1957), Skoog and Armstrong (1970) and Akiyoshi *et al*. (1983) established the importance of PGRs (auxins and cytokinins) in plant tissue culture to induce and initiate cell division and callus formation.

Many cactus species produce an excess of auxin *in vitro* which stimulates callus production (Clayton *et al*., 1990). Optimum callus formation of *Opuntia ficus-indica* was observed on MS medium supplemented with 0.9 μM 6-furfurylaminopurine (FAP) + 2.3 μM 2,4-D + 1.0 μM 4-amino 3,5,6-trichloropicolinic acid + 400 mg/l casein hydrolysate (Llamoca-Zárate *et al*., 1999). De Oliveira *et al*. (1995) suggested MS medium supplemented with 18.1 μM 2,4-D + 18.6 or 27.9 μM Kinetin was suitable for callus induction of *Cereus peruvianus* which showed the production of green callus. Further studies on *Cereus peruvianus* revealed that the callus obtained from explants cultured on MS medium containing B5 vitamins supplemented with 4.0 mg/l 2,4-D + 4.0 mg/l Kinetin was used for alkaloid production (De Oliveira and da Silva Machado, 2003). Callus proliferation in *Coryphantha macromeris* was observed when explants were cultured on MS medium supplemented with 44 μM BA + 0.5 μM 2,4-D + 0.4 mg/l thiamine HCl + 100 mg/l i-inositol (Smith *et al*., 1991) while in *Coryphantha elephantidens*, callus was produced in MS medium supplemented with 9.05 μM 2,4-D +

2.3 μM Kinetin (Wakhlu and Bhau, 2000). Wakhlu and Bhau recorded both friable (whitish) and compact callus (green) for explants treated with 9.05 μM 2,4-D + 2.3 μM Kinetin and 9.05 μM 2,4-D applied singly. Optimum callus production in *Mammillaria elongate* was observed in MS supplemented with 0.89 μM BA + 1.07 μM NAA (Papafotiou *et al*., 2001) whereas for *Notocactus magnificus*, which is commonly known as blue cactus, the optimum callus production was obtained on MS medium supplemented with 0.5 μM 2,4-D + 4.4 μM BA + 0.4 mg/l thiamine HCl + 100 mg/l i-inositol (De Medeiros *et al*., 2006). Zhang *et al.* (2013) established the optimum medium for callus induction of *Agave* hybrid, No. 11648 with the highest rate of compact callus (light yellowgreen) obtained in MS supplemented with 2.0 mg/l BA + 0.2 mg/l NAA. In *Selenicereus megalanthus* (yellow pitahaya), callus was induced in MS medium supplemented with 2,4-D, 2,4-D + BAP, TDZ, TDZ + BAP (Caetano Nunez *et al*., 2014). Treatments with 2,4-D and 2,4-D + BAP produced light green to white callus while treatments with TDZ and TDZ + BAP produced green-purple callus.

Previous studies indicated that several factors that influence the induction of callus including genotype, type of explant, nutrient of the medium, PGR, light and temperature. Principally, all the factors could contribute to the induction of different types of callus in a great range of colours. Two types of callus that mostly obtained are compact and friable in structures. Normally, the colour of callus produced is akin to the explant's colour but sometimes the colours are absolutely different.

In this study, the effects of various combinations and concentrations of PGRs were observed in the induction of coloured callus of *Echinocereus cinerascens*. Further investigations on the growth and development of callus were recorded. The ideal requirement for the production of coloured callus was subsequently identified.

7.2 MATERIALS AND METHODS

7.2.1 Source of Explants

In general, methods applied for the production of coloured callus were almost similar to the production of direct *in vitro* regeneration, where the explant preparation technique was slightly modified. The explants (6-month-old plantlets with 6 cm height and 1 cm diameter) were peeled before sectioning into square size in order to obtain optimum production of coloured callus. This procedure was quite difficult since there would be slippery conditions with the sticky mucilage produced by the explants. Small pieces of explants were transferred cautiously to avoid injury as the explants became more friable. The explants were cultured on the media prepared in sterile containers. Production of coloured callus was observed after 2 months for the mean (%) explants forming callus (green/ yellow/ pink).

7.2.2 Preparation of Culture Media

Preparation of culture medium for the production of coloured callus was similar with the procedures in the production of direct *in vitro* regeneration. Preparation of MS medium includes the addition of 4.4 g/l of MS medium including vitamin (commercial powder) and 30 g/l of sucrose in a conical flask filled with 800 ml of distilled water. The conical flask was placed on the hot plate and the mixture was subsequently stirred with a magnetic stirrer to obtain a homogenized medium solution. The medium solution was refilled with distilled water up to 1 litre. Next, the pH medium was adjusted to 5.7 with either 1.0 N sodium hydroxide (NaOH) or 1.0 N hydrochloride acid (HCl). Subsequently, the medium solution was solidified with 8 g/l agar technical (Agar No.3) and then added with selected synthetic hormones and vitamin. Finally, the medium was autoclaved for 20 minutes at temperature of 121 °C, pressure of 104 kPa (15 psi). After autoclaved, sterilized medium was transferred to the laminar air flow cabinet and 20 ml were dispensed into each 60 ml sterile universal container.

7.2.3 Culture Conditions

Standard culture condition applied in the production of direct *in vitro* regeneration was also applied in the production of coloured callus. Apparatus needed for culturing were cleaned and wrapped with aluminium foils before subjected to autoclave for 20 minutes to be sterilized. The culturing process was done under sterile condition, in the laminar flow cabinet that has been exposed to UV light for 15 minutes to disinfect and avoid infections. After culturing, the cultures were kept and maintained in the culture room at 25 ± 2 °C under 16 hours light provided by cool daylight or fluorescent light (36W), PHILIPS.

7.2.4 Effects of NAA and BAP Applied Singly and in Combination on Coloured Callus Induction

All treatments or concentrations of NAA and BAP applied singly and in combinations tested throughout this study were prepared as listed below;

1. MS basal (medium without hormone/ control)
2. MS + 0.5 mg/l NAA
3. MS + 1.0 mg/l NAA
4. MS + 1.5 mg/l NAA
5. MS + 2.0 mg/l NAA
6. MS + 0.5 mg/l BAP
7. MS + 1.0 mg/l BAP
8. MS + 1.5 mg/l BAP
9. MS + 2.0 mg/l BAP
10. MS + 0.5 mg/l NAA + 0.5 mg/l BAP
11. MS + 0.5 mg/l NAA + 1.0 mg/l BAP
12. MS + 0.5 mg/l NAA + 1.5 mg/l BAP
13. MS + 0.5 mg/l NAA + 2.0 mg/l BAP

14. MS + 1.0 mg/l NAA + 0.5 mg/l BAP
15. MS + 1.0 mg/l NAA + 1.0 mg/l BAP
16. MS + 1.0 mg/l NAA + 1.5 mg/l BAP
17. MS + 1.0 mg/l NAA + 2.0 mg/l BAP
18. MS + 1.5 mg/l NAA + 0.5 mg/l BAP
19. MS + 1.5 mg/l NAA + 1.0 mg/l BAP
20. MS + 1.5 mg/l NAA + 1.5 mg/l BAP
21. MS + 1.5 mg/l NAA + 2.0 mg/l BAP
22. MS + 2.0 mg/l NAA + 0.5 mg/l BAP
23. MS + 2.0 mg/l NAA + 1.0 mg/l BAP
24. MS + 2.0 mg/l NAA + 1.5 mg/l BAP
25. MS + 2.0 mg/l NAA + 2.0 mg/l BAP

7.2.5 Effects of 2,4-D, 2,4,5-T and 4CPA Applied Singly on Coloured Callus Induction

All treatments or concentrations of 2,4-D, 2,4,5-T and 4CPA applied singly which were tested throughout this study were prepared as listed below;

1. MS basal (medium without hormone/ control)
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 + 0.1 mg/l 2,4,5-T
7. MS + 0.5 mg/l 2,4,5-T
8. MS + 1.0 mg/l 2,4,5-T
9. MS + 1.5 mg/l 2,4,5-T
10. MS + 0.1 mg/l 4CPA

11. MS + 0.5 mg/l 4CPA
12. MS + 1.0 mg/l 4CPA
13. MS + 1.5 mg/l 4CPA

7.2.6 Effects of 2,4-D and BAP or 2,4-D and Thiamine HCl Applied in Combinations on Coloured Callus Induction

All treatments or concentrations of 2,4-D and BAP or 2,4-D and thiamine HCl applied in combinations tested throughout this study were prepared as listed below;

1. MS basal (medium without hormone/ control)
2. MS + 0.1 mg/l 2,4-D + 0.1 mg/l BAP
3. MS + 0.1 mg/l 2,4-D + 0.5 mg/l BAP
4. MS + 0.1 mg/l 2,4-D + 0.5 mg/l thiamine HCl
5. MS + 0.5 mg/l 2.4-D + 0.1 mg/l BAP
6. MS + 0.5 mg/l 2.4-D + 0.5 mg/l BAP
7. MS + 0.5 mg/l 2.4-D + 0.5 mg/l thiamine HCl
8. MS + 1.0 mg/l 2,4-D + 0.1 mg/l BAP
9. MS + 1.0 mg/l 2,4-D + 0.5 mg/l BAP
10. MS + 1.0 mg/l 2,4-D + 0.5 mg/l thiamine HCl
11. MS + 1.5 mg/l 2,4-D + 0.1 mg/l BAP
12. MS + 1.5 mg/l 2,4-D + 0.5 mg/l BAP
13. MS + 1.5 mg/l 2,4-D + 0.5 mg/l thiamine HCl

7.2.7 Effects of 2,4-D, BAP and Thiamine HCl Applied in Combinations on Coloured Callus Induction

All treatments or concentrations of 2,4-D, BAP and thiamine HCl applied in combinations tested throughout this study were prepared as listed below;

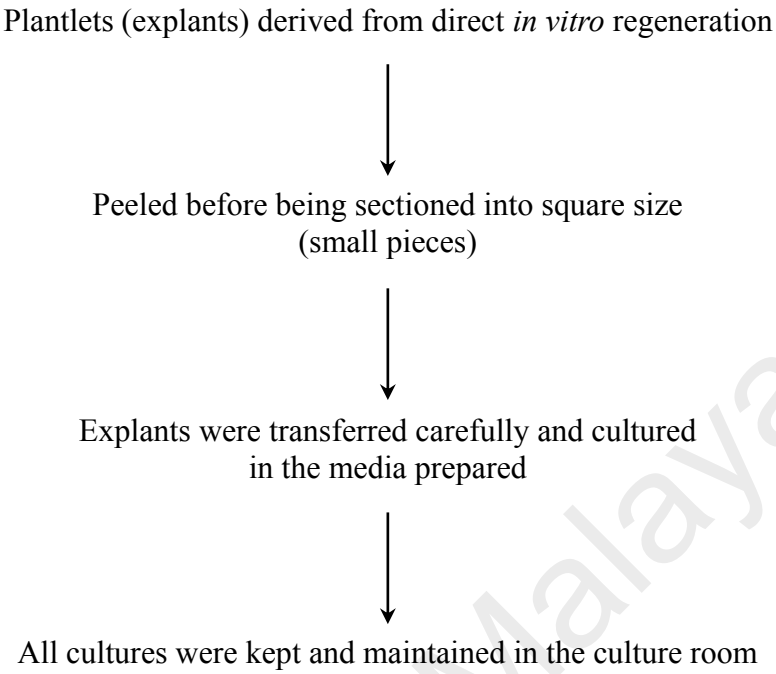
1. MS basal (medium without hormone/ control)
2. MS + 0.1 mg/l 2,4-D + 0.1 mg/l BAP + 0.1 mg/l thiamine HCl

3. MS + 0.1 mg/l 2,4-D + 0.1 mg/l BAP + 0.5 mg/l thiamine HCl
4. MS + 0.1 mg/l 2,4-D + 0.5 mg/l BAP + 0.1 mg/l thiamine HCl
5. MS + 0.1 mg/l 2,4-D + 0.5 mg/l BAP + 0.5 mg/l thiamine HCl
6. MS + 0.1 mg/l 2,4-D + 1.0 mg/l BAP + 0.1 mg/l thiamine HCl
7. MS + 0.1 mg/l 2,4-D + 1.0 mg/l BAP + 0.5 mg/l thiamine HCl
8. MS + 0.5 mg/l 2,4-D + 0.1 mg/l BAP + 0.1 mg/l thiamine HCl
9. MS + 0.5 mg/l 2,4-D + 0.5 mg/l BAP + 0.1 mg/l thiamine HCl
10. MS + 0.5 mg/l 2,4-D + 1.0 mg/l BAP + 0.1 mg/l thiamine HCl
11. MS + 0.5 mg/l 2,4-D + 0.1 mg/l BAP + 0.5 mg/l thiamine HCl
12. MS + 0.5 mg/l 2,4-D + 0.5 mg/l BAP + 0.5 mg/l thiamine HCl
13. MS + 0.5 mg/l 2,4-D + 1.0 mg/l BAP + 0.5 mg/l thiamine HCl
14. MS + 1.0 mg/l 2,4-D + 0.1 mg/l BAP + 0.1 mg/l thiamine HCl
15. MS + 1.0 mg/l 2,4-D + 0.5 mg/l BAP + 0.1 mg/l thiamine HCl
16. MS + 1.0 mg/l 2,4-D + 1.0 mg/l BAP + 0.1 mg/l thiamine HCl
17. MS + 1.0 mg/l 2,4-D + 0.1 mg/l BAP + 0.5 mg/l thiamine HCl
18. MS + 1.0 mg/l 2,4-D + 0.5 mg/l BAP + 0.5 mg/l thiamine HCl
19. MS + 1.0 mg/l 2,4-D + 1.0 mg/l BAP + 0.5 mg/l thiamine HCl

7.2.8 Data Analysis

Data obtained were statistically analysed using Duncan's Multiple Range Test (DMRT). Means with different letters in the same column differ significantly at p=0.01.

7.2.9 Experimental Outline



7.3 RESULTS

The explants were peeled before being sectioned into small pieces (Figure 7.1) to obtain optimum production of coloured callus. Callus developed and formed on the cut surfaces of the explants in all treatments. Initiation of coloured callus from explants was detected as early as the first week of culture. Callus textures were examined and the observation confirmed that all coloured callus produced were compact.

7.3.1 Effects of NAA and BAP Applied Singly and in Combination on Coloured Callus Induction

Analysis of the results (Table 7.1 and 7.2) showed that the addition of hormones such as NAA and BAP to MS medium, either singly or in combinations, plays an important role in the production of coloured callus. More explants produced green callus (83-100%) when cultured in media with hormones applied singly compared to explants cultured in hormone combinations (30-67%), while 83% of explants produced green callus in control. The highest rate of green callus production (Figure 7.2), 100%, was observed in the treatments with 1.5 or 2.0 mg/l NAA only and with 0.5, 1.5, or 2.0 mg/l BAP only (Table 7.1). However, more explants produced yellow callus in hormone combinations (27-100% compared to 63% in control) while in treatments with hormones applied singly, only13-33% produced yellow callus. All explants (100%) produced yellow callus (Figure 7.3) in media with the hormone combinations such as 1.0 mg/l NAA + 2.0 mg/l BAP, 1.5 mg/l NAA + 2.0 mg/l BAP, 2.0 mg/l NAA + 1.5 mg/l BAP, and 2.0 mg/l NAA + 2.0 mg/l BAP. Production of pink callus was less than green and yellow callus. In treatments with hormones applied singly, 7-63% of explants produced pink callus, whereas 0% in control, and 3-40% of explants produced pink callus in treatments with hormone combinations. Even though the highest percentage of pink callus (Figure 7.4) was 63% observed in MS medium fortified with 2.0 mg/l NAA, however, a few treatments with hormones applied singly resulted in 0% of pink callus.

Interestingly, dramatic changes of coloured callus were monitored and it was noted that coloured callus varied with age. This particular aspect could be seen clearly in the conversion of green to pink callus within 2 months of culture and the pink callus would revert to green (Figure 7.5) after 3 months.

Table 7.1: Effects of different concentrations of single hormones (NAA or BAP) on the mean number of explants producing coloured callus (%).

	Green	Yellow	Pink
MS + Hormones (mg/l)	Mean explants produced callus (%)	Mean explants produced callus (%)	Mean explants produced callus (%)
Control	$83 \pm 0.07_{b}$	$63 \pm 0.09_{a}$	$0 \pm 0.00_{b}$
0.5 NAA	$93 \pm 0.05_{ab}$	$13 \pm 0.06_{b}$	$0 \pm 0.00_{b}$
1.0 NAA	$93 \pm 0.05_{ab}$	$17 \pm 0.07_{b}$	$7 \pm 0.05_{b}$
1.5 NAA	$100 \pm 0.00_{a}$	$33 \pm 0.09_{b}$	$13 \pm 0.06_{b}$
2.0 NAA	$100 \pm 0.00_{a}$	$13 \pm 0.06_{b}$	$63 \pm 0.09_{a}$
0.5 BAP	$100 \pm 0.00_{a}$	$13 \pm 0.06_{b}$	$0 \pm 0.00_{b}$
1.0 BAP	$83 \pm 0.07_{b}$	$20 \pm 0.07_{b}$	$7 \pm 0.05_{b}$
1.5 BAP	$100 \pm 0.00_{a}$	$13 \pm 0.06_{b}$	$7 \pm 0.05_{b}$
2.0 BAP	$100 \pm 0.00_{a}$	$13 \pm 0.06_{b}$	$0 \pm 0.00_{b}$

Data represents mean value ± standard error (SE) with 30 explants in each treatment. Means with different letters in the same column are significantly different at p=0.01according to Duncan's multiple range test (DMRT).

Table 7.2: Effects of different concentrations and combinations of hormones (NAA and BAP) on the mean number of explants producing coloured callus (%).

MS + Hormones (mg/l)	Green	Yellow	Pink
	Mean explants produced callus (%)	Mean explants produced callus (%)	Mean explants produced callus (%)
control	$83 \pm 0.07_{a}$	$63 \pm 0.09_{cd}$	$0 \pm 0.00_{b}$
0.5 NAA + 0.5 BAP	$33 \pm 0.09_{b}$	$50 \pm 0.09_{de}$	$7 \pm 0.05_{b}$
0.5 NAA + 1.0 BAP	$30 \pm 0.09_{b}$	$93 \pm 0.05_{ab}$	$13 \pm 0.06_{ab}$
0.5 NAA + 1.5 BAP	$50 \pm 0.09_{ab}$	$67 \pm 0.09_{bcd}$	$27 \pm 0.08_{ab}$
0.5 NAA + 2.0 BAP	$30 \pm 0.09_{b}$	$97 \pm 0.03_{a}$	$7 \pm 0.05_{b}$
1.0 NAA + 0.5 BAP	$47 \pm 0.09_{ab}$	$53 \pm 0.09_{cde}$	$27 \pm 0.08_{ab}$
1.0 NAA + 1.0 BAP	$43 \pm 0.09_{b}$	$97 \pm 0.03_{a}$	$27 \pm 0.08_{ab}$
1.0 NAA + 1.5 BAP	$67 \pm 0.09_{ab}$	$73 \pm 0.08_{abcd}$	$40 \pm 0.09_{a}$
1.0 NAA + 2.0 BAP	$47 \pm 0.09_{ab}$	$100 \pm 0.00_{a}$	$7 \pm 0.05_{b}$
1.5 NAA + 0.5 BAP	$43 \pm 0.09_{b}$	$27 \pm 0.08_{e}$	$7 \pm 0.05_{b}$
1.5 NAA + 1.0 BAP	$33 \pm 0.09_{b}$	$50 \pm 0.09_{de}$	$20 \pm 0.07_{ab}$
1.5 NAA + 1.5 BAP	$67 \pm 0.09_{ab}$	$80 \pm 0.07_{abc}$	$20 \pm 0.07_{ab}$
1.5 NAA + 2.0 BAP	$30 \pm 0.09_{b}$	$100 \pm 0.00_{a}$	$3 \pm 0.03_{b}$
2.0 NAA + 0.5 BAP	$60 \pm 0.09_{ab}$	$57 \pm 0.09_{cd}$	$20 \pm 0.07_{ab}$
2.0 NAA + 1.0 BAP	$53 \pm 0.09_{ab}$	$57 \pm 0.09_{cd}$	$17 \pm 0.07_{ab}$
2.0 NAA + 1.5 BAP	$57 \pm 0.09_{ab}$	$100 \pm 0.00_{a}$	$27 \pm 0.08_{ab}$
2.0 NAA + 2.0 BAP	$67 \pm 0.09_{ab}$	$100 \pm 0.00_{a}$	$27 \pm 0.08_{ab}$

Data represents mean value ± standard error (SE) with 30 explants in each treatment. Means with different letters in the same column are significantly different at p=0.01according to Duncan's multiple range test (DMRT).

7.3.2 Effects of 2,4-D, 2,4,5-T and 4CPA Applied Singly on Coloured Callus Induction

Analysis of coloured callus on single hormones (Table 7.3) such as 2,4-D, 2,4,5-T and 4CPA revealed that explants subjected to all treatments tested produced green and yellow callus in the range of 27-100% and 20-100%, respectively. It was found that 100% of explants produced green callus in all media treated with (0.1, 0.5, 1.0 or 1.5 mg/l) 2,4-D, in two media treated with (0.1 or 0.5 mg/l) 2,4,5-T, and also in a medium treated with 1.0 mg/l 4CPA. In contrast, 100% of yellow callus was observed only when the explants were subjected to a medium treated with 1.5 mg/l 4CPA. Unfortunately, not even one of the treatments produced 100% of the pink callus. The highest production of pink callus was only 27%, which was detected when explants were cultured in media treated with 0.5 mg/l 2,4-D and 1.0 mg/l 2,4,5-T (Figure 7.6). Even though the explants produced pink callus in almost all treatments tested, however the result was unsatisfactory, with 7-27% only. Therefore, further studies were carried out to investigate the effects of combinations of hormones, and also the addition of vitamin in media for the production of coloured callus.

Table 7.3: Effects of different concentrations of single hormones (2,4-D or 2,4,5-T or 4CPA) on the mean number of explants producing coloured callus (%).

	Green	Yellow	Pink
MS + hormones (mg/l)	Mean explant produced callus (%)	Mean explant produced callus (%)	Mean explant produced callus (%)
control	83 ± 0.07 a	63 ± 0.09 b	0 ± 0.00 b
0.1 2,4-D	100 ± 0.00 a	33 ± 0.09 bc	0 ± 0.00 b
0.5 2,4-D	100 ± 0.00 a	53 ± 0.09 bc	27 ± 0.08 a
1.0 2,4-D	100 ± 0.00 a	20 ± 0.07 d	7 ± 0.05 ab
1.5 2,4-D	100 ± 0.00 a	40 ± 0.09 bc	7 ± 0.05 ab
0.1 2,4,5-T	100 ± 0.00 a	60 ± 0.09 b	13 ± 0.06 ab
0.5 2,4,5-T	100 ± 0.00 a	37 ± 0.09 bc	20 ± 0.07 ab
1.0 2,4,5-T	87 ± 0.06 a	53 ± 0.09 bc	27 ± 0.08 a
1.5 2,4,5-T	27 ± 0.08 b	63 ± 0.09 b	20 ± 0.07 ab
0.1 4CPA	83 ± 0.07 a	40 ± 0.09 bc	23 ± 0.08 ab
0.5 4CPA	90 ± 0.06 a	67 ± 0.09 b	7 ± 0.05 ab
1.0 4CPA	100 ± 0.00 a	50 ± 0.09 bc	0 ± 0.00 b
1.5 4CPA	40 ± 0.09 b	100 ± 0.00 a	0 ± 0.00 b

Data represents mean value ± standard error (SE) with 30 explants in each treatment. Means with different letters in the same column are significantly different at p=0.01according to Duncan's multiple range test (DMRT).

7.3.3 Effects of 2,4-D and BAP or 2,4-D and Thiamine HCl Applied in Combinations on Coloured Callus Induction

Analysis on the effects of 2,4-D applied in combination of BAP or thiamine HCl (Table 7.4) in the production of coloured callus showed that the explants in all treatments produced green (33-100%) and yellow (13-100%) callus which were comparable with the previous study (effects of 2,4-D, 2,4,5-T, 4CPA applied singly). The previous study indicated that explants produced 100% green callus in all media treated with 2,4-D applied singly. Additionally, it was found that the highest production of green callus, 100% were also detected in all media with 2,4-D and thiamine HCl in combinations, such as MS medium supplemented with 0.1, 0.5, 1.0 or 1.5 mg/l 2,4-D + 0.5 mg/l thiamine HCl. Meanwhile, the highest production of yellow callus, 100% was detected in MS media supplemented with 1.0 mg/l 2,4-D + 0.1 mg/l BAP. Besides, the observation also recorded almost all media combinations showed that less than 50% of explants produced yellow callus. Aside from green callus, the pink callus could also be seen in all media with 2,4-D and thiamine HCl in combinations while only in two media supplemented with 2,4-D and BAP in combination (0.1 or 0.5 mg/l 2,4-D + 0.1 mg/l BAP). In general, the production of pink callus was in the range of 7-33% which showed a slight increase from the previous study (effects of 2,4-D, 2,4,5-T, 4CPA applied singly). As noted, the highest production of pink callus, 33% was obtained when the explants were subjected to the MS medium fortified with 1.0 mg/l 2,4-D + 0.5 mg/l thiamine HCl. Investigation on the dramatic changes of coloured callus verified that similar occurrence was studied in all the production of pink callus (Figure 7.7). As noted previously (effects of NAA and BAP applied singly and in combinations), the explants that produced green callus turned to pink callus after 2 months. Nevertheless, the pink callus faded by time and eventually appears green after 4 months.

Table 7.4: Effects of different concentrations and combinations of hormones (2,4-D and BAP) or combinations of hormone and vitamin (2,4-D and thiamine HCl) on the mean number of explants producing coloured callus (%).

	Green	Yellow	Pink
MS + hormones (mg/l)	Mean explant produced callus (%)	Mean explant produced callus (%)	Mean explant produced callus (%)
control	83 ± 0.07ab	63 ± 0.09 bcd	0 ± 0.00 c
0.1 2,4-D + 0.1 BAP	93 ± 0.05 a	33 ± 0.09 de	23 ± 0.08 ab
0.1 2,4-D + 0.5 BAP	67 ± 0.09 bc	43 ± 0.09 cde	0 ± 0.00 c
0.1 2,4-D + 0.5 thiamine	100 ± 0.00 a	13 ± 0.06 e	13 ± 0.06 abc
0.5 2.4-D + 0.1 BAP	87 ± 0.06 ab	30 ± 0.09 de	30 ± 0.09 a
0.5 2.4-D + 0.5 BAP	40 ± 0.09 d	83 ± 0.07 ab	0 ± 0.00 c
0.5 2.4-D + 0.5 thiamine	100 ± 0.00 a	47 ± 0.09 cde	7 ± 0.05 bc
1.0 2,4-D + 0.1 BAP	33 ± 0.09 d	100 ± 0.00 a	0 ± 0.00 c
1.0 2,4-D + 0.5 BAP	87 ± 0.06 ab	47 ± 0.09 cde	0 ± 0.00 c
1.0 2,4-D + 0.5 thiamine	100 ± 0.00 a	47 ± 0.09 cde	33 ± 0.09 a
1.5 2,4-D + 0.1 BAP	77 ± 0.08 ab	77 ± 0.08 abc	0 ± 0.00 c
1.5 2,4-D + 0.5 BAP	47 ± 0.09 cd	47 ± 0.09 cde	0 ± 0.00 c
1.5 2,4-D + 0.5 thiamine	100 ± 0.00 a	27 ± 0.08 e	27 ± 0.08 ab

Data represents mean value ± standard error (SE) with 30 explants in each treatment. Means with different letters in the same column are significantly different at p=0.01according to Duncan's multiple range test (DMRT). thiamine, thiamine HCl

7.3.4 Effects of 2,4-D, BAP and Thiamine HCl Applied in Combinations on Coloured Callus Induction

Analysis on the effects of 2,4-D, BAP and thiamine HCl applied in combinations (Table 7.5) discovered that 100% of green, yellow and pink callus were produced when the explants were subjected to the media tested. The production of green, yellow and pink callus were comparable with the results in previous studies (effects of 2,4-D, 2,4,5-T, 4CPA applied singly, effects of 2,4-D and BAP or 2,4-D and thiamine HCl applied in combinations**)** which indicated that all treatments produced green and yellow callus while pink callus was produced in several treatments only. Observations recorded that the explants produced green callus in the range of 60-100% (Figure 7.8), 7-100% of yellow (Figure 7.9) and pink callus (Figure 7.10), respectively. It was found that all treatments produced 100% green callus except for the explants subjected to the MS medium supplemented with 0.1 mg/l 2,4-D + 0.5 mg/l BAP + 0.1 mg/l thiamine HCl that produced 60% green callus, which 23% lesser than control. Although this treatment showed the lowest production of green callus, it promoted the highest production of yellow callus (100%). Meanwhile, the highest production of pink callus (100%) was observed when the explants were cultured in two treatments, MS medium supplemented with 0.1 mg/l 2,4-D + 1.0 mg/l BAP + 0.5 mg/l thiamine HCl, and MS medium supplemented with 1.0 mg/l 2,4-D + 0.1 mg/l BAP + 0.1 mg/l thiamine HCl.

Table 7.5: Effects of different concentrations and combinations of hormones (2,4-D and B AP) and vitamin (thiamine HCl) on the mean number of explants producing coloured callus (%).

	Green	Yellow	Pink
MS + hormones (mg/l)	Mean explant produced callus (%)	Mean explant produced callus (%)	Mean explant produced callus (%)
Control	83 ± 0.07b	63 ± 0.09 b	0 ± 0.00 e
0.1 2,4-D + 0.1 BAP + 0.1 thiamine	100 ± 0.00 a	13 ± 0.06 de	0 ± 0.00 e
0.1 2,4-D + 0.1 BAP + 0.5 thiamine	100 ± 0.00 a	40 ± 0.09 bcd	0 ± 0.00 e
0.1 2,4-D + 0.5 BAP + 0.1 thiamine	60 ± 0.09 c	100 ± 0.00 a	0 ± 0.00 e
0.1 2,4-D + 0.5 BAP + 0.5 thiamine	100 ± 0.00 a	27 ± 0.08 cde	43 ± 0.09 cd
0.1 2,4-D + 1.0 BAP + 0.1 thiamine	100 ± 0.00 a	7 ± 0.05 e	77 ± 0.08 b
0.1 2,4-D + 1.0 BAP + 0.5 thiamine	100 ± 0.00 a	20 ± 0.07 cde	100 ± 0.00 a
0.5 2,4-D + 0.1 BAP + 0.1 thiamine	100 ± 0.00 a	7 ± 0.05 e	53 ± 0.09 c
0.5 2,4-D + 0.5 BAP + 0.1 thiamine	100 ± 0.00 a	33 ± 0.09 cde	33 ± 0.09 cd
0.5 2,4-D + 1.0 BAP + 0.1 thiamine	100 ± 0.00 a	20 ± 0.07 cde	0 ± 0.00 e
0.5 2,4-D + 0.1 BAP + 0.5 thiamine	100 ± 0.00 a	47 ± 0.09 bc	20 ± 0.07 de
0.5 2,4-D + 0.5 BAP + 0.5 thiamine	100 ± 0.00 a	13 ± 0.06 de	0 ± 0.00 e
0.5 2,4-D + 1.0 BAP + 0.5 thiamine	100 ± 0.00 a	13 ± 0.06 de	23 ± 0.08 de
1.0 2,4-D + 0.1 BAP + 0.1 thiamine	100 ± 0.00 a	7 ± 0.05 e	100 ± 0.00 a
1.0 2,4-D + 0.5 BAP + 0.1 thiamine	100 ± 0.00 a	13 ± 0.06 de	40 ± 0.09 cd
1.0 2,4-D + 1.0 BAP + 0.1 thiamine	100 ± 0.00 a	33 ± 0.09 cde	7 ± 0.05 e
1.0 2,4-D + 0.1 BAP + 0.5 thiamine	100 ± 0.00 a	13 ± 0.06 de	83 ± 0.07 ab
1.0 2,4-D + 0.5 BAP + 0.5 thiamine	100 ± 0.00 a	7 ± 0.05 e	20 ± 0.07 de
1.0 2,4-D + 1.0 BAP + 0.5 thiamine	100 ± 0.00 a	17 ± 0.07 de	0 ± 0.00 e

Data represents mean value ± standard error (SE) with 30 explants in each treatment. Means with different letters in the same column are significantly different at p=0.01according to Duncan's multiple range test (DMRT). thiamine, thiamine HCl

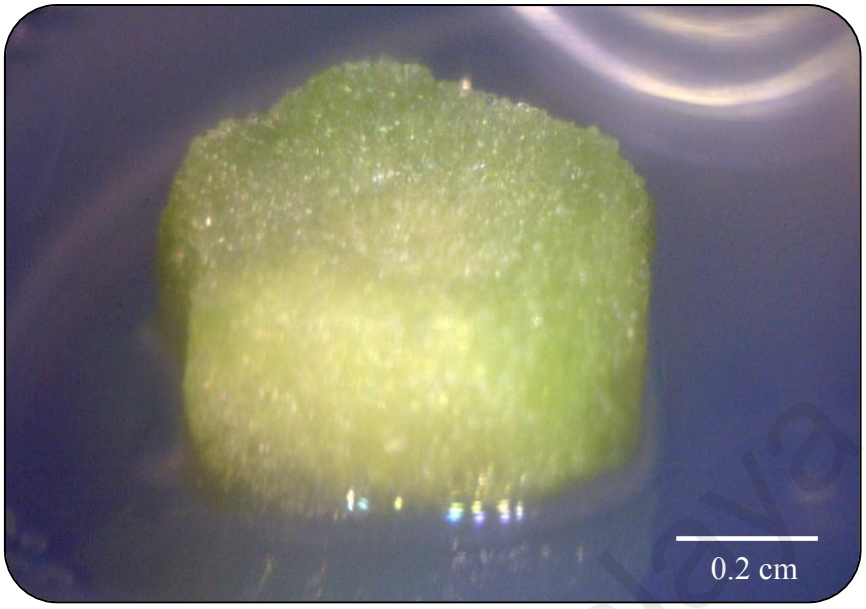


Figure 7.1: Stem explant (peeled before section into small pieces) of *Echinocereus cinerascens* for coloured callus induction.

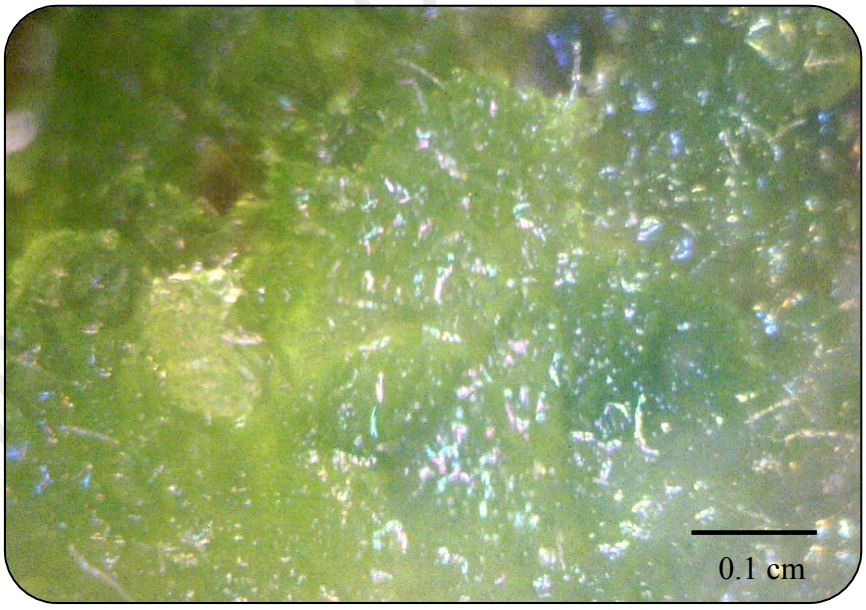


Figure 7.2: Green callus obtained in MS medium with 2.0 mg/l BAP after 2 months of culture.

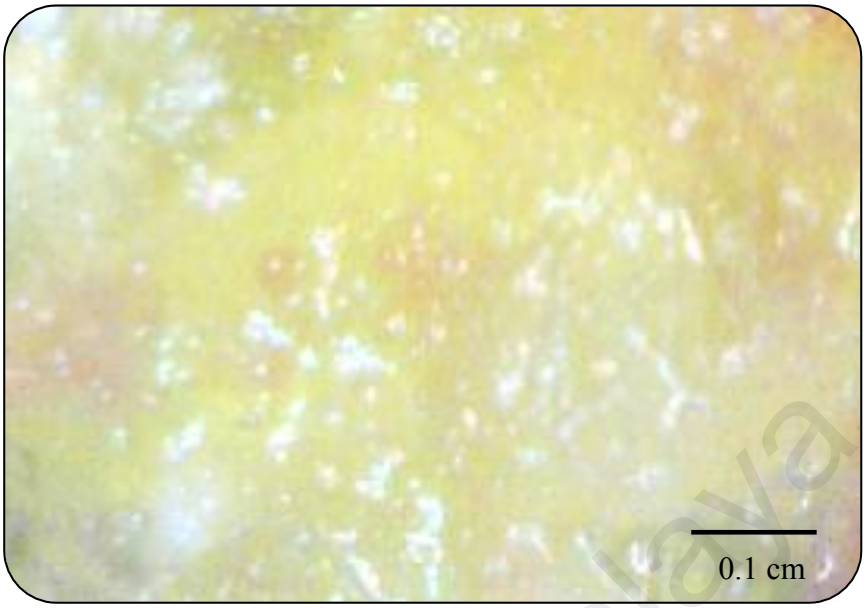


Figure 7.3: Yellow callus obtained in MS medium with 2.0 mg/l NAA + 2.0 mg/l BAP after 2 months of culture.

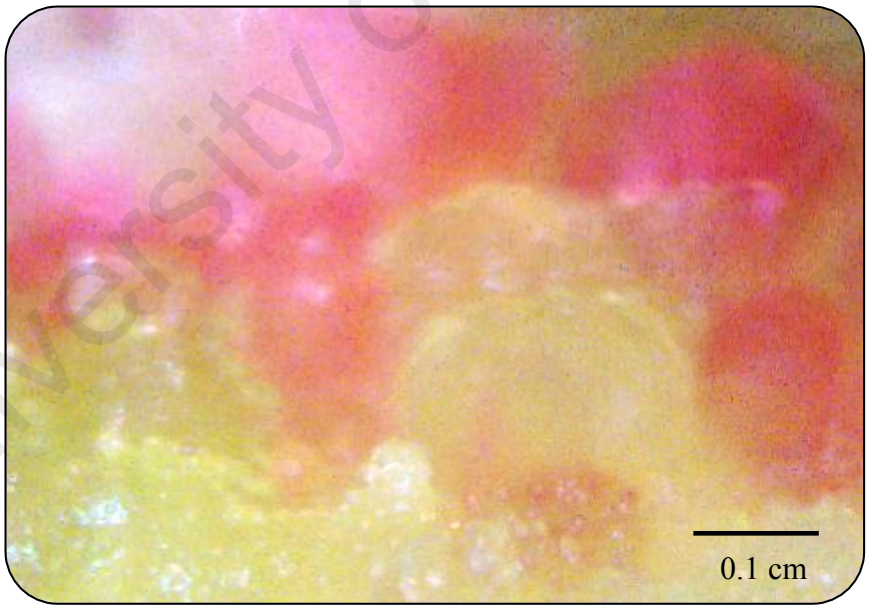


Figure 7.4: Combination of pink and yellow callus obtained in MS medium with 2.0 mg/l NAA after 2 months of culture.

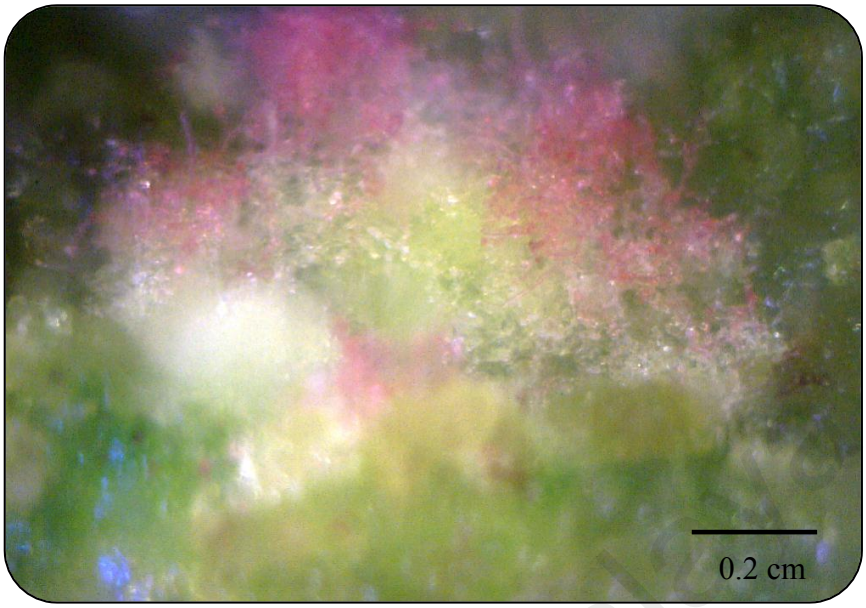


Figure 7.5: Pink callus appeared in MS medium with 2.0 mg/l NAA after 2 months faded by time and displayed green after 4 months of culture.

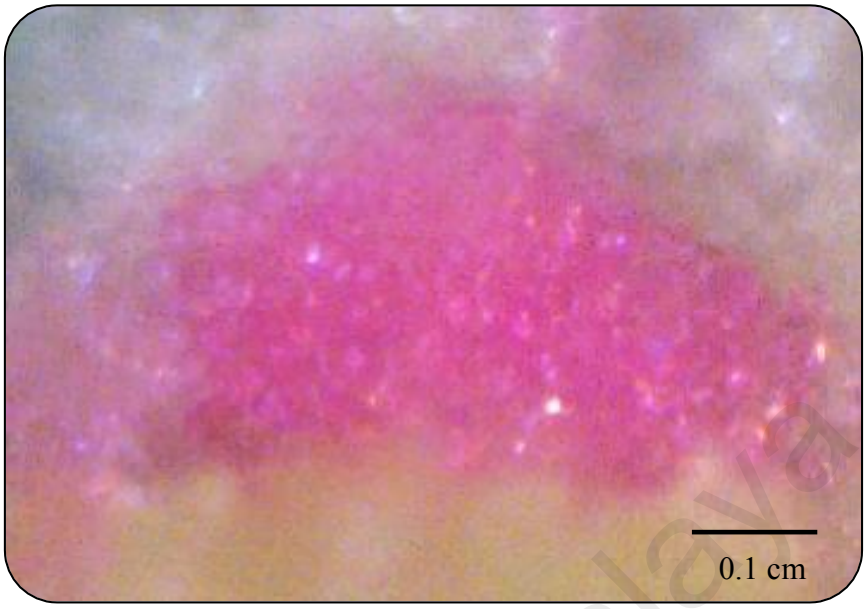


Figure 7.6: Combination of pink and yellow callus obtained in MS medium with 1.0 mg/l 2,4,5-T after 2 months of culture.

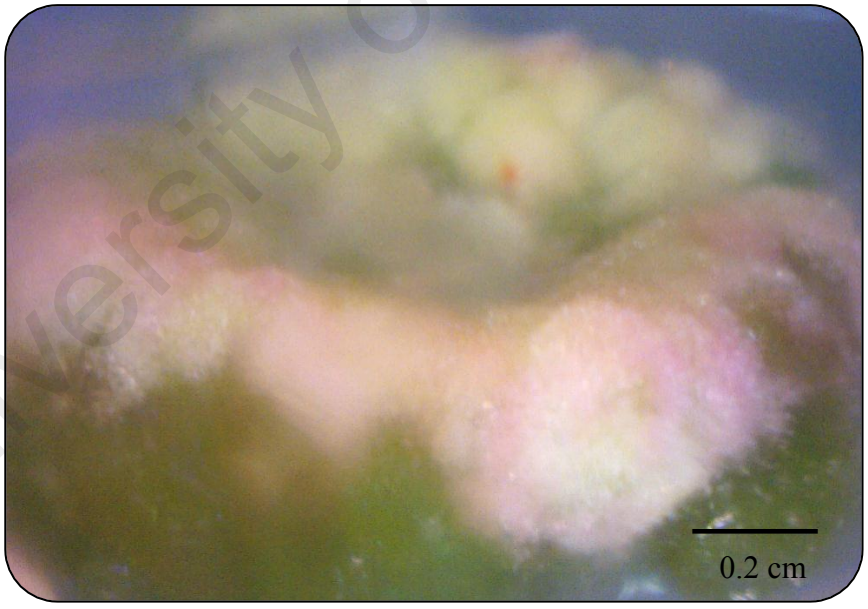


Figure 7.7: Pink callus appears in MS medium with 1.0 mg/l 2,4-D + 0.5 mg/l thiamine HCl after 2 months faded by time and displayed green after 4 months of culture.

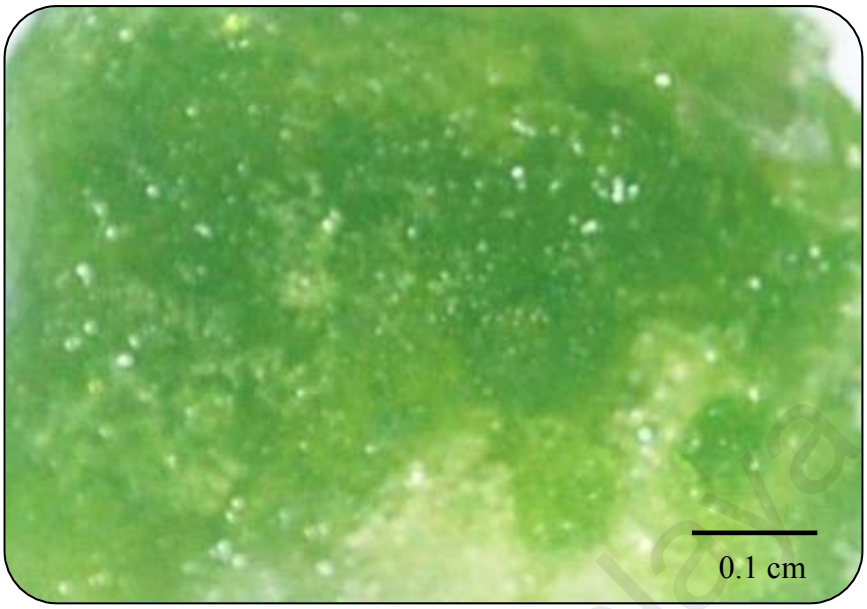


Figure 7.8: Green callus obtained in MS medium with 0.1 mg/l 2,4-D + 0.1 mg/l BAP + 0.1 mg/l thiamine HCl after 2 months of culture.

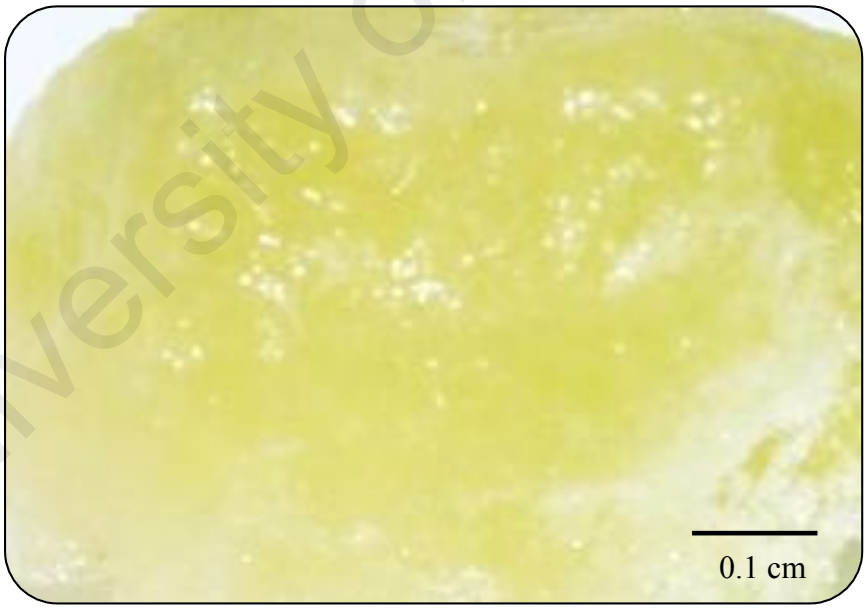


Figure 7.9: Yellow callus obtained in MS medium with 0.1 mg/l 2,4-D + 0.5 mg/l BAP + 0.1 mg/l thiamine HCl after 2 months of culture.

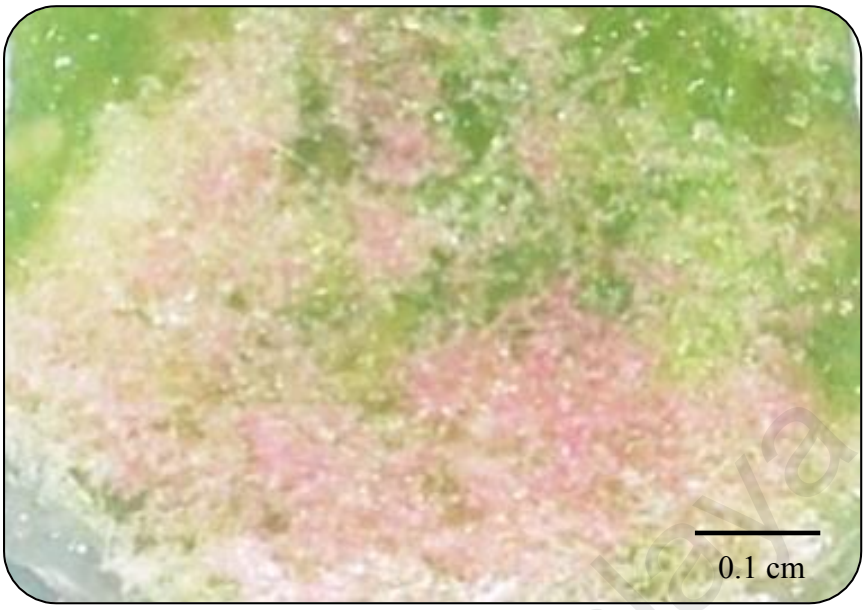


Figure 7.10: Pink callus appears in MS medium with 1.0 mg/l 2,4-D + 0.1 mg/l BAP + 0.1 mg/l thiamine HCl after 2 months faded by time and displayed green after 4 months of culture.

7.4 SUMMARY OF RESULTS

1. Production of coloured callus was affected by concentrations and combinations of plant growth regulators or hormones and vitamin applied in the media.
2. The explants produced 100% of green callus in MS medium supplemented with hormones applied singly such as 4CPA (1 treatment), BAP (3 treatments), 2,4,5-T (2 treatments), NAA (2 treatments), and 2,4-D (4 treatments). Besides, media supplemented with 2,4-D + thiamine HCl (4 treatments) and 2,4-D + BAP + thiamine HCl (17 treatments) also produced 100% of green callus.
3. The explants produced 100% of yellow callus in MS medium supplemented with hormones applied singly such as 4CPA (1 treatment), others in media supplemented with NAA + BAP (4 treatments), 2,4-D + BAP (1 treatment), and 2,4-D + BAP + thiamine HCl (1 treatment).
4. Conversely, the highest production of pink callus, 100% was obtained only when the explants were subjected to MS medium supplemented with 2,4-D + BAP + thiamine HCl (2 treatments).
5. Therefore, MS medium supplemented with 2,4-D + BAP + thiamine HCl applied in combination was identified as the optimum medium for the production of coloured callus since 100% production of green, yellow and pink callus were found in several treatments tested with different concentrations of 2,4-D + BAP + thiamine HCl.
6. Surprisingly, dramatic changes of coloured callus were clearly observed in the conversion of green to pink callus within 2 months of culture and the pink callus faded by time and eventually appears green after 4 months.
7. Observations recorded that green and yellow callus produced after 2 months of culture retain their colour.

CHAPTER 8

PIGMENT EXTRACTION AND DETECTION FROM *IN VITRO* PLANTLETS AND CALLUS OF *Echinocereus cinerascens*

8.1 EXPERIMENTAL AIMS

Majority of the plant species possess valuable compounds which provide tremendous prospects for new drug discoveries because of the unmatched availability of chemical diversity (Cos *et al.*, 2006). These plant species gained critical attention among researches around the world due to their potential application in health benefits, pharmaceutical studies and drug development. Data recorded by the World Health Organization (WHO) revealed that more than 80% of the world's population relies on traditional (herbal) medicine for primary healthcare. Consumption and development of traditional medicines in Asia represent the great interaction of human with the environment aimed to treat chronic and infectious diseases. Researches in related fields indicated that phytochemicals derived from plants commonly nontoxic, which are safe to be consumed orally or applied as poultices. Furthermore, studies on natural pigments which very well known as phytochemicals rich in nutrients and valuable properties have become increasingly popular.

Sun *et al.* (2005) listed benefits of natural pigments to human health such as promoted antioxidant activity, anticancer, antimicrobial, anti-inflammatory etc. According to Rao and Shen (2002), the major interest in carotenoids is not only due to their provitamin A activity but also to their antioxidant action by scavenging oxygen radicals and reducing oxidative stress. Moreover, research suggests that high dietary consumption of carotenoids could prevent lung cancer (Smith, 1998; Wright *et al.*, 2003) and prostate cancer (DePrimo *et al.*, 2001; Giovannucci *et al.*, 2002). Additionally, studies have reported strong correlation between carotenoid intake and reduced risk of diseases such as cancer, atherogenesis, bone calcification, eye

degeneration and neuronal damages (Cantuti-Castelveri *et al*., 2000; Ferguson, 1997; Yamaguchi and Uchiyama, 2003). These indicate that natural pigments can be used commercially in agriculture, medicine, cosmetic or even in the food industry (natural colourant).

Natural pigments promoted high potential applications in health benefits especially for prevention of various diseases. Therefore, this study focuses on the extraction and detection of pigments from *in vitro* plantlets and callus of *Echinocereus cinerascens* by UV-VIS spectrophotometer and HPLC analysis to determine valuable pigments in the samples. UV-VIS spectrophotometer analysis presents an absorption spectrum which provides the first clues for identification of pigments (Appendix V). This method is relatively simple and very useful for rapid estimation of pigments. Normally, the pigments are extracted using different organic solvents such as acetone, methanol, ethanol and diethyl ether. Previous studies indicated that acetone is the most frequently used solvent (Ritchie, 2006), that produces sharper absorption peaks compared to the others. Nevertheless, it is more volatile and flammable. In addition, it is also considered to be a less efficient solvent for pigment extraction (Ritchie, 2006). Hence, in this study different types of organic solvents were tested to determine the most efficient solvent for pigment extraction of *in vitro* plantlets and callus of *Echinocereus cinerascens*.

Subsequently, HPLC analysis was performed since spectroscopic method enables the estimation of total and approximate amount of carotenoids, but not the amounts of individual carotenoids (Mantoura and Llewellyn, 1983). Rodriguez-Amaya and Kimura (2004) suggested reversed-phase HPLC on C18 columns was preferred for separating a mixture of carotenoids, which allows detection and quantification of individual carotenoid (Ferruzzi *et al*., 1998; Unlu *et al*., 2005). HPLC analysis presents a chromatogram of respective carotenoids which detected in the sample. The identified

carotenoid is measured individually according to the linear equation derived from the calibration curve that has been constructed. Several pigments such as chlorophylls (chlorophyll a, chlorophyll b) and carotenoids (xanthophylls and carotenes) together with their chemical structures are included as references (Appendix VI).

8.2 MATERIALS AND METHODS

8.2.1 Source of Explants

Fresh samples of *in vitro* plantlets (from *in vitro* regeneration) and various colour of callus such as green, yellow and pink callus (from coloured callus induction) were used as sources of explants. The explants were cut into small pieces before grinding with solvent extractions to obtain supernatants which contain pigments of interest that were analysed using UV-VIS spectrophotometer. Beside UV-VIS spectrophotometer, pigments were also analysed by HPLC. Conversely, in HPLC analysis, the explants (*in vitro* plantlets and green callus) were sliced into small pieces, freeze dried and then ground into powder form. Afterwards, the powders were solvent-extracted and subjected to several procedures to collect pigments of interest for analysis.

8.2.2 Extraction of Pigments for Spectrophotometer Analysis

Fresh samples of *in vitro* plantlets and coloured callus (green, yellow and pink) were harvested and weighed out to 0.1 or 0.4 g respectively. These samples were immersed separately in 10 ml of various solvent extractions, 100% acetone, 80% acetone, 95% ethanol, 100% methanol and 90% methanol. Subsequently, a small amount of $MgCO_3$ and sand were added to the mixtures to enhance the grinding process in order to extract pigments from the samples. The samples were ground gently with chilled mortar and pestle since the pigments are heat sensitive. This experiment was conducted under dim light due to the pigments' sensitivity to light. The mixtures were kept in the centrifuge tubes which were wrapped with aluminium foil and left in -20 °C refrigerator overnight. The next day, the mixtures were centrifuged at 5000 rpm, 24 °C for 10 min to obtain clear coloured supernatants. The clear coloured supernatants were collected and analysed by Shimadzu UV-1650 PC spectrophotometer for detection of pigments through absorbance spectra. Absorbance readings were taken immediately after the supernatants were collected and 3 months after the supernatants were stored in

-20 °C refrigerator. The experiment was performed thrice at room temperature. Pigment content was calculated following Lichtenthaler formula (Appendix VII) to determine pigment content in the samples and their degradation.

8.2.2.1 Analysis of Pigments Extracted From *In Vitro* Plantlets and Callus (Green) in Different Solvent Extractions

Initially, 0.1 g of fresh samples of *in vitro* plantlets and green callus were extracted using various solvent extractions including 100% acetone, 80% acetone, 95% ethanol, 100% methanol and 90% methanol. Comparisons were made on the wavelength (nm) of peak detected, pigment content (mg/g) such as chlorophyll a, chlorophyll b, total chlorophyll, total carotenoid, total chlorophyll + carotenoid, ratio of chlorophyll a/ chlorophyll b, and ratio of chlorophyll/ carotenoid from the samples (*in vitro* plantlets and green callus) tested. Analysis on the results identified the most efficient solvent extraction for each of the pigments detected.

8.2.2.2 Analysis of Pigments Extracted From Coloured Callus (Green, Yellow, Pink) in 95% Ethanol

In this study, 0.4 g of the coloured callus (green, yellow and pink) were freshly harvested and subsequently extracted using 95% ethanol to obtained pigments of interest. The supernatants which contain pigments of interest were subjected to UV-VIS spectrophotometric analysis. Absorbance results were taken immediately after the supernatants were collected and 3 months after the supernatants were stored in -20 °C refrigerator. Observations recorded comparison of pigment content (mg/g) in different coloured callus including chlorophyll a, chlorophyll b, total chlorophyll, total carotenoid, total chlorophyll + carotenoid, ratio of chlorophyll a/ chlorophyll b, and ratio of chlorophyll/ carotenoid. Besides, stability or degradation of pigments was also investigated.

8.2.3 Extraction of Carotenoids for HPLC Analysis

Fresh samples of *in vitro* plantlets and callus (green) were harvested and sliced into small pieces before subjected to freeze-dried process. After freeze-dried, the samples were ground into a powder form. In this study, the dried samples were extracted according to procedure established by Othman (2009) with some modifications.

Initially, 1.0 g of dried sample was rehydrated with 1.0 ml of distilled water in a clean centrifuge tube. Then, 20 ml of a solvent mixture (Acetone: Methanol, 7:3) was added and then, the centrifuge tube containing sample and solvent was kept overnight in the dark at room temperature to provide an efficient condition for extraction. Next, the tube was vortexed and centrifuged at 5000g (Thermo Scientific Sorvall Biofuge Primo R, Germany) for 2 minutes to collect the supernatant which contains pigments of interest. This procedure was repeated for several times until the sample was completely colourless. The supernatants were mixed together and transferred to a new centrifuge tube wrapped with aluminium foil. Afterwards, an equal volume of hexane and distilled water was added in the supernatants before the mixture was vortexed and centrifuged to separate carotenoids. The upper layer containing carotenoids was pipetted into a small vial to be dried under a gentle stream of oxygen-free nitrogen. Lastly, the vial was capped and sealed with parafilm, then immediately stored at -20 ºC to prevent oxidation and degradation of carotenoids or subsequently processed for further analysis.

8.2.3.1 Saponification of Carotenoid Extract

The dried carotenoid extract in a small vial was re-suspended in 500 µl of ethyl acetate (HPLC grade) and then centrifuged. After being centrifuged, 50 µl of ethyl acetate was collected and subjected to saponification. Saponification was applied to remove hydrolysed carotenoid esters and chlorophylls that might interfere with the

chromatographic separation and shorten the HPLC column's life (Rodriguez-Amaya and Kimura, 2004).

In saponification, 350 µl of a mixture of acetonitrile and water (9:1) was added in a container filled with 50 µl of ethyl acetate consisted carotenoids, which made up the total volume of solution to 400 µl. After that, the solution was added with 400 µl (an equal volume) of methanolic potassium hydroxide (10% w/v). Then, the solution was vortexed and left overnight in the dark at room temperature. The procedure was continued by adding 2 ml of a mixture of hexane and butylated hydroxytoluene (0.1 % BHT) and 2 ml of sodium chloride (10% NaCl). Next, the solution was centrifuged at 5000g for 2 minutes to separate carotenoids. The upper layer of supernatant in yellow colour (carotenoids in hexane) was collected while the residue (chlorophyll and polar compounds) was re-extracted with 2 ml of a mixture of hexane and 0.1% BHT. Re-extraction was done for several times until the upper layer became colourless. Once the yellow supernatant (carotenoids) was completely extracted, a small amount of distilled water was added to the collected supernatant to remove an excess alkali and cleaved esters. This mixture was centrifuged and once again the supernatant was collected. The supernatant was pipetted into a small new centrifuge tube to be dried under a gentle stream of oxygen free nitrogen and then kept in -20 ºC or processed for subsequent studies.

8.2.3.2 Analysis of Carotenoids Extracted From *In Vitro* Plantlets and Callus

Analysis of carotenoids was performed by HPLC (Agilent model 1100 series) system equipped with a binary pump auto sampler injector, micro vacuum degassers, thermostat column compartment and a diode array detector, according to the procedure established by Morris *et al*. (2004) with some modifications. A ZORBAX Eclipse SB-C18 column fitted with an analytical guard column (4.6 x 150 nm x 5 µm) was preferred for the chromatographic separation with the temperature of the column was maintained

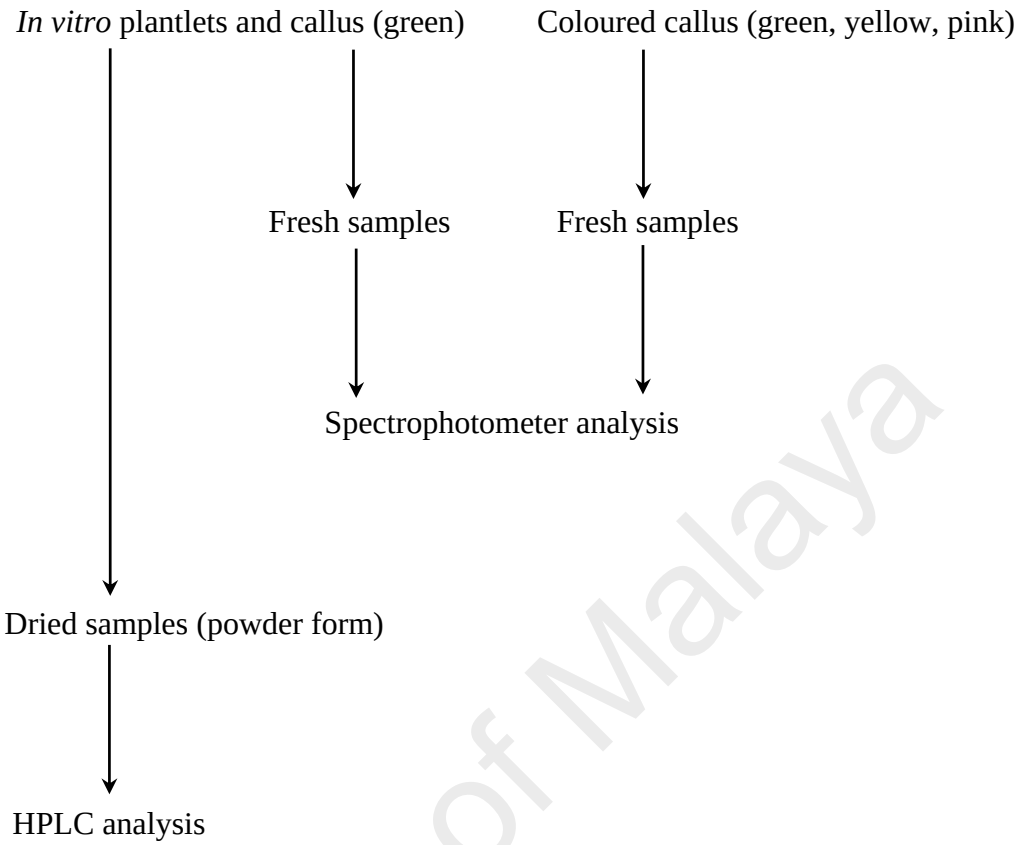
at 20 ºC. The separation of different carotenoids was performed using the solvent gradient that has been developed. Solvent A was consisted a mixture of acetonitrile and distilled water with ratio 9:1 (v/v) while solvent B was ethyl acetate. In this study, the carotenoids were separated using the solvent gradient as follows: 0-40% solvent B (0-20 min), 40-60% solvent B (20-25 min), 60-100% solvent B (25-25.1 min), 100% solvent B (25.1-35 min), 100-0% solvent B (35-35.1 min). Injection volume of sample was 10 µl with the solvent flow rate was adjusted at 1.0 ml/min. The column was allowed to re-equilibrate in 100% solvent A for 10 min prior to the next injection.

In this study, the concentrations of the respective carotenoids were calculated through the calibration curve as described by Morris *et al.* (2004), Kimura and Rodriquez-Amaya, (2002). Identity of each carotenoid was verified by the spectral characteristics depicted at the specific absorption maxima and retention time according to procedures established by Britton *et al*. (1995). Compounds were identified by co-chromatography with standards and also by elucidation of their spectral characteristics using a photo diode array detector. Detection of carotenoid peak was studied in the range of 350 to 550 nm wavelengths. Each of carotenoid concentration was measured per 1.0 g dry weight (mg/g DW). Analysis by HPLC was repeated thrice for both samples tested (*in vitro* plantlets and callus) and the mean of identified carotenoid content was recorded.

8.2.4 Data Analysis

Most of the data obtained in pigment detection were statistically analysed using Duncan's Multiple Range Test (DMRT) except for the result on comparison of pigment content from *in vitro* plantlets and callus (detected by HPLC) which was analysed using independent sample T-test (p=0.01). Means with different letters in the same column differ significantly at p=0.01 for DMRT.

8.2.5 Experimental Outline



8.3 RESULTS

8.3.1 Spectrophotometer Analysis

By using UV-VIS spectrophotometer, the intensity of pigment colour was detected and depicted through visible spectrum. Generally, a pigment presents a shape of spectrum and peak that is unique for a particular solvent. In this study, different types of solvent extractions were tested to investigate the most efficient solvent for pigment extraction of both *in vitro* plantlets and callus. Besides, the pigment content was measured according to Lichtenthaler (1987) using 'Spectroscopic quantification equation' derived from Lambert-Beer law (Wellburn, 1994; Lichtenthaler and Buschmann, 2001) to identify the highest mean of pigments and their ratios. In addition, further study on stability or degradation of pigments was also examined.

8.3.1.1 Analysis of Pigments Extracted From *In Vitro* Plantlets in Different Solvent Extractions

Analysis on pigments extracted from various solvent extractions revealed that two types of pigments detected in *in vitro* plantlets of *Echinocereus cinerascens* were chlorophylls and carotenoids. The pigments were identified through the shapes of absorption spectra which were depicted in various solvent extractions tested (Figure 8.1). Quantitative analysis was done to distinguish the pigment content and their ratios in each of the solvent extraction.

Comparison of chlorophyll a, chlorophyll b, total chlorophyll, total carotenoid and total pigment content which were measured in different solvent extractions (Table 8.1) indicated that 100 % Methanol was the most efficient solvent extraction for *in vitro* plantlets with the highest total pigment content, 64.10 mg/g. It was found that total chlorophyll content was the highest in 100 % Methanol (55.71 mg/g). The greatest contribution was obtained from chlorophyll a content, 40.75 mg/g which was half of the total pigment content in 100 % Methanol. However, the highest chlorophyll b content

was observed in 90 % Methanol (24.87 mg/g). Meanwhile, the highest total carotenoid content was obtained in 100 % acetone (10.55 mg/g). In fact, total pigment content in 100% Acetone was the second highest (after 100 % Methanol), followed by 90 % Methanol, 95 % Ethanol and lastly 80 % Acetone with the total pigment content of 55.23 mg/g, 48.57 mg/g and 39.83 mg/g, respectively. Thus, 100 % acetone was also considered as a good candidate for pigment extraction of *in vitro* plantlets as it showed higher total pigment content (59.70 mg/g).

Analysis on the ratio of pigment content from *in vitro* plantlets measured in various solvent extractions (Table 8.2) showed the highest ratio of chlorophyll a to chlorophyll b content was 3.23 obtained in 100% Acetone while the lowest ratio detected were 1.20 and 1.10 which were observed in 95% Ethanol and 90% Methanol, respectively. In contrast, the highest ratio of chlorophyll to carotenoid content was 19.77 obtained in 95% Ethanol, followed by 90% Methanol with the ratio of 17.30. Meanwhile, the lowest ratio of chlorophyll to carotenoid content, 4.66 was observed in 100% Acetone.

Table 8.1: Comparison of chlorophyll, carotenoid and total pigment content (mg/g fresh weight) for *in vitro* plantlets of *Echinocereus cinerascens* measured in different solvent extractions.

Solvent	Pigment content (mg/g)				
Extraction	Ca	Cb	C(a+b)	C(x+c)	Total
100% Acetone	37.52 ± 0.03b	11.63 ± 0.04d	49.15 ± 0.03c	10.55 ± 0.03a	59.70 ± 0.01b
80% Acetone	22.61 ± 0.02e	11.10 ± 0.01e	33.71 ± 0.01e	6.13 ± 0.04c	39.83 ± 0.02e
95% Ethanol	25.18 ± 0.01d	21.05 ± 0.05b	46.23 ± 0.03d	2.34 ± 0.03e	48.57 ± 0.02d
100% Methanol	40.75 ± 0.03a	14.96 ± 0.03c	55.71 ± 0.06a	8.39 ± 0.02b	64.10 ± 0.05a
90% Methanol	27.34 ± 0.02c	24.87 ± 0.02a	52.21 ± 0.02b	3.02 ± 0.04d	55.23 ± 0.03c

Data represents mean value ± standard error (SE) for three replicates of each treatment. Means with different letters in the same column are significantly different at p = 0.01 according to Duncan's multiple range test (DMRT). Ca, chlorophyll a; Cb, chlorophyll b; C(a+b), total chlorophyll a and b; C(x+c), total carotenoid (xanthophyll and carotene)

Table 8.2: Ratio of pigment content (mg/g fresh weight) for *in vitro* plantlets of *Echinocereus cinerascens* measured in different solvent extractions.

	Pigment content (mg/g)	
Solvent Extraction	Ca/Cb ratio	C(a+b)/C(x+c) ratio
100% Acetone	3.23 ± 0.04a	4.66 ± 0.09e
80% Acetone	2.04 ± 0.03c	5.50 ± 0.05d
95% Ethanol	1.20 ± 0.01d	19.77 ± 0.02a
100% Methanol	2.72 ± 0.03b	6.64 ± 0.03c
90% Methanol	1.10 ± 0.03d	17.30 ± 0.03b

Data represents mean value ± standard error (SE) for three replicates of each treatment. Means with different letters in the same column are significantly different at p = 0.01 according to Duncan's multiple range test (DMRT). Ca, chlorophyll a; Cb, chlorophyll b; C(a+b), total chlorophyll a and b; C(x+c), total carotenoid (xanthophyll and carotene)

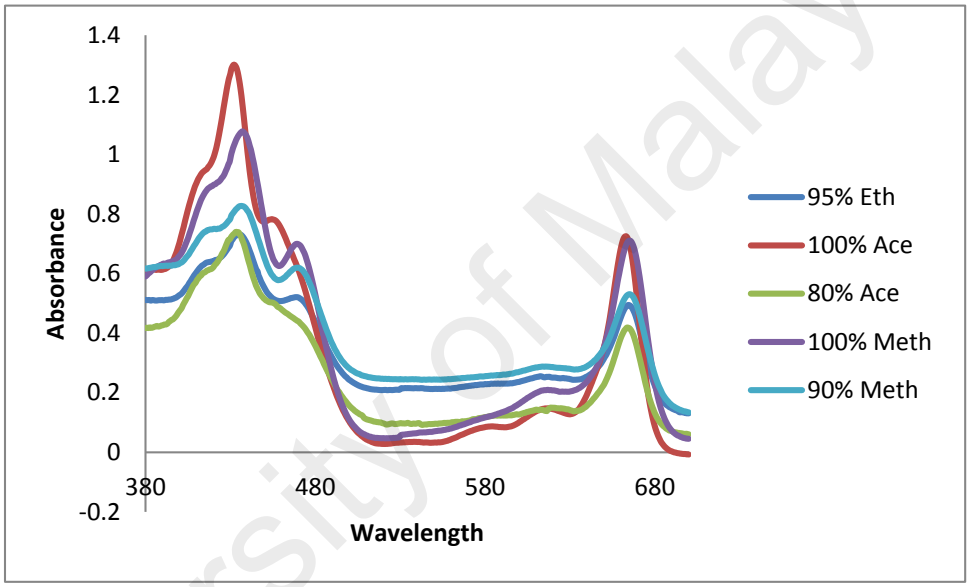


Figure 8.1: Absorption spectra of pigments from *in vitro* plantlets in various solvent extractions.

8.3.1.2 Analysis of Pigments Extracted From Callus (Green) in Different Solvent Extractions

Analysis on pigments extracted from callus in different solvent extractions (Figure 8.2) presented quite similar shapes of absorption spectra which depicted the peaks in both red and blue regions. The results indicated that each of solvent extraction contains a mixture of chlorophyll and carotenoid as the pigments were identified according to the peak of wavelength detected in the samples.

The pigments extracted from callus were measured and comparison of pigment content including chlorophyll a, chlorophyll b, total chlorophyll, total carotenoid and total pigment content in different solvent extractions were recorded (Table 8.3). Interestingly, 80% Acetone showed the highest chlorophyll a and chlorophyll b content with 22.61 mg/g and 11.10 mg/g, respectively, whereas 90% Methanol showed the lowest chlorophyll a and chlorophyll b content which correspond to 11.42 mg/g and 1.23 mg/g. Therefore, the highest total chlorophyll content (33.71 mg/g) was observed in 80% Acetone while the lowest total chlorophyll content (12.65 mg/g) was observed in 90% Methanol. Nevertheless, the highest total carotenoid content (7.40 mg/g) was obtained in 100% Acetone whilst the lowest total carotenoid content (3.03 mg/g) was obtained in 100% Methanol. Overall, the highest total pigment content was 39.84 mg/g, observed in 80% Acetone, followed by 100% Acetone, 100% Methanol, 95% Ethanol and lastly 90% Methanol that corresponded to 36.33 mg/g, 24.93 mg/g, 22.00 mg/g and 16.18 mg/g, respectively. As a result, 80% Acetone was identified as the most efficient solvent extraction for callus.

Analysis on the ratio of pigment content from callus measured in different solvent extractions (Table 8.4) indicated that the highest ratio of chlorophyll a to chlorophyll b with 9.28 was observed in 90% Methanol. However, the lowest ratio of chlorophyll a to chlorophyll b with 2.04 was observed in 80% Acetone. In contrast, the

highest and lowest ratio of chlorophyll to carotenoid was observed in 100% and 90% Methanol with the ratio of 7.23 and 3.58, respectively.

Table 8.3: Comparison of chlorophyll, carotenoid and total pigment content (mg/g fresh weight) for callus of *Echinocereus cinerascens* measured in different solvent extractions.

Solvent Extraction	Pigment content (mg/g)				
	Ca	Cb	C(a+b)	C(x+c)	Total
100% Acetone	22.34 ± 0.04b	6.59 ± 0.05c	28.93 ± 0.02b	7.40 ± 0.02a	36.33 ± 0.02b
80% Acetone	22.61 ± 0.05a	11.10 ± 0.01a	33.71 ± 0.01a	6.13 ± 0.04b	39.84 ± 0.05a
95% Ethanol	14.12 ± 0.05d	4.07 ± 0.03d	18.19 ± 0.06d	3.81 ± 0.01c	22.00 ± 0.08d
100% Methanol	15.05 ± 0.02c	6.85 ± 0.03b	21.90 ± 0.02c	3.03 ± 0.07e	24.93 ± 0.02c
90% Methanol	11.42 ± 0.05e	1.23 ± 0.05e	12.65 ± 0.03e	3.53 ± 0.02d	16.18 ± 0.01e

Data represents mean value ± standard error (SE) for three replicates of each treatment. Means with different letters in the same column are significantly different at p = 0.01 according to Duncan's multiple range test (DMRT). Ca, chlorophyll a; Cb, chlorophyll b; C(a+b), total chlorophyll a and b; C(x+c), total carotenoid (xanthophyll and carotene)

Table 8.4: Ratio of pigment content (mg/g fresh weight) for callus of *Echinocereus cinerascens* measured in different solvent extractions.

Solvent Extraction	Pigment content (mg/g)	
	Ca/Cb ratio	C(a+b)/C(x+c) ratio
100% Acetone	3.39 ± 0.02b	3.91 ± 0.01d
80% Acetone	2.04 ± 0.02d	5.50 ± 0.01b
95% Ethanol	3.47 ± 0.03b	4.77 ± 0.03c
100% Methanol	2.20 ± 0.01c	7.23 ± 0.02a
90% Methanol	9.28 ± 0.05a	3.58 ± 0.04e

Data represents mean value ± standard error (SE) for three replicates of each treatment. Means with different letters in the same column are significantly different at p = 0.01 according to Duncan's multiple range test (DMRT). Ca, chlorophyll a; Cb, chlorophyll b; C(a+b), total chlorophyll a and b; C(x+c), total carotenoid (xanthophyll and carotene).

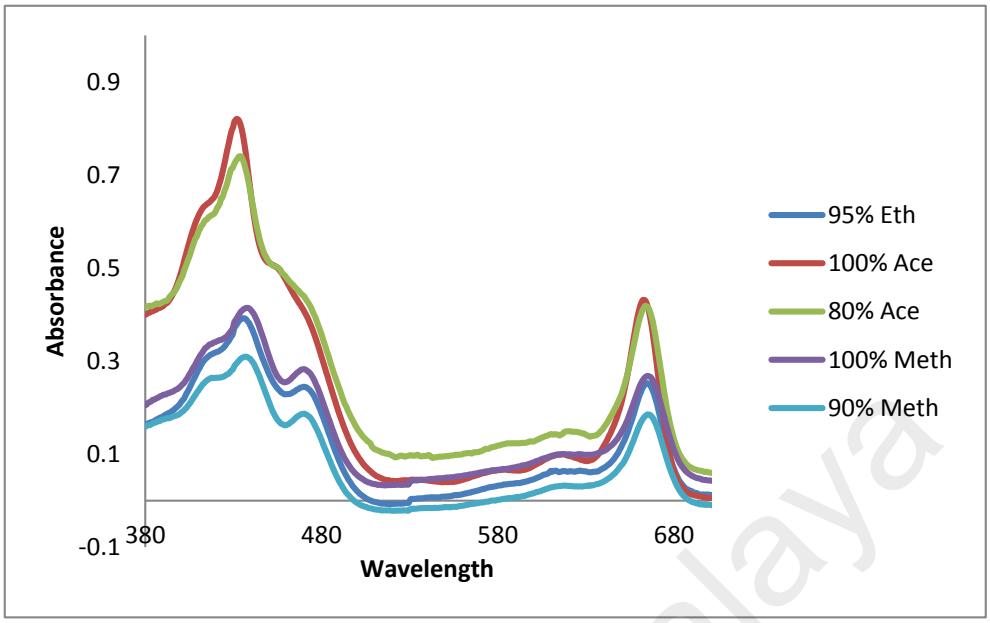


Figure 8.2: Absorption spectra of pigments from fresh callus in various solvent extractions.

8.3.1.3 Analysis of Pigments Extracted From Coloured Callus (Green, Yellow, Pink) in 95% Ethanol

Analysis of the absorption spectra (Figure 8.3a,b,c) detected two natural pigments extracted with 95% ethanol from fresh samples of coloured callus of *Echinocereus cinerascens,* namely chlorophylls and carotenoids. Natural pigments are not stable and degraded with time as shown in absorption spectra measured before and after 3 months of storage at -20 °C.

Results on pigment content showed parameters assessed, chlorophyll a, chlorophyll b, total chlorophyll, total carotenoid, and total pigment measured before and after extracts were stored for 3 months at -20 °C (Table 8.5). The highest pigment content for all parameters examined was observed in green callus before the extracts were stored while the lowest pigment content was observed in both pink and yellow callus after the extracts were stored. The results suggested that pigment content were higher in green callus extracts compared to pink and yellow callus extracts. Moreover, it was found that the highest total pigment content calculated was 83.28 mg/g, in green (B) callus while the lowest was 10.55 mg/g, in pink (A) callus extracts. Total chlorophyll and pigment content for green (B) and yellow (B) callus extracts degraded by 17 mg/g approximately, whereby showed half reduction of pigments in pink (B) callus extracts. However, total carotenoid content for green (B) callus and yellow (B) callus extracts degraded by 1 mg/g only, while pink (B) callus extracts degraded almost 4 mg/g.

Comparison on mean degradation of pigment (%) among the coloured callus extracts (Table 8.6) indicated that both the highest degradation of chlorophyll and carotenoid were observed from pink callus with 85.19 % and 39.79%, respectively, which followed by yellow (70.13%, 12.71%) and green callus (22.93%, 9.02%). Furthermore, the data showed degradation of chlorophyll was higher than carotenoid. These results clarified that carotenoid is more stable compared to chlorophyll. Besides,

the data also recorded ratio of chlorophyll a to chlorophyll b and ratio of chlorophyll to carotenoid, measured before and after extracts were stored for 3 months in a refrigerator at -20 °C (Table 8.6). Both parameters were essential in order to indicate the functional pigment equipment, light adaptation and as an indicator of the greenness of plants as well. The ratio of chlorophyll a to chlorophyll b evaluated showed that after storage, a higher ratio was observed than before the extracts were stored. In contrast, the ratio of chlorophyll to carotenoid analysed before was higher than after the extracts were stored. The highest ratio of chlorophyll a to chlorophyll b was 3.97 obtained in yellow (A) callus extracts, quite similar with the ratio of green (A) callus extracts, while the highest ratio of chlorophyll to carotenoid was 5.11 obtained in green (B) callus extracts. The lowest ratio of chlorophyll a to chlorophyll b was 0.52 noted in pink (B) callus extracts, which showed no significant difference with the ratio of yellow (B) callus extracts. Meanwhile, the lowest ratio of chlorophyll to carotenoid was 0.83 noted in pink (A) callus extracts. The results revealed that green callus extracts possess a normal ratio (4.2-5.0), comparable to most sun leaves and sun-exposed plants, while others with a lower ratio (≤ 3.5) may promote the occurrence of senescence, stress, and damage to the plant or photosynthetic apparatus.

Table 8.5: Comparison of chlorophyll, carotenoid and total pigment content (mg/g fresh weight) for coloured callus of *Echinocereus cinerascens* measured before and after 3 months storage at -20 °C.

	Pigment content (mg/g)				
Coloured callus	Ca	Cb	Ca+Cb	C(x+c)	Total
Green (B)	$46.08 \pm 0.04_a$	$23.58 \pm 0.26_a$	$69.65 \pm 0.51_a$	$13.63 \pm 0.14_a$	$83.28 \pm 0.19_a$
Green (A)	$42.50 \pm 0.66_b$	$11.18 \pm 0.10_d$	$53.68 \pm 0.10_b$	$12.40 \pm 0.04_b$	$66.08 \pm 0.04_b$
Pink (B)	$11.08 \pm 0.03_c$	$21.20 \pm 0.46_b$	$32.28 \pm 0.24_c$	$9.60 \pm 0.17_c$	$41.88 \pm 0.16_c$
Pink (A)	$2.09 \pm 0.07_f$	$1.88 \pm 0.03_e$	$4.78 \pm 0.03_f$	$5.78 \pm 0.01_d$	$10.55 \pm 0.03_f$
Yellow (B)	$9.40 \pm 0.03_d$	$14.30 \pm 0.09_c$	$23.70 \pm 0.13_d$	$5.90 \pm 0.19_d$	$29.60 \pm 0.36_d$
Yellow (A)	$5.65 \pm 0.05_e$	$1.43 \pm 0.03_e$	$7.08 \pm 0.03_e$	$5.15 \pm 0.09_e$	$12.23 \pm 0.11_e$

Data represents mean value ± standard error (SE) for three replicates of each treatment. Means with different letters in the same column are significantly different at p=0.01according to Duncan's multiple range test (DMRT). Ca, chlorophyll a; Cb, chlorophyll b; Ca+Cb, total chlorophyll a and b; C(x+c), total carotenoid (xanthophyll and carotene); B, before; A, after.

Table 8.6: Mean degradation of pigment (%) and ratio of pigment content (mg/g fresh weight) for coloured callus of *Echinocereus cinerascens* measured before and after 3 months storage at -20 °C.

	Mean degradation of pigment (%)		Pigment content (mg/g)	
Coloured callus	Ca+Cb	C(x+c)	Ca/Cb ratio	Ca+Cb/C(x+c) ratio
Green (B)	$22.93 \pm 0.10_c$	$9.02 \pm 0.02_c$	$1.96 \pm 0.02_c$	$5.11 \pm 0.02_a$
Green (A)			$3.80 \pm 0.03_b$	$4.33 \pm 0.02_b$
Pink (B)	$85.19 \pm 0.05_a$	$39.79 \pm 0.02_a$	$0.52 \pm 0.01_e$	$3.36 \pm 0.03_d$
Pink (A)			$1.55 \pm 0.05_d$	$0.83 \pm 0.00_f$
Yellow (B)	$70.13 \pm 0.05_b$	$12.71 \pm 0.01_b$	$0.66 \pm 0.00_e$	$4.03 \pm 0.11_c$
Yellow (A)			$3.97 \pm 0.04_a$	$1.38 \pm 0.02_e$

Data represents mean value ± standard error (SE) for three replicates of each treatment. Means with different letters in the same column are significantly different at p=0.01according to Duncan's multiple range test (DMRT). Ca, chlorophyll; Cb, chlorophyll b; Ca+Cb, total chlorophyll a and b; C(x+c), total carotenoid (xanthophyll and carotene); B, before; A, after.

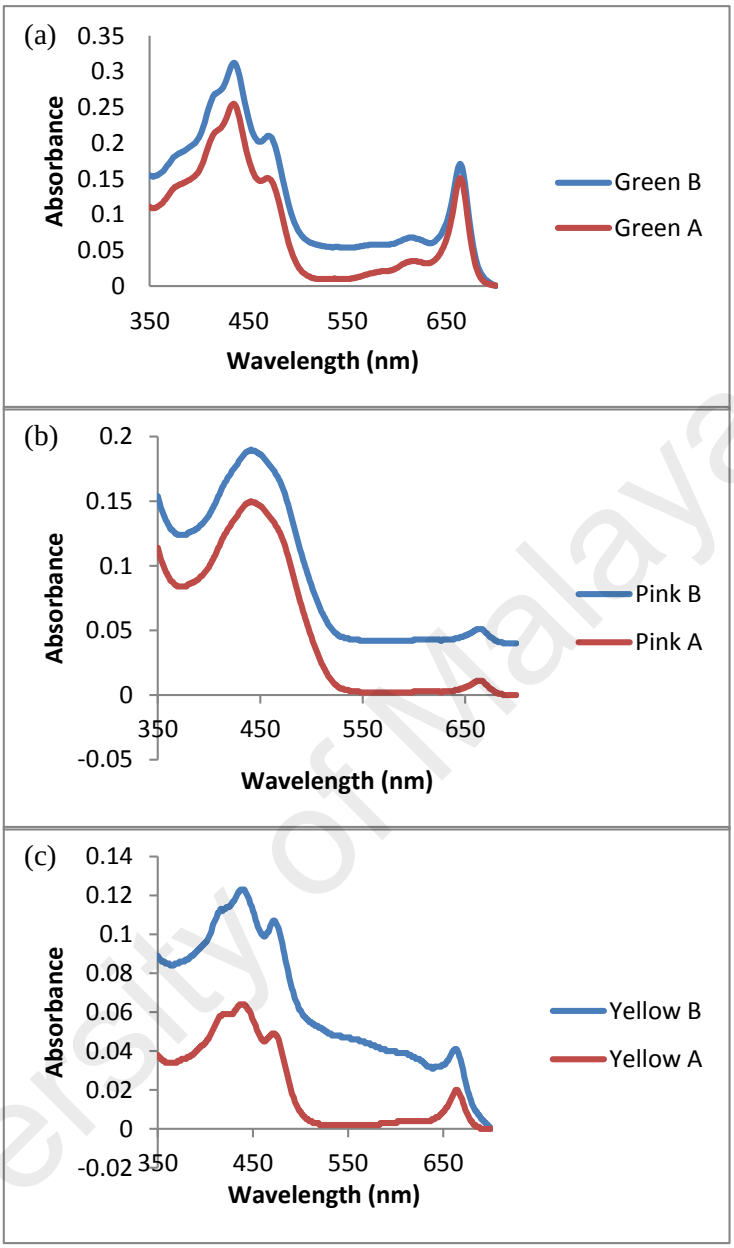


Figure 8.3: Comparison between absorption spectra of pigments in 95% Ethanol extracted from fresh samples of (a) green callus, (b) pink callus and (c) yellow callus measured before and after storage for 3 months at -20 °C. B, before; A, after.

8.3.2 HPLC Analysis

Carotenoids extracted from *in vitro* plantlets and callus samples that have been saponified (Figure 8.4) were directly injected into the HPLC system. By HPLC analysis, individual carotenoids were quantified using the equation derived from the calibration curve constructed. Calibration curve was constructed for each of identified carotenoids such as neoxanthin at 438 nm wavelength, violaxanthin at 441 nm wavelength, lutein at 447 nm wavelength and β-carotene at 452 nm wavelength. Subsequently, a linear regression equation and a correlation coefficient of each calibration curve were obtained (Table 8.7). Linear regression equations with specific correlation coefficients of the identified carotenoids were including $y = 18.825x – 8.141$, $R^2 = 0.9858$ (neoxanthin), $y = 196.05x – 40.373$, $R^2 = 0.9961$ (violaxanthin), $y = 3160.60x – 1347.7$, $R^2 = 0.9959$ (lutein) and lastly $y = 798.07x – 106.45$, $R^2 = 0.9950$ (β-carotene).

Table 8.7: Linear regression equation and correlation coefficient from a calibration curve constructed.

Parameter	Calibration curve			
	Neoxanthin	Violaxanthin	Lutein	β-carotene
Linear regression equation	y = 18.825x – 8.141	y = 196.05x – 40.373	y = 3160.60x – 1347.7	y = 798.07x – 106.45
Correlation coefficient (r)	0.9858	0.9961	0.9959	0.9950

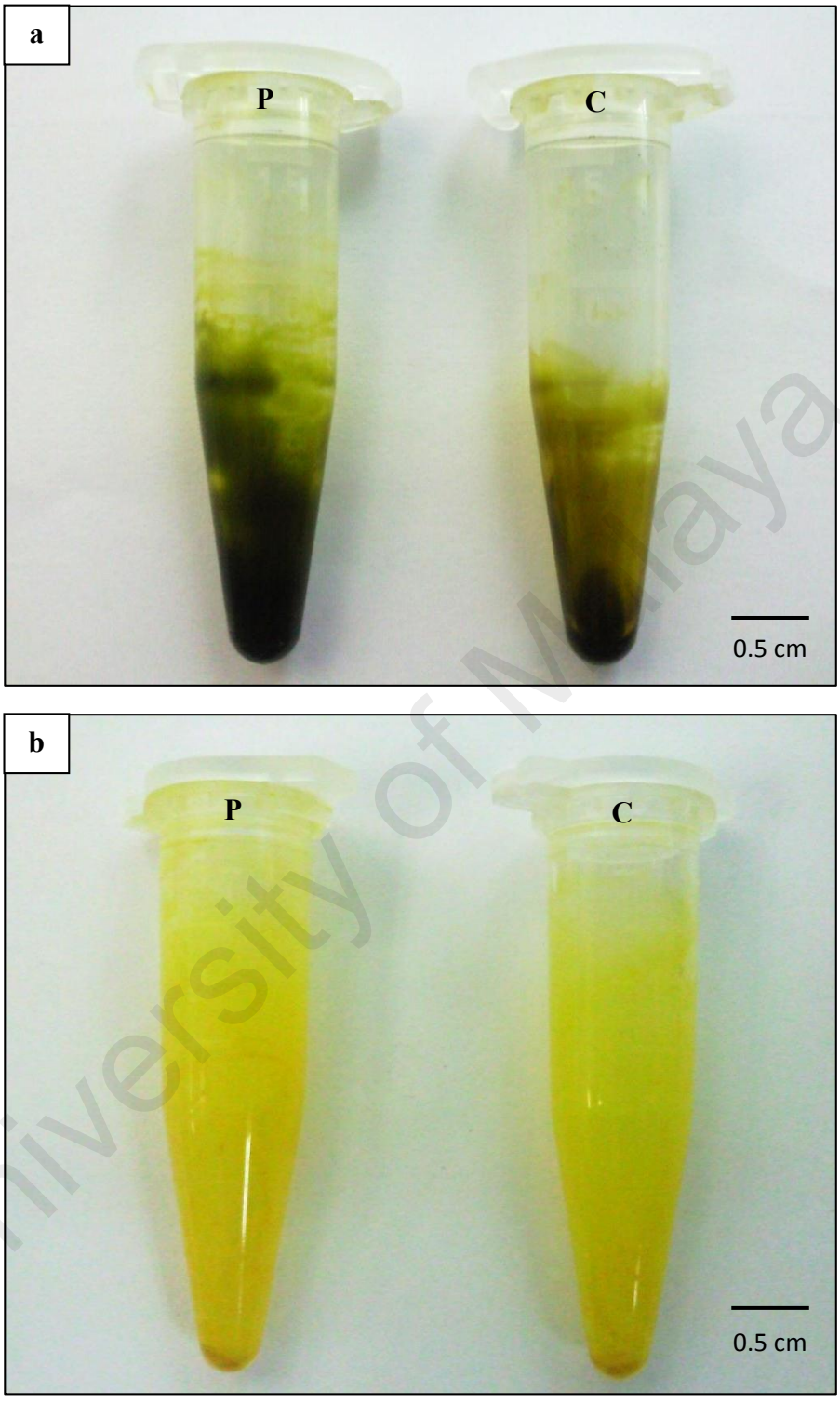


Figure 8.4: *In vitro* plantlets (P) and callus (C) extracts (a) before and (b) after saponification.

8.3.2.1 Analysis of Carotenoids Extracted From *In Vitro* Plantlets and Callus

HPLC chromatogram of carotenoids extracted from *in vitro* plantlets (Figure 8.5) and callus (Figure 8.6) revealed several identified carotenoids, while some others were detected but unable to be identified due to the absence of commercial standards. Carotenoids which were identified in *in vitro* plantlets include neoxanthin, violaxanthin, lutein and β-carotene, whereas in callus were lutein and β-carotene only.

Comparison of carotenoid content was measured in *in vitro* plantlets and callus (Table 8.8). Observations recorded that neoxanthin and violaxanthin were only detected in *in vitro* plantlets with 5.34 mg/g and 0.44 mg/g, respectively. Meanwhile, lutein and β-carotene were detected in both *in vitro* plantlets and callus. The results showed that concentration of lutein in *in vitro* plantlets, 1.28 mg/g was higher compared to callus, 1.09 mg/g. Similarly, it was found that the concentration of β-carotene in *in vitro* plantlets was significantly higher than callus. Concentration of β-carotene calculated in *in vitro* plantlets was 2.16 mg/g which was almost 2-fold higher than in callus (1.41 mg/g). Notably, the total concentration of identified carotenoids in *in vitro* plantlets (9.22 mg/g) was also higher than callus (2.49 mg/g).

Table 8.8: Comparison of carotenoid content (mg/g dry weight) in *in vitro* plantlets and callus of *Echinocereus cinerascens*.

Type of sample	Pigment content, mg/g (Mean ± SE)				Total
	Neoxanthin	Violaxanthin	Lutein	β-carotene	
In vitro plantlets	5.34 ± 0.05	0.44 ± 0.00	1.28 ± 0.01	2.16 ± 0.04	9.22
Callus	n.d.	n.d.	1.09 ± 0.00	1.41 ± 0.00	2.49
			Sig. different	Sig. different	

n.d. not detected, Sig. significantly different at p = 0.01 (Independent Samples T-test)

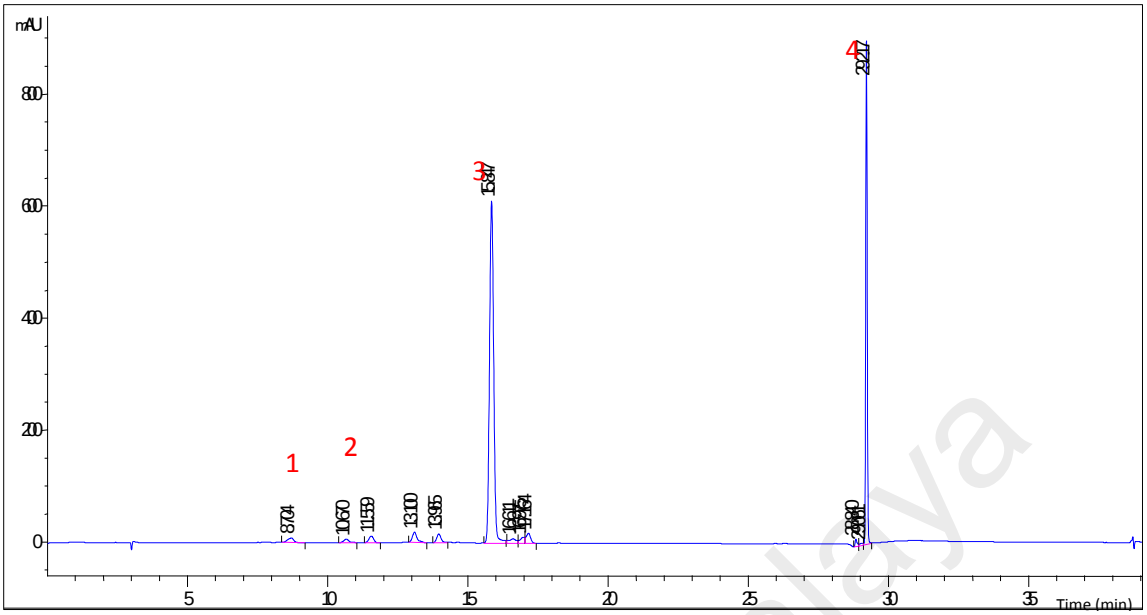


Figure 8.5: HPLC chromatogram of *in vitro* plantlets of *Echinocereus cinerascens.* Carotenoids identified: 1 = neoxanthin (RT 8.7), 2 = violaxanthin (RT 10.7), 3 = lutein (RT 15.8), 4 = β-carotene (RT 29.2).

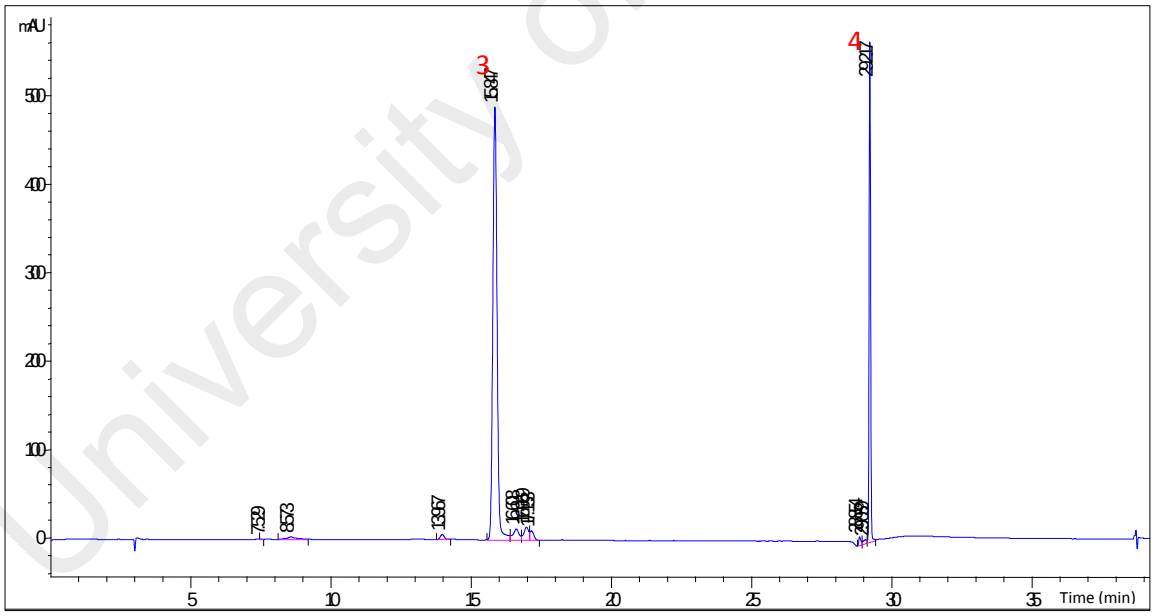


Figure 8.6: HPLC chromatogram of callus of *Echinocereus cinerascens.* Carotenoids identified: 3 = lutein (RT 15.8), 4 = β-carotene (RT 29.2).

8 .4 SUMMARY OF RESULTS

1. Pigments of *in vitro* plantlets and callus of *Echinocereus cinerascens* were detected using UV-VIS spectrophotometer and HPLC analysis.
2. UV-VIS spectrophotometer depicted the absorption spectra of pigments which included chlorophyll a, chlorophyll b and carotenoid (xanthophyll + carotenes).
3. The highest total pigment extracted for *in vitro* plantlets was 64.10 mg/g, obtained from 100% Methanol whereas the highest total pigment extracted for callus was 39.84 mg/g, obtained from 80% Acetone. Therefore, the most efficient solvent extraction for *in vitro* plantlets was 100% Methanol while for callus was 80% Acetone.
4. Analysis on coloured callus (green, yellow and pink) revealed that the highest pigment content was recorded for green callus (83.28 mg/g), before the extracts were stored (3 months at -20 ºC) while the lowest pigments content was recorded for pink callus (10.55 mg/g) after the extracts were stored (3 months at -20 ºC).
5. Although the results showed higher chlorophyll than carotenoid content in the samples tested, but the degradation of chlorophyll was higher compared to carotenoid. The highest chlorophyll content was 69.65 mg/g, recorded for green callus before the extracts were stored. Meanwhile, the highest degradation of chlorophyll was 85.19%, observed from pink callus extracts.
6. Comparison of the results indicated that *Echinocereus cinerascens* consist of chlorophyll as the major pigment and carotenoid as the minor pigment. However, it was found that carotenoid is more stable compared to chlorophyll since degradation of chlorophyll was higher than carotenoid.

7. Ratio of chlorophyll a to chlorophyll b showed that after storage, a higher ratio was observed than before the extracts were stored. In contrast, the ratio of chlorophyll to carotenoid analysed before storage was higher than after the extracts were stored.
8. Through HPLC analysis, several carotenoids were detected and identified in *in vitro* plantlets including neoxanthin (5.34 mg/g), violaxanthin (0.44 mg/g), lutein (1.28 mg/g) and β-carotene (2.16 mg/g) whereas in callus were lutein (1.09 mg/g) and β-carotene (1.41 mg/g) only.
9. The highest carotenoid content in *in vitro* plantlets was neoxanthin, followed by β-carotene, lutein and lastly violaxanthin. Meanwhile, the highest carotenoid content in callus was β-carotene, followed by lutein.

CHAPTER 9

CELLULAR BEHAVIOUR IN MERISTEMATIC ROOT CELLS OF *Echinocereus cinerascens*

9.1 EXPERIMENTAL AIMS

Plant organs (shoot, root, flower and so on) consist of several types of tissues that work together to perform particular functions. Meristem tissues like roots or shoot tips which are actively dividing cells undergo diploid chromosomal complement whereas non-meristem tissues like stems, flowers and others may have varied chromosomal numbers triggered by somatic chromosomal inconsistency (Partanen, 1963). Partanen suggested that plant species are commonly characterized by their morphology and chromosomal stability. According to Van't Hof (1974a), cell division and cell differentiation are controlled by factors that operate during G1 and G2 phase. In root meristem cells, the duration of S phase is determined by the amount of nuclear DNA (Van't Hof, 1974b).

Each of plant species has a characteristic average amount of DNA and it has been established that the more DNA a cell has in its nucleus, the longer time it takes for cell cycle (Van't Hof and Sparrow, 1963; Van't Hof, 1965; 1974b). The larger cells divide earlier because they grow faster and the faster growing cells will grow more (Donnan and John, 1983). Cavalier-Smith (1985) reported the larger cells appear to grow at a greater rate than smaller cells. This proved that the cell size is influenced by the time for the cell division. Other studies (Woodward *et al*., 1961; Lyndon, 1967) indicated that nuclear volume varies at a different time or in different tissues of the same species without corresponding changes in the DNA content. In meristem cells, the nuclear volume increases during the cell cycle by a factor of five or six rather than a factor of two. The possibility of growth in nuclear volume plays a key role in the G1/S

phase transition which suggested by the finding in plant meristem cells that the larger nucleus of the two sister cells divides first (Armstrong, 1983).

In this study, the root meristem (root tip) was selected for cellular behaviour studies of *Echinocereus cinerascens* since it has extensively been preferred and used as standard tissue in these related works. Besides, the root meristem structure is stable and it also has several advantages in cycling-cell studies (Giménez-Martín *et al*., 1977). In addition, the root meristem is easy to produce and handle as it can be grown in a simple mineral medium (MS basal medium) or a moist cotton wools which makes it is an ideal candidate for this study. Root meristem is responsible for all primary root growth where the cells are actively dividing. Analysis on cellular behaviour of the root meristem offers important knowledge and discovery of the behaviour of cells in other tissues.

Studies on cellular behaviour involve determination of standard primary root length that plays an important role for subsequent experiments. Primary roots have generally been divided into several areas namely cell division, cell elongation and cell maturation (Mauseth, 1998). The area in primary root which cells are actively dividing (root tip), that has the ability to regulate the growth of roots, was subjected to several procedures cytological studies such as mitotic index (MI), chromosome counts, nuclear areas, cell areas and ratio of nuclear to cell areas for all the samples tested. Comparisons were made for all the samples to observe cellular changes or differences between *in vivo* grown seedlings, *in vitro* plantlets and *ex vitro* plants (the *in vitro* plantlets that have been transferred to *ex vitro* condition). Indeed, this study offers an alternative method to detect any occurrence of somaclonal variation in the samples.

9.2 MATERIALS AND METHODS

9.2.1 Standard Growth of Primary Root (*In Vivo*)

In vivo grown seedlings were selected for determination of standard primary root growth. The standard primary root growth was determined by growing 100 seeds of *Echinocereus cinerascens* on moist cotton wools. Observation was recorded on the mean primary root length daily until a certain period where most of the secondary roots were developed. The mean primary root length (cm) versus time (day) was plotted and a linear regression analysis was used to find the relationship between the parameters (root length and time) using a formula given:

$$Y = b + mX, \text{ where}$$

m = gradient or slope of linear regression

b = constant or intercept at Y-axis

9.2.2 Cytological Studies of Primary Roots of *In Vivo* (Seedlings), *In Vitro* (Plantlets) and *Ex Vitro* (Plants) Samples

Once the standard growth was established, further studies on the investigation of cytological parameters such as measurement of mitotic index, chromosome counts, nuclear areas, cell areas and ratio of nuclear to cell areas were carried out using the standard length of primary root growth as a reference. Procedures for the induction of primary roots applied in this study were varied according to the treatments tested.

Procedures for *in vivo* (seedlings) primary root induction were similar to the preparation for the standard growth of primary roots, where the seeds were grown on moist cotton wools. In contrast, primary root induction for *in vitro* (plantlets) was done by growing the *in vitro* plantlets (4-month-old) on MS basal medium (*in vitro*). Meanwhile, for *ex vitro* (plants) primary root induction, the procedures were quite similar to both treatments of *in vivo* and *in vitro* primary roots induction. *Ex vitro* primary roots were induced by transferring and growing the *in vitro* plantlets (4-month-

old) on moist cotton wools. Primary roots of *in vivo* (seedlings), *in vitro* (plantlets) and *ex vitro* (plants), were allowed to develop in the culture room at 25 ± 2 °C under 16 hours light provided by cool daylight or fluorescent light (36W), PHILIPS for a few weeks. The primary roots that reached the standard of primary root length (0.534 cm on day 9) were harvested. The root tips were collected (between 0830-0930 am hours) and washed thoroughly with tap water to remove all the contaminants before being used in the following step (preparation of temporary slides using squash technique).

9.2.3 Preparation of Temporary Slides using Squash Technique

The samples with actively growing root tips were fixed in Farmer's solution (absolute ethanol:glacial acetic acid with a ratio of 3:1, v/v) in a clean glass container wrapped with aluminium foil and subsequently kept in the refrigerator at 4 ± 1 ºC for 24 hours (overnight). After that, the samples were washed in 2-3 changes of distilled water before being hydrolysed in 5N HCl at room temperature for 30 minutes with occasional agitation. After hydrolysed, the samples were washed in distilled water for approximately 1 minute to remove any remaining acid. Then, the samples were stained with Feulgen or Schiff's reagent for 1 hour in the dark at room temperature. Next, the samples were washed with several changes of tap water for 5 minutes and kept in distilled water afterwards while temporary slides were prepared.

In preparation of temporary slide, the sample with Feulgen-stained root tip was initially placed onto a clean glass slide. Feulgen-stained root tip was cut in approximately 0.1 cm size, dissected into small pieces using dissecting needles and then squashed under a cover slip in a drop of 45% (v/v) acetic acid. The cover slip was sealed with nail varnish immediately after the slide was gently heated (by passing over a spirit lamp for a few seconds) to prevent the sample from drying as the observation is being made. The slide was viewed under a light microscope (Axioskop Zeiss, Germany) attached to AxioCam MRc video camera. Observations involved measurement of

mitotic index, chromosome counts, mean nuclear and cell areas, and also the ratio of nuclear to cell areas which were analyzed using AxioVision 4.7 software.

9.2.3.1 Measurement of Mitotic Index (MI)

Mitotic index is the percentage of cells that are undergoing mitosis including the cells in prophase, metaphase, anaphase and telophase which are measured by scoring these cells from five slides of each sample in a series of random transect across the slides. Each slide represents a replicate with a total number of 400 cells. Therefore, the data recorded the mean mitotic index calculated from 2000 cells for each sample tested. The calculation of mitotic index was done according to the formula given:

$$\text{Mitotic index (MI)} = \frac{\text{number of cells undergoing mitosis}}{\text{total number of cells}} \times 100$$

9.2.3.2 Chromosome Counts

Screening process was done to select 30 cells in metaphase with good chromosome spread suitable for chromosome count. The selection was done on all the cells from five slides of each the sample tested. Data analysis recorded the mean of chromosome counts and the range of chromosome counts as well.

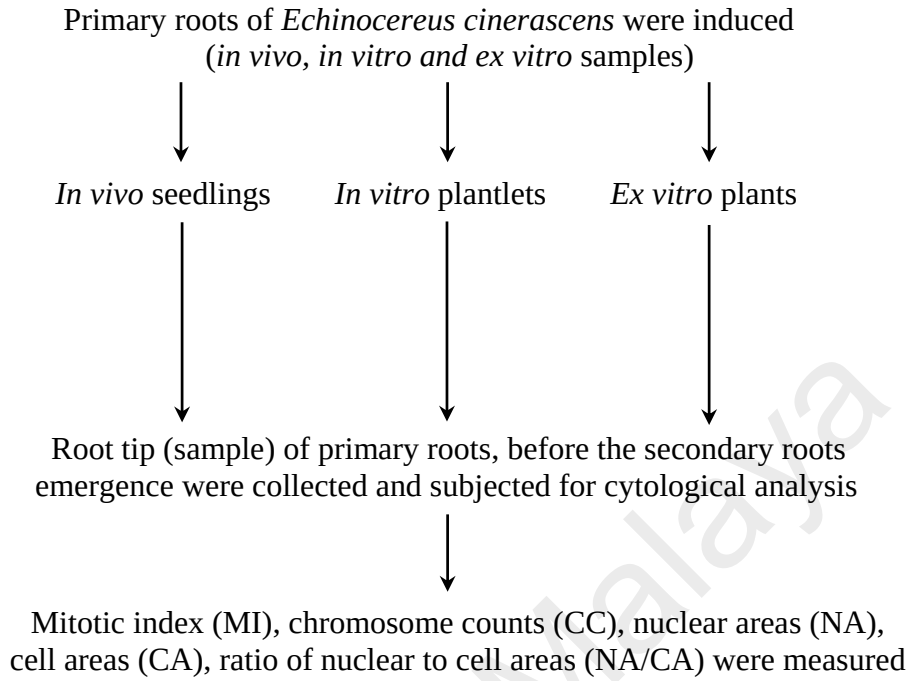
9.2.3.3 Mean Nuclear and Cell Areas

Mean nuclear and cell areas were measured from a total of 250 cells in prophase from 5 slides for each of the sample tested. Besides, data analysis also recorded the ratio of nuclear to cell areas.

9.2.4 Data Analysis

Data obtained were statistically analyzed using Duncan's Multiple Range Test (DMRT). Means with different letters in the same column differ significantly at p=0.01 and p=0.05.

9.2.5 Experimental Outline



9.3 RESULTS

9.3.1 Standard Growth of Primary Root

Observation was done on the growth of *Echinocereus cinerascens* seedlings *in vivo* (Figure 9.1) and the mean of primary root length from 100 seedlings was recorded (Table 9.1) to study the standard growth of primary roots. Several primary roots of seedlings grown on moist cotton wools appear on day 2. Nevertheless, the primary roots mostly developed on day 3, with mean root length of 0.01 cm. The primary roots grew further and the observations showed that the primary root length gradually increased until day 9 as it reached steady-state phase. Moreover, it was found that the secondary root emerged on day 10. Thus, the standard root length was determined on day 9, with mean primary root length of 0.53 cm. The standard root length (0.53 cm) was selected and was used in subsequent experiments (cytological analysis).

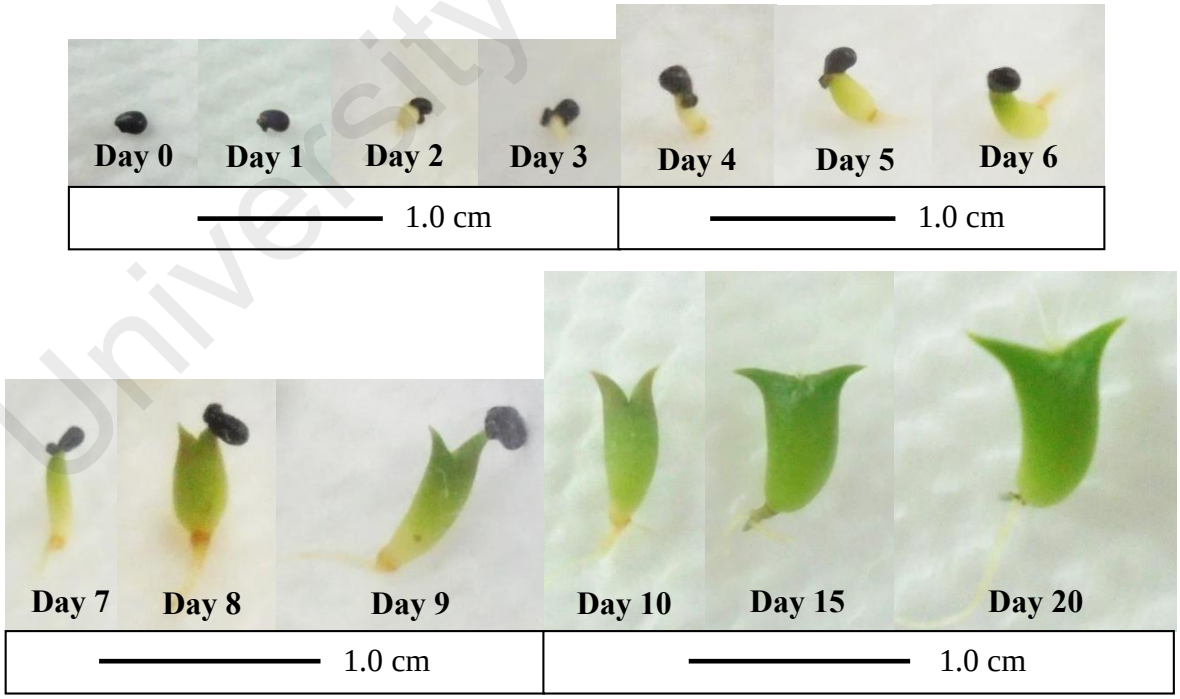


Figure 9.1: Germination stages of *Echinocereus cinerascens* seedlings grown *in vivo* (on moist cotton wools) in the culture room at 25 ± 2 ºC, under 16 hours light.

Table 9.1: The mean primary root length of seedlings grown *in vivo* (on moist cotton wools).

Day	Mean root length (cm) ± SE	Root emergence
1	0.00 ± 0.00 d	-
2	0.00 ± 0.00 d	Primary root
3	0.01 ± 0.01 d	Primary root
4	0.03 ± 0.01 d	Primary root
5	0.17 ± 0.02 c	Primary root
6	0.25 ± 0.02 c	Primary root
7	0.37 ± 0.03 b	Primary root
8	0.49 ± 0.03 a	Primary root
9	0.53 ± 0.03 a	Primary root
10	0.54 ± 0.04 a	Secondary root

Data represents mean value ± standard error (SE) with 30 explants in each treatment. Means with different letters in the same column are significantly different at p=0.05 according to Duncan's multiple range test (DMRT).

The data from Table 9.1 (the mean primary root length of seedlings grown on moist cotton wools) was plotted in a graph (standard growth graph). The graph (Figure 9.2) presented a linear regression line, which showed the relationship between the mean of root length (cm) versus time (day). There is a positive linear association between the two parameters. It was found that the mean of primary root length (cm) increased with time (day) and the rate of primary root elongation was 0.0768 cm per day as clarified by the standard growth graph in a linear regression line of y = 0.0768x – 0.1984, with the correlation coefficient, R^2 = 0.9437. The R^2 value obtained was approximately 1.0 (perfect positive correlation) which indicated that there is a positive linear correlation between the mean of primary root length (cm) and the time (day) required for the roots to grow.

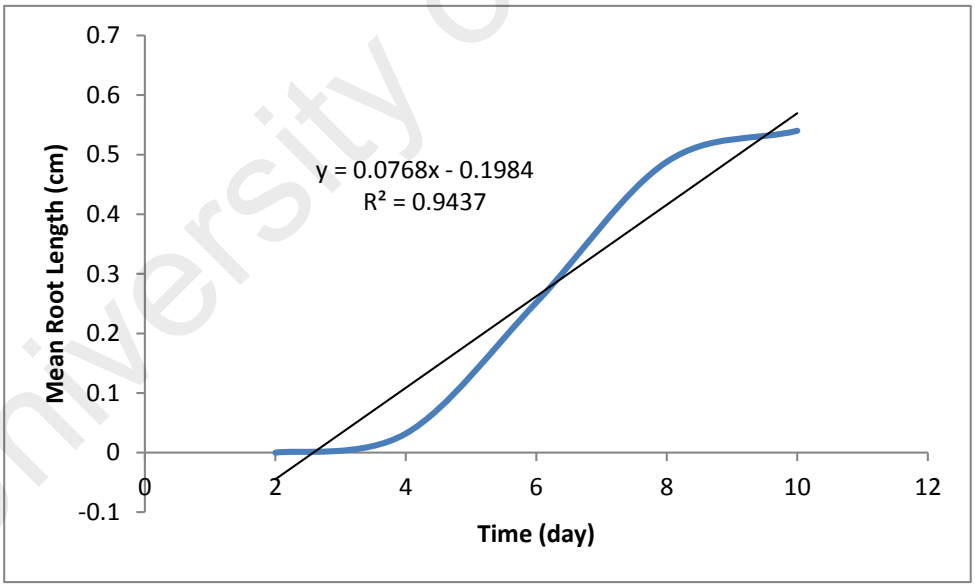


Figure 9.2: The standard growth of primary root of seedlings grown *in vivo* (on moist cotton wools).

9.3.2 Stages of Mitosis in *In Vivo* Meristematic Root Cells (Root tips)

Primary root tips of seedlings grown *in vivo* (Figure 9.3) were harvested before secondary root emergence. Then, the samples (primary root tips) were used to prepare temporary slides for cytological analysis. The slides viewed under a light microscope revealed the stages of mitosis in *in vivo* meristematic root cells (Figure 9.4).

Mitosis is a part of the cell cycle which plays the key roles of cell division. The stages of mitosis include interphase, prophase, metaphase, anaphase and telophase. During interphase, a nuclear envelope encloses the nucleus. A cell grows and copies its chromosomes in order to prepare for cell division. Generally, interphase can be divided into 3 phases namely G1 phase (first gap - cell grows), S phase (synthesis – cell copies its chromosomes) and G2 phase (second gap – cell prepares to divide). After completing all the phases, the cell will go through Mitotic phase (prophase, metaphase, anaphase, telophase).

Interphase is followed by prophase. During prophase, the chromatin becomes tightly coiled and condenses into discrete chromosomes which observable with a light microscope. The chromosomes appear as two identical sister chromatids that joined at the centromeres (homologous chromosome).

After prophase is metaphase. During metaphase, the homologous chromosomes become more condense and align at the metaphase plate by the centromeres. In fact, the centromeres arranged themselves on the metaphase plate, an imaginary line in the middle of two centrosome poles. Besides, the kinetochores of sister chromatids for each of the chromosome are attached to kinetochore microtubules coming from the opposite poles.

Anaphase occurs afterwards where each of the chromosome (sister chromatids) separates and moves toward opposite poles as the kinetochore microtubules shorten. Since microtubules are attached to the centromere, therefore the chromosome (daughter

chromosomes) moves centromere first. By the end of anaphase, both daughter chromosomes reached to the opposite ends of the cell with equivalent and complete collections of chromosomes.

The next phase in cell division is telophase. During telophase, the two nuclei form in the cell while the nuclear envelopes arise to enclose nucleus. Besides, the chromosomes become less condensed and lastly, cytoplasm was divided by cytokinesis where the two daughter cells are produced.

Similar observations were recorded for stages of mitosis in primary root tip of plantlets grown *in vitro* and also plants grown *ex vitro*. Consequently, further studies on cytological parameters were done to investigate the similarities and differences between all the samples (primary root of *in vivo* seedlings, *in vitro* plantlets, *ex vitro* plants).

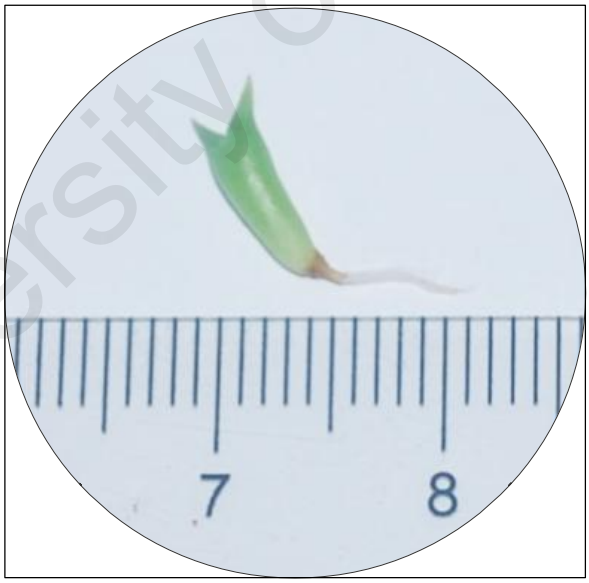


Figure 9.3: Primary root tip of *in vivo* seedling (9 days) grown on moist cotton wools.

9.3.2.1 Stages of mitosis for *in vivo* primary root tips before secondary root emergence

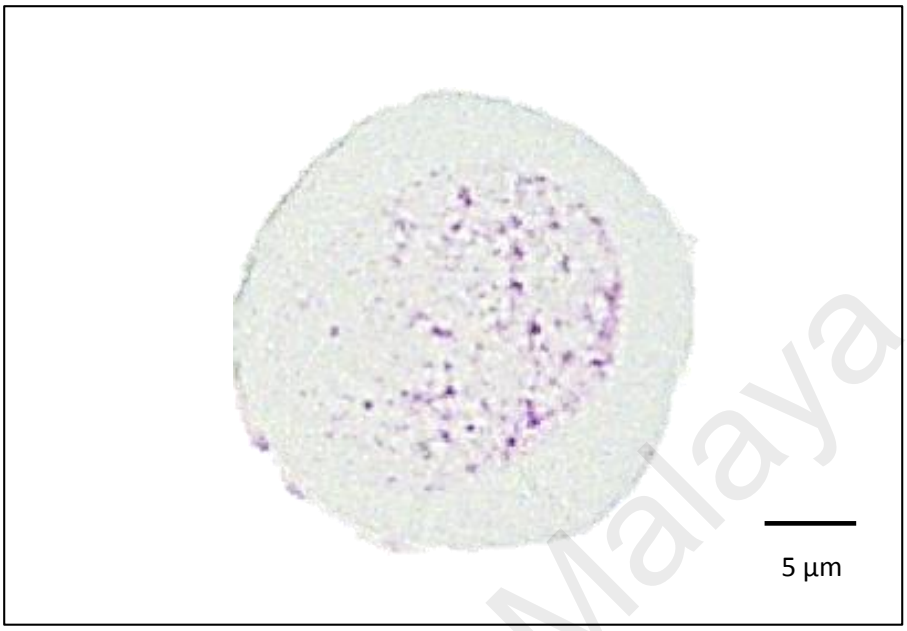


Figure 9.4 (a): Interphase observed from *in vivo* primary root tip before secondary root emergence.

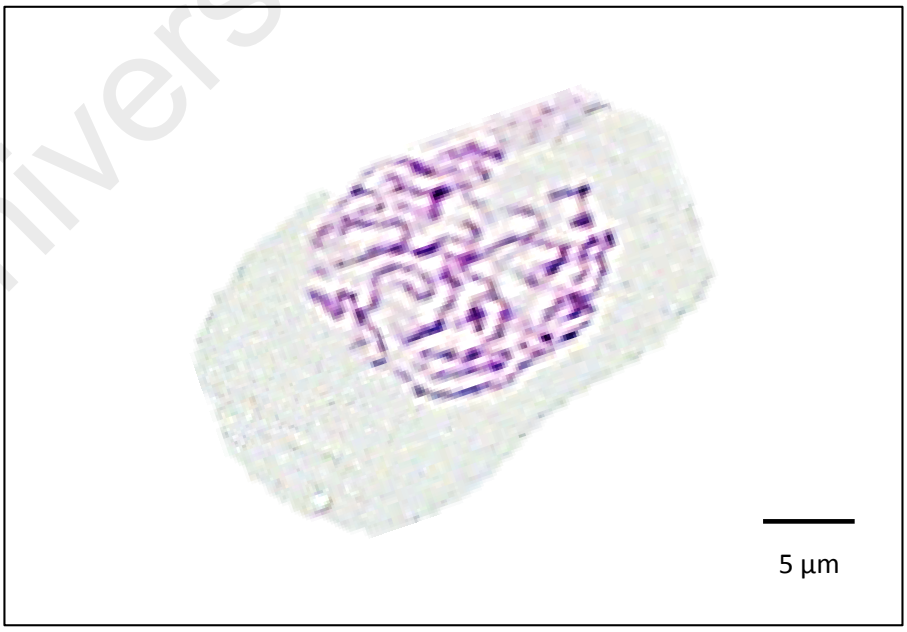


Figure 9.4 (b): Prophase observed from *in vivo* primary root tip before secondary root emergence.

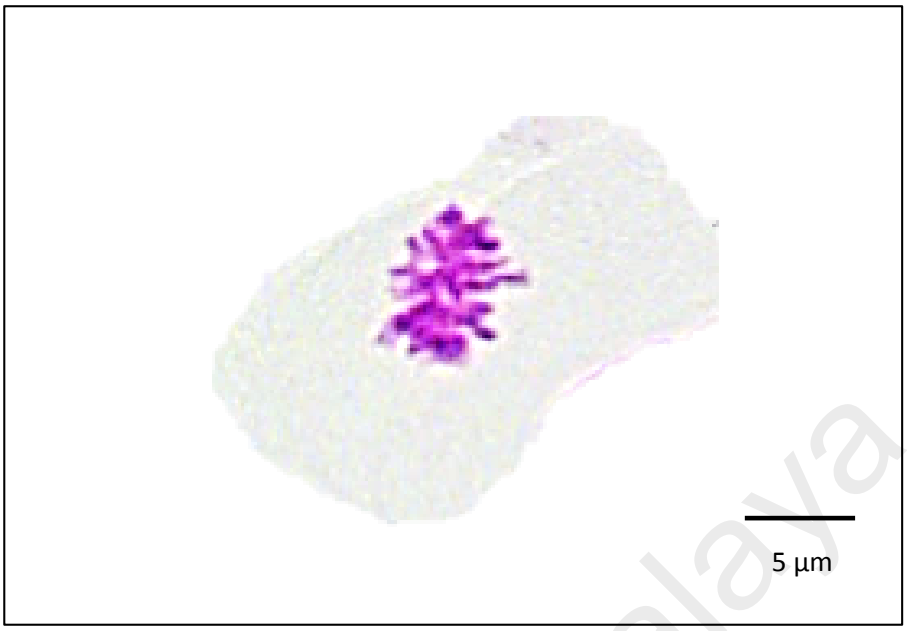


Figure 9.4 (c): Metaphase observed from *in vivo* primary root tip before secondary root emergence.

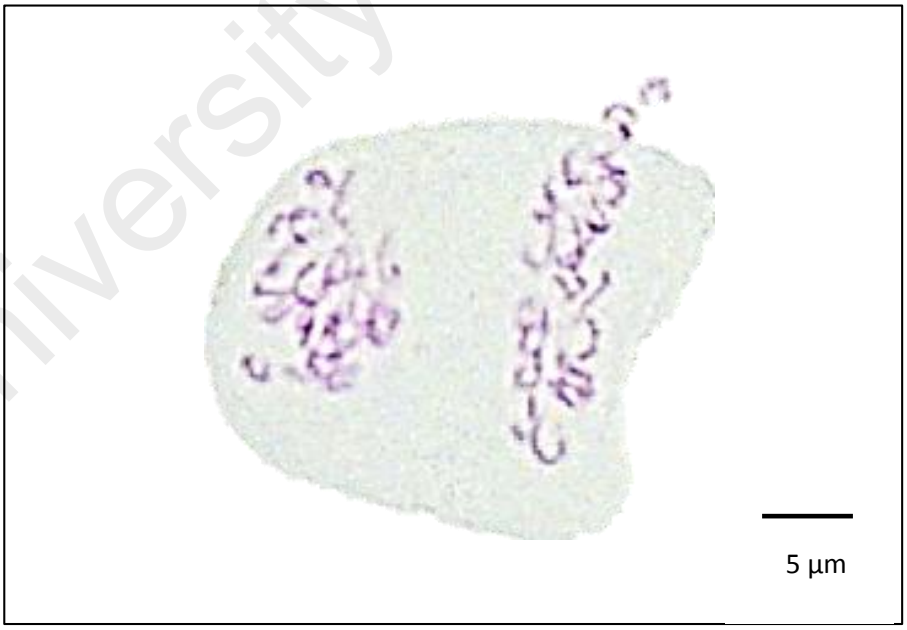


Figure 9.4 (d): Anaphase observed from *in vivo* primary root tip before secondary root emergence.

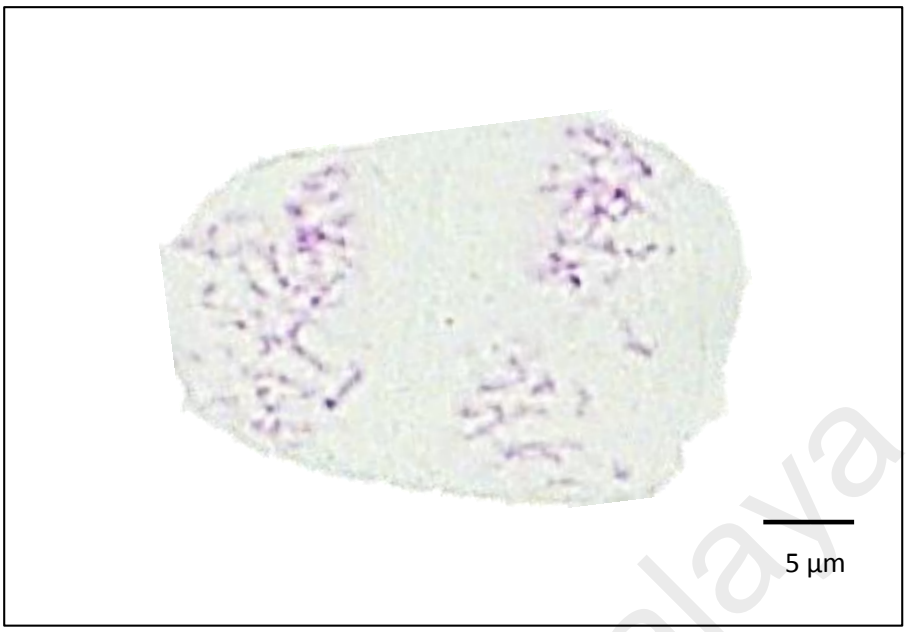


Figure 9.4 (e): Early telophase observed from *in vivo* primary root tip before secondary root emergence.

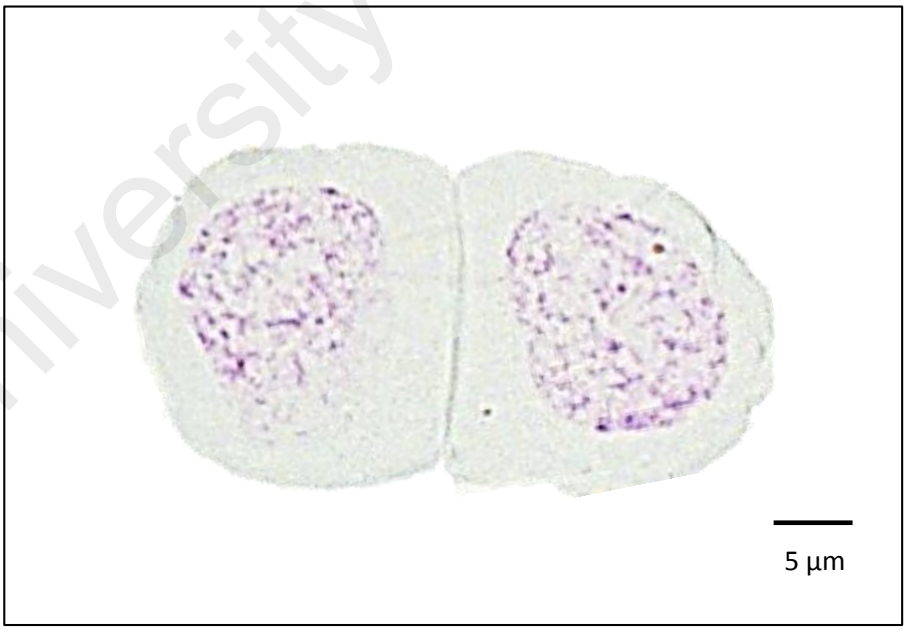


Figure 9.4 (f): Telophase observed from *in vivo* primary root tip before secondary root emergence.

9.3.3 Stages of Mitosis in *In Vitro* Meristematic Root Cells (Root tips)

Primary root tips of plantlets grown *in vitro* (Figure 9.5) were harvested before secondary root emergence. Subsequently, temporary slides were prepared which revealed the stages of mitosis in *in vitro* meristematic root cells (Figure 9.6).

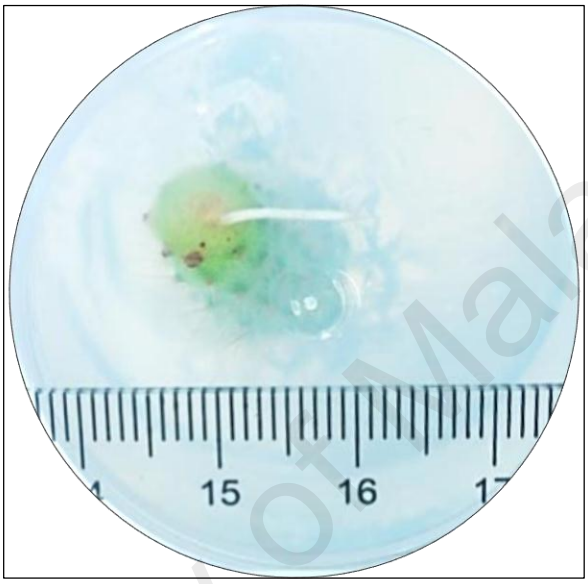


Figure 9.5: Primary root tip of *in vitro* plantlet (1 month) grown on MS basal medium.

9.3.3.1 Stages of mitosis for *in vitro* primary root tips before secondary root emergence

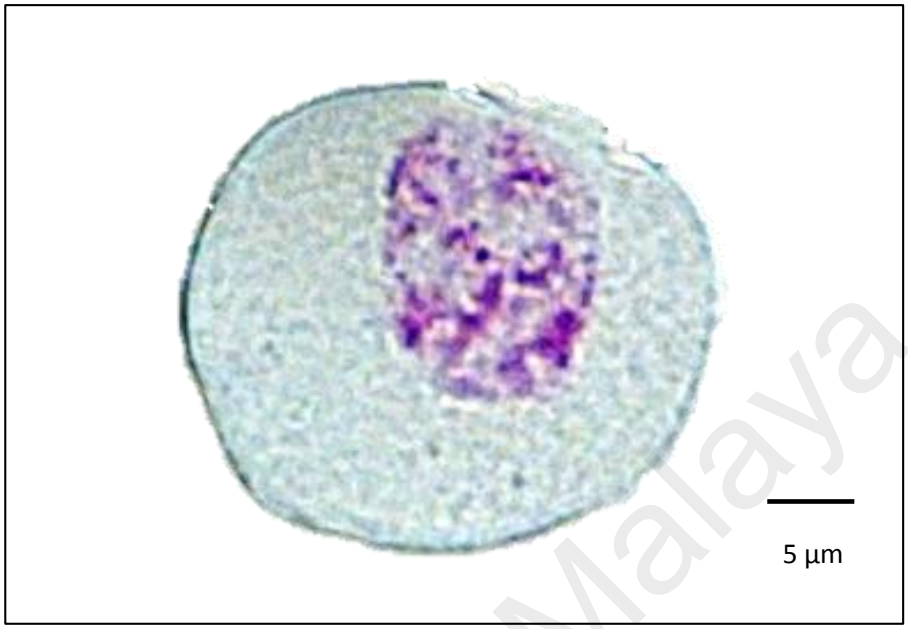


Figure 9.6 (a): Interphase observed from *in vitro* primary root tip before secondary root emergence.

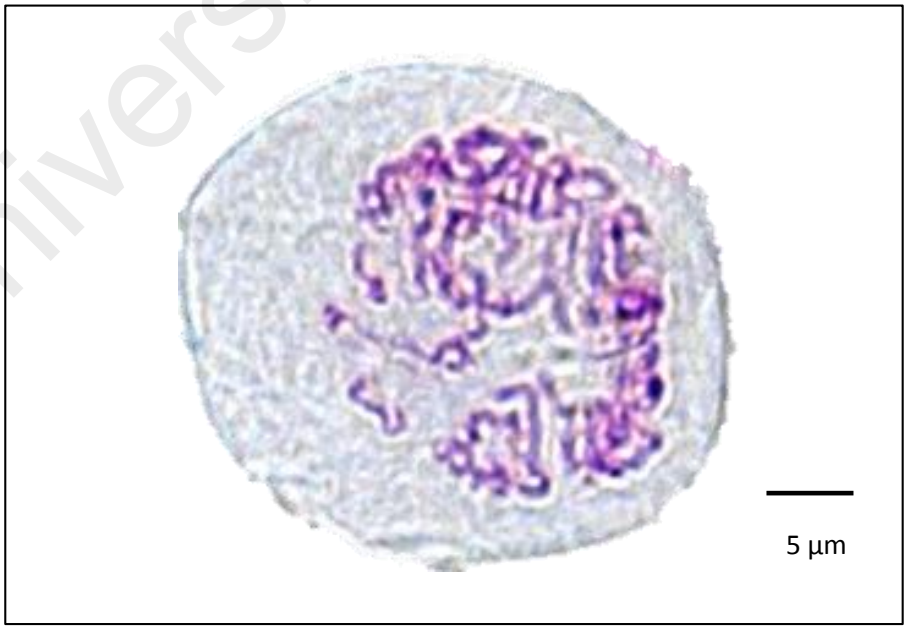


Figure 9.6 (b): Prophase observed from *in vitro* primary root tip before secondary root emergence.

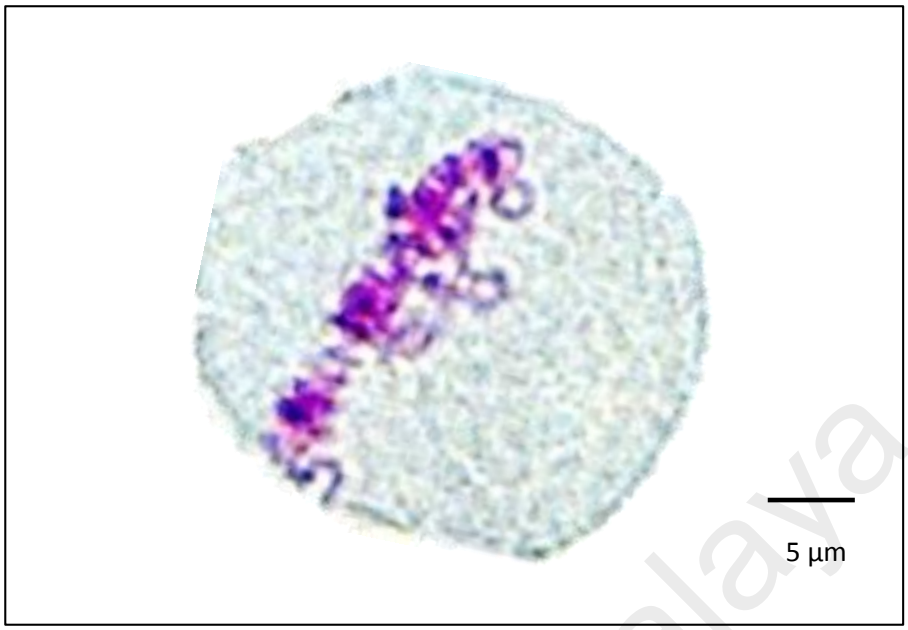


Figure 9.6 (c): Metaphase observed from *in vitro* primary root tip before secondary root emergence.

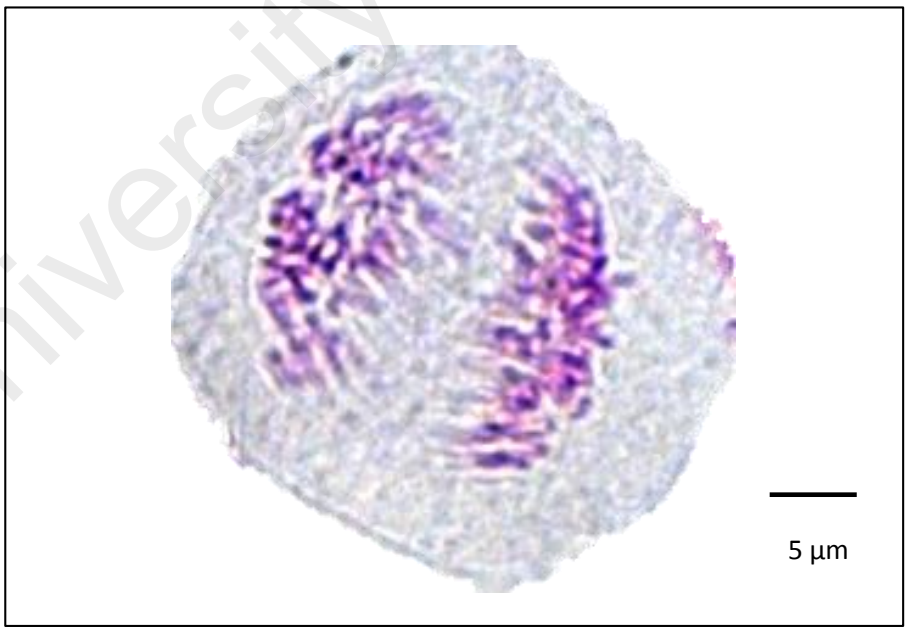


Figure 9.6 (d): Anaphase observed from *in vitro* primary root tip before secondary root emergence.

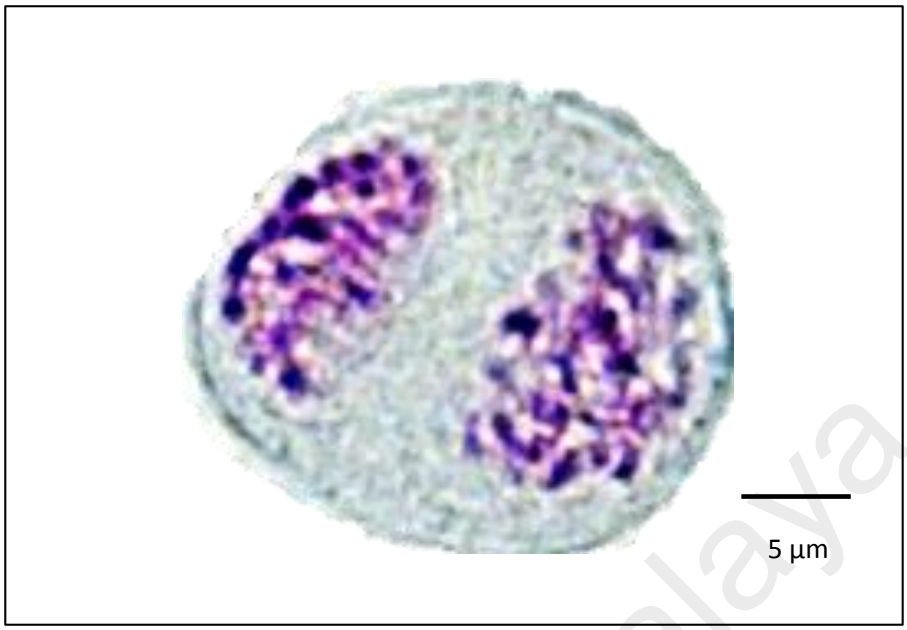


Figure 9.6 (e): Early telophase observed from *in vitro* primary root tip before secondary root emergence.

Figure 9.6 (f): Telophase observed from *in vitro* primary root tip before secondary root emergence.

9.3.4 Stages of Mitosis in *Ex Vitro* Meristematic Root Cells (Root tips)

Temporary slides were also prepared for primary root tips of plantlets grown *ex vitro* (Figure 9.7) which were harvested before secondary root emergence. The stages of mitosis in *ex vitro* meristematic root cells were viewed under a light microscope. The results presented all the phases including interphase, prophase, metaphase, anaphase and telophase (Figure 9.8) which comparable with the phases observed in *in vivo* and *in vitro* samples. Therefore, comparisons were made between all the samples by analysing several cytological studies including measurement of mitotic index, chromosome counts, nuclear areas, cell areas and ratio of nuclear to cell areas.

Figure 9.7: Primary root tips of *ex vitro* plants (1 month) grown on moist cotton wools.

9.3.4.1 Stages of mitosis for *ex vitro* primary root tips before secondary root emergence

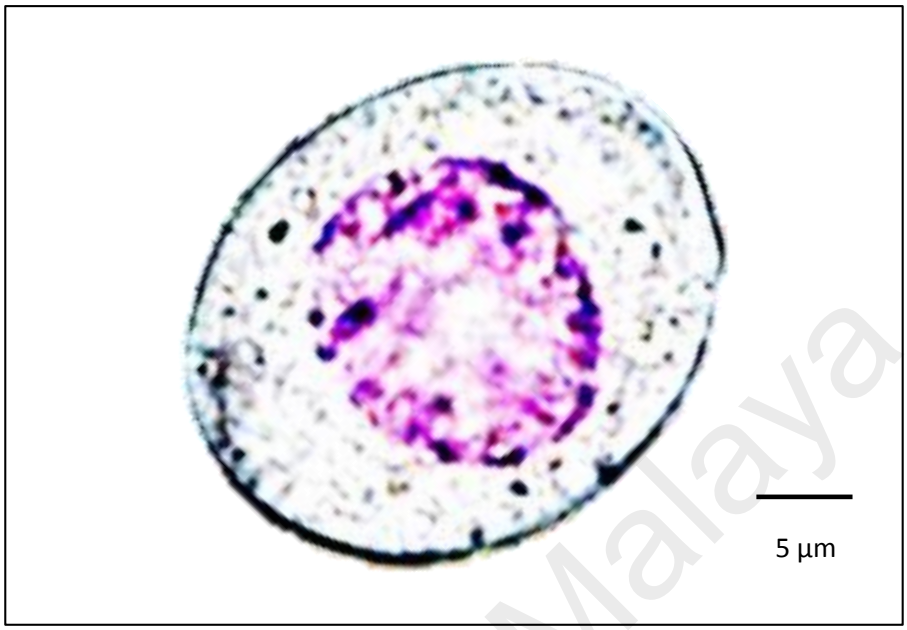


Figure 9.8 (a): Interphase observed from *ex vitro* primary root tip before secondary root emergence.

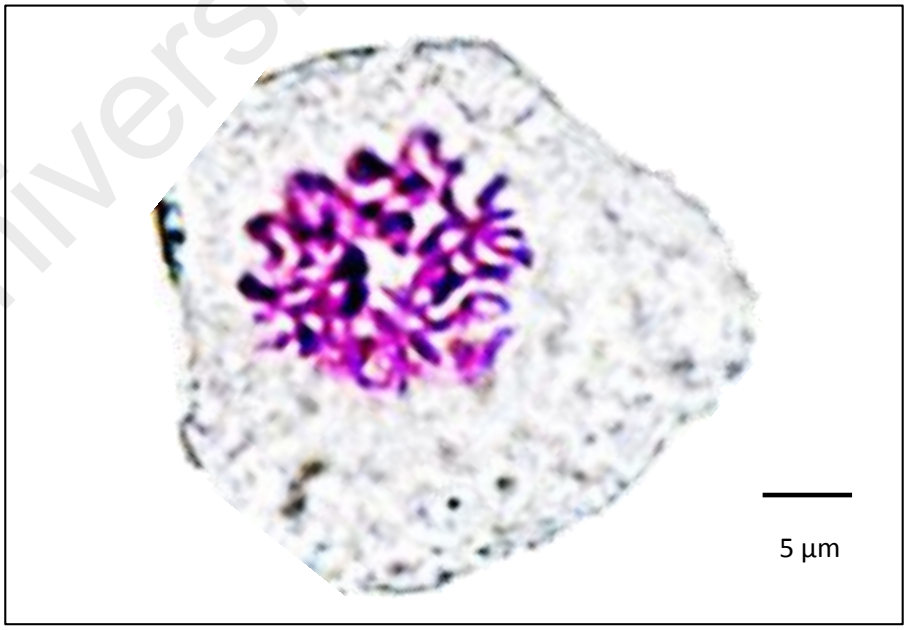


Figure 9.8 (b): Prophase observed from *ex vitro* primary root tip before secondary root emergence.

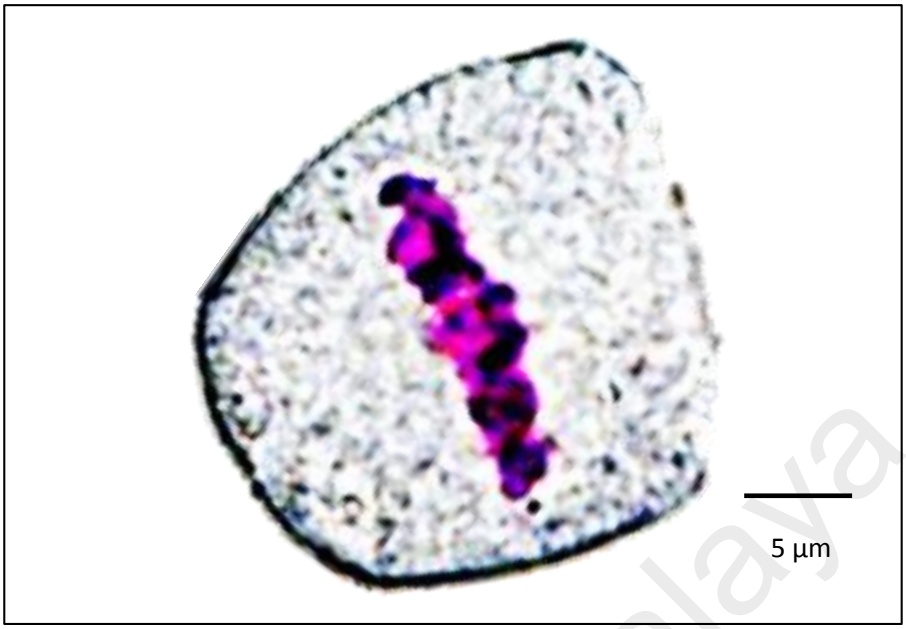


Figure 9.8 (c): Metaphase observed from *ex vitro* primary root tip before secondary root emergence.

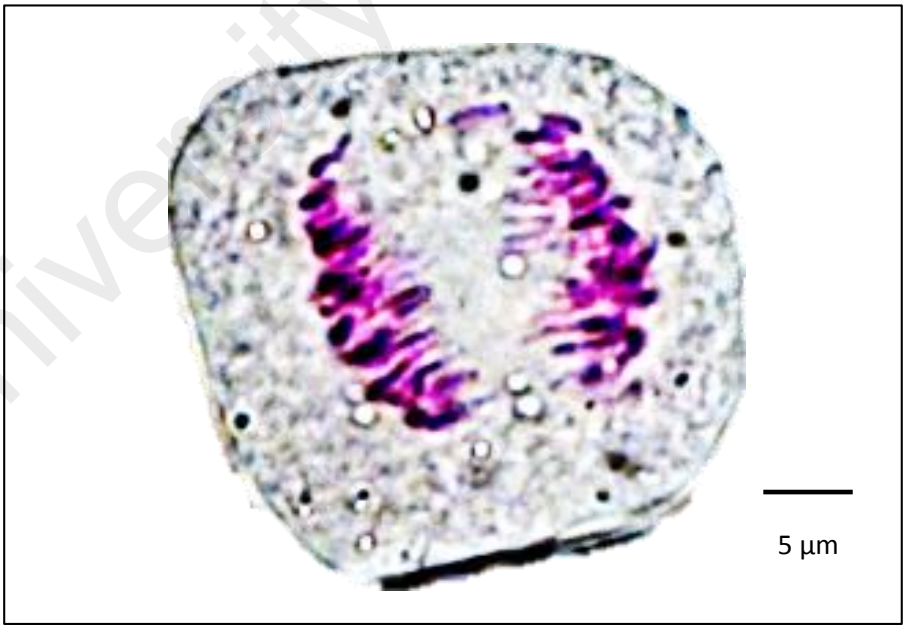


Figure 9.8 (d): Anaphase observed from *ex vitro* primary root tip before secondary root emergence.

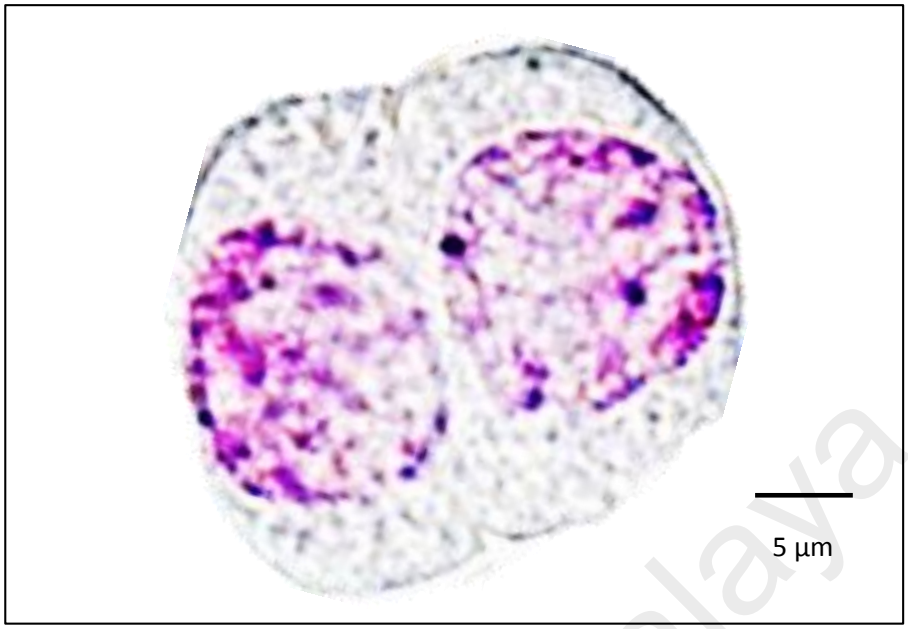


Figure 9.8 (e): Early telophase observed from *ex vitro* primary root tip before secondary root emergence.

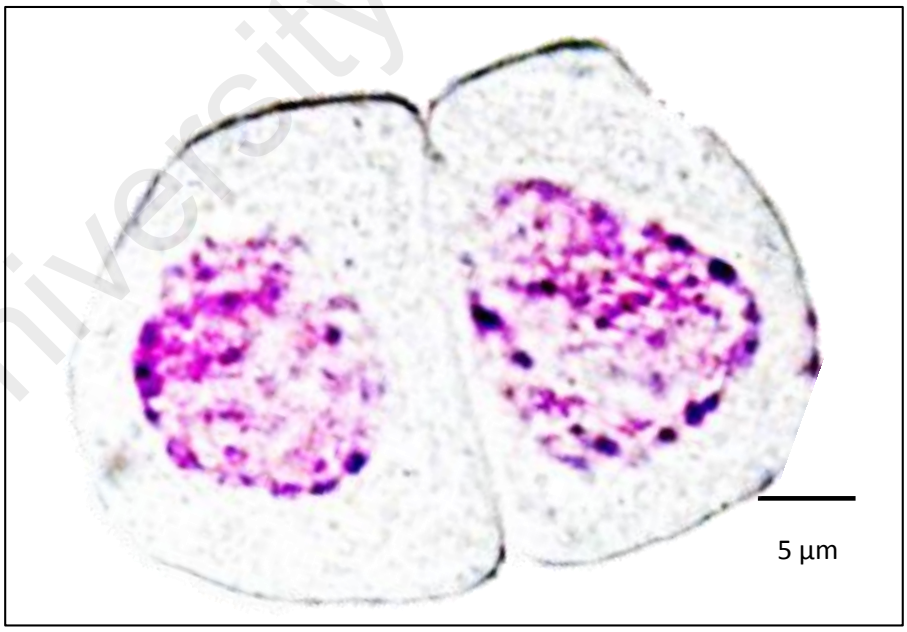


Figure 9.8 (f): Telophase observed from *ex vitro* primary root tip before secondary root emergence.

9.3.5 Measurement of Cellular Parameter

Analysis on cellular parameters was done by measuring the mitotic index, chromosome counts, nuclear areas, cell areas, and ratio of nuclear to cell areas of root tip meristem. Investigation of the parameters may facilitate significant knowledge in cellular behaviour of root tip which will lead to the understanding of the behaviour of cells in other tissues. The studies revealed that cellular behaviour of primary root of *in vitro* plantlets and *ex vitro* plants were almost similar, with no significant difference in most of the cellular behaviour parameters measured (Table 9.2, Table 9.3). However, analysis of results for all the samples verified that cellular behaviour for the primary root of *in vitro* plantlets and *ex vitro* plants were significantly different with the primary root of *in vivo* seedlings, as a control (Table 9.2, Table 9.3).

9.3.5.1 Mitotic Index (MI)

Mean mitotic index of primary root tip of seedlings grown *in vivo* was significantly higher than the mean mitotic index of primary root tip of plantlets grown *in vitro* and primary root tip of plants grown *ex vitro* (Table 9.2). The result verified that the mean mitotic index of primary root tip of seedlings grown *in vivo* was the highest (38.80%), followed by plantlets grown *in vitro* (26.40%) and primary root tip of plants grown *ex vitro* (19.00%). Though, the mean mitotic index of primary root tip seem to decrease as the *in vitro* plantlets were transferred to *ex vitro* condition, statistical analysis proved that there was no significant difference between the mean mitotic index of *in vitro* and *ex vitro* samples.

9.3.5.2 Chromosome Counts

Mean chromosome counts for primary root tip of seedlings grown *in vivo,* plantlets grown *in vitro* and plants grown *ex vitro* were measured. Observation on chromosome counts was hardly made since the chromosomes were not clearly separated due to poor slide preparation. Therefore, the chromosome counts were analysed mainly

using Image Analyzer software (Axio Vision 4.7) where majority of the chromosome counts were in the range of 20-22 (Figure 9.9). The mean chromosome counts calculated for *in vivo*, *in vitro* and *ex vitro* samples were 20.33, 21.93 and 21.40, respectively (Table 9.2). Analysis on the results verified that the mean chromosome counts for *in vitro* sample was not significantly different from the mean chromosome counts for *ex vitro* sample but, significantly different with the mean chromosome counts for *in vivo* sample. Nevertheless, there was no difference between the mean chromosome counts for *in vivo* and *ex vitro* samples statistically.

9.3.5.3 Mean Nuclear and Cell Areas

Similarly, the mean nuclear and cell areas of primary root tip of plants grown *ex vitro* were higher compared to the mean nuclear and cell areas of primary root tip of plantlets grown *in vitro* and seedlings grown *in vivo* (Table 9.3). The highest mean nuclear and cell area evaluated for *ex vitro* sample was 286.71 μm^2 and 845.11 μm^2. Meanwhile, the mean nuclear and cell areas for *in vitro* sample was 256.39 μm^2 and 747.81 μm^2 whereas for *in vivo* sample was 184.20 μm^2 and 523.70 μm^2, respectively. The results indicate that both *in vitro* and *ex vitro* samples were not significantly different, but there was significant difference with the *in vivo* samples. The mean ratio of nuclear to cell areas for all the samples was retained, with no significant difference statistically.

Table 9.2: Comparison of the mean mitotic index (%) and chromosome counts for primary root tip of seedlings grown *in vivo*, plantlets grown *in vitro* and plants grown *ex vitro* of *Echinocereus cinerascens*.

Treatment	Mean MI (%) ± SE	Mean CC ± SE
In vivo (seedlings)	38.80 ± 2.75 a	20.33 ± 0.44 b
In vitro (plantlets)	26.40 ± 0.93 b	21.93 ± 0.20 a
Ex vitro (plants)	19.00 ± 0.71 b	21.40 ± 0.32 ab

Data represents mean value ± standard error (SE) for five replicates of each treatment. Means with different letters in the same column are significantly different at p = 0.01 according to Duncan's multiple range test (DMRT). MI, mitotic index; CC, chromosome counts.

Table 9.3: Comparison of the mean nuclear areas (NA), cell areas (CA) and the mean ratio of nuclear to cell areas (NA/CA) for primary root tip of seedlings grown *in vivo*, plantlets grown *in vitro* and plants grown *ex vitro* of *Echinocereus cinerascens*.

Treatment	Mean NA ± SE	Mean CA ± SE	Mean Ratio NA/CA ± SE
In vivo (seedlings)	184.20 ± 9.51 b	523.70 ± 28.07 b	0.35 ± 0.01 a
In vitro (plantlets)	256.39 ± 9.25 a	747.81 ± 31.58 a	0.34 ± 0.01 a
Ex vitro (plants)	286.71 ± 6.19 a	845.11 ± 27.87 a	0.34 ± 0.01 a

Data represents mean value ± standard error (SE) for five replicates of each treatment. Means with different letters in the same column are significantly different at p = 0.01 according to Duncan's multiple range test (DMRT). NA, nuclear areas; CA, cell areas.

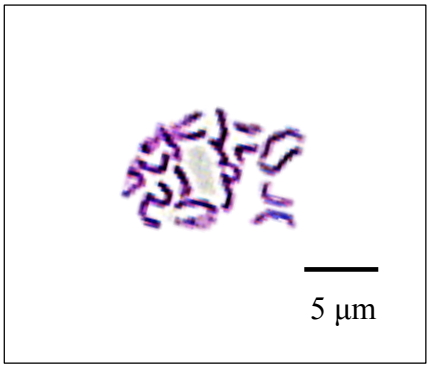


Figure 9.9 (a): 2n = 20 chromosomes obtained from the primary root tip of seedling grown *in vivo* of *Echinocereus cinerascens*.

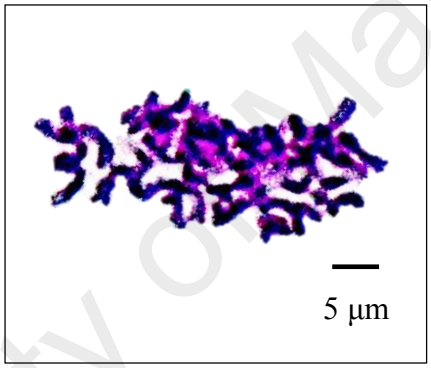


Figure 9.9 (b): 2n = 22 chromosomes obtained from the primary root tip of plantlet grown *in vitro* of *Echinocereus cinerascens*.

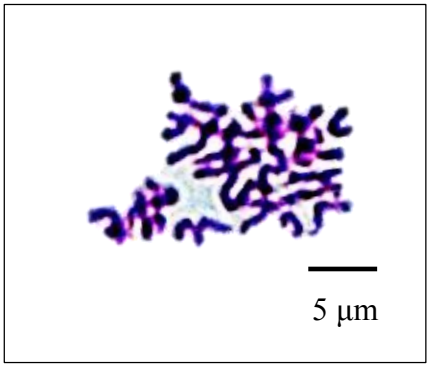


Figure 9.9 (c): 2n = 22 chromosomes obtained from the primary root tip of plant grown *ex vitro* of *Echinocereus cinerascens*.

9.4 SUMMARY OF RESULTS

1. The rate of primary root elongation was 0.0768 cm per day as shown by the standard growth graph in a linear regression line of $y = 0.0768x – 0.1984$, with the correlation coefficient, $R^2 = 0.9437$.
2. The standard root length was obtained on day 9, with mean primary root length of 0.53 cm where the optimum primary root growth was detected.
3. Observation on temporary slides prepared viewed under a light microscope found that all the phases such as interphase, prophase, metaphase, anaphase and telophase were comparable for primary root tip of *in vivo*, *in vitro* and *ex vitro* samples.
4. The highest mean mitotic index was observed in *in vivo* primary root tip with 38.80%, which was significantly higher than the mean mitotic index of *in vitro* and *ex vitro* primary root tip. The mean mitotic index of *in vitro* and *ex vitro* primary root tip was 26.40% and 19.00%, respectively.
5. Majority of the chromosome counts analysed were slightly different, in the range of 20-22. The mean chromosome counts calculated for *in vivo, in vitro* and *ex vitro* primary root tip were 20.33, 21.93 and 21.40, respectively.
6. Analysis of the results also indicated that the mean nuclear (286.71 μm^2) and cell areas (845.11 μm^2) of *ex vitro* primary root tip was higher than the mean nuclear (256.39 μm^2) and cell areas (747.81 μm^2) of *in vitro* primary root tip with no difference statistically. In contrast, both of the results were significantly different with the mean nuclear (184.20 μm^2) and cell areas (523.70 μm^2) of *in vivo* primary root tip.
7. Nevertheless, the ratio of nuclear to cell areas for primary root of *in vivo*, *in vitro* and *ex vitro* were not significantly different.

CHAPTER 10

DISCUSSION

The present work deals mainly with establishing an efficient and reproducible micropropagation or *in vitro* regeneration procedure for an endangered cactus species, *Echinocereus cinerascens* since it offers an alternative for the production of thousands of plants from a single explant in a short period of time. It has been proven that *in vitro* regeneration approaches are tremendously essential for the conservation of endangered plant species (Coelho *et al*., 2012; Debnath, 2004), propagation of elite superior varieties for trade, high production of secondary metabolites etc. Successful *in vitro* plant regeneration coupled with genetic engineering work has a great potential in advanced research of plant biotechnology to produce superior plants which are pathogen-free or disease-resistant, plants with higher content of valuable compounds and other desirable characteristics.

Numerous factors have been reported to influence the success of *in vitro* plant propagation including starting culture materials (age and types of explants), culture conditions (culture room temperature, photoperiod and pH of medium), plant growth regulators applied (concentrations and combinations of auxin and cytokinin) etc. (Chen *et al.*, 2012; Jia *et al*., 2014; Zhang *et al.*, 2013). Trigano and Gray (2000) reported that explant responses were preferentially stimulated by different concentrations of endogenous and exogenous hormones. Additionally, the source of exogenous regulators (auxin and cytokinin) was needed for the maximum production of shoots that interacted strongly with the species (Clayton *et al.*, 1990). This suggest that the optimum medium for *in vitro* plant regeneration differed for each of plant species due to the great variation in the requirements of exogenous regulators.

A number of studies on the effects of plant growth regulators (exogenous auxin and cytokinin) in *in vitro* regeneration of cactus species have been successfully established. However, to date, the *in vitro* regeneration of *Echinocereus cinerascens* has not been reported. Hence, this research was performed and the observations revealed the effects of plant growth regulators on shoot formation, regeneration and coloured callus production of *Echinocereus cinerascens in vitro* (Elias *et al*., 2015). Besides, other critical areas of research were included the production of somatic embryos, synthetic seeds, analysis of pigment content and cytological studies of *Echinocereus cinerascens* since nowadays the intention is directed not only to produce plants on a large scale but also to reveal and introduce multipurpose plants that could give maximum benefits. Recently, increased awareness of the impressive benefits of multipurpose plants for the medicinal, food and textile industries, as well as ornamental horticulture has spurred research projects in numerous related fields. Through the development of technology and advanced research, many plants are being explored and exploited for their benefits, including cactus species.

Chapter 3 presented the effects of plant growth regulators applied singly and in combinations on direct *in vitro* regeneration of *Echinocereus cinerescens*. Stem explants were cultured in MS medium supplemented with various concentrations and combinations of plant growth regulators and subsequently, all cultured explants were allowed to develop and maintained in the culture room at 25 ± 2 °C under 16 hours light provided by cool daylight or fluorescent light (36W), PHILIPS.

Observations after 4 months recorded media treated with Kinetin alone produced higher mean number of shoots, followed by BAP, control, IBA and lastly NAA (Table 3.1 and 3.5). In media with hormone combinations, Kinetin + IBA showed higher mean number of shoots compared to NAA + BAP (Table 3.2 and 3.6). Nevertheless, it was found that the highest mean number of shoots was 4.37, produced in MS medium

supplemented with 2.0 mg/l Kinetin + 1.0 mg/l IBA (Table 3.6). This result suggest the formation of shoots in *Echinocereus cinerascens* requires a combination of cytokinin and auxin to exhibit optimum production of shoots. This finding is supported by other studies carried out by Martìnez-Vázquez and Rubluo (1989) and Infante (1992) that stated in certain genera and species of cactus, a single culture medium may not be suitable for optimum shoot production. Previous study reported by Perez-Molphe-Balch *et al*. (1998) discovered encouraging effects of hormone combinations on axillary proliferation of cacti, in which among all cactus species studied, *Echinocereus dubis* gave 4.87 shoots per explant, while *Echinocereus pectinatus* formed 3.86 shoots per explant in medium with 1.0 mg/l BA + 0.01 mg/l NAA. Additionally, Hubstenberger *et al*. (1992) also stated that the production of axillary shoots was affected by the combination of plant growth regulators applied, which is unique for each species. Conversely, several studies revealed direct regeneration of other cactus species through the initiation of axillary buds from areoles structure cultured in media with high or moderate concentrations of cytokinin (BA or Kinetin) and zero auxins (NAA or IAA) (Aliyu and Mustapha, 2007; Pérez-Molphe-Batch *et al*., 2012). The studies indicated that *in vitro* plant regeneration could also be achieved in media added with cytokinin applied singly.

In general, analysis on the shoot sizes of *Echinocereus cinerascens* revealed that height and diameter of shoots produced were standard and uniform in all media, regardless of control and media with auxin applied singly (Table 3.3, 3.4, 3.7 and 3.8). Most of the shoots produced about 1 cm height and diameter as they reached 4 months old. Similar analysis was done by Pérez-Molphe-Balch *et al*. (2002) where shoots with homogeneous size were produced when lateral and transverse explants were cultured while shoots with heterogeneous size were observed from apical explants. However, observations recorded that the greatest shoot height of *Echinocereus cinerascens* was

2.72 cm observed in MS medium supplemented with 2.0 mg/l Kinetin + 0.5 mg/l IBA (Table 3.8) whereas the greatest shoot diameter was 1.30 cm observed in MS supplemented with 2.0 mg/l Kinetin (Table 3.7).

Further analysis on the production of shoots monthly indicated that media added with hormones applied singly such as BAP and Kinetin produced higher number of shoots after 1 month of culture (Figure 3.4 and 3.7). In contrast, media added with hormone combinations, NAA + BAP and Kinetin + IBA produced higher number of shoots after 2 and the following months (Figure 3.5 and 3.8). Based on the data recorded, the highest production of shoots after 4 months was 131 shoots, observed in MS medium supplemented with 2.0 mg/l Kinetin + 1.0 mg/l IBA (Figure 3.8). Therefore, MS medium supplemented with 2.0 mg/l Kinetin + 1.0 mg/l IBA was considered as the optimum medium for direct *in vitro* regeneration of *Echinocereus cinerascens*.

Ultrastructural studies performed on *in vivo, in vitro* and *ex vitro* samples using a light microscope, SEM and FESEM revealed interesting structures in morphology and anatomy of *Echinocereus cinerascens.* All the samples showed similar structures of apical shoots, vascular bundles, starch grains and types of stomata, areoles, ribs and spines however with slightly different in the mean number of stomata per area (mm^2), mean size of stomata, length and width of stomata opening (Table 3.9, 3.10 and 3.11). Nevertheless, statistical analysis verified that there was no significant difference between the samples tested, which is in agreement with results of Batagin-Piotto *et al*. (2012) who described that no visible alterations in the structural characteristics examined on leaves of *Bactris gasipaes* under different cultivation conditions (*in vivo, in vitro* and *ex vitro*). Observations on morphological features of *Echinocereus cinerascens* including epidermis surfaces, viewed using SEM verified that the structures of stomata were anisocytic, as most of the samples depicted the stomata surrounded by

three subsidiary cells of which one was distinctly smaller than the other two (Zarinkamar, 2007). According to Janu and Raghuvanshi (2011), stomata in cactus species (*Mammillaria* spp.) were nocturnal in nature. They claimed that each of the stomata had a pair of kidney-shaped guard cells that consists of a nucleus and a number of chloroplasts. Ting (1985) and Nobel (1988) also reported similar finding where the cacti exhibited nocturnal stomata opening.

Stoma has been considered as one of the main structures in plants that are widely dispersed on epidermis surfaces. The epidermis surfaces consist of epidermal cells which vary amongst different species. Results on the ultrastructure of *Echinocereus cinerascens* revealed that stomata were randomly scattered on epidermis surfaces and the epidermal cells observed were sinuous. Similarly, microscopic studies on other cactus species such as *Mammillaria* spp. Detected that the epidermal cells were normally sinuous while *Mammillaria comptrotricha* presented straight-walled epidermis cells (Janu and Raghuvanshi, 2011). In addition, the mean number of stomata per area (mm^2) recorded in *Echinocereus cinerascens* was in the range of 14-15 (Table 3.9), which was comparable with *Mammillaria* spp. that showed a range of 13-24 stomata per unit area. Further analysis regarding the number of stomata present on the epidermis surfaces revealed stomata per unit area of other plants was higher than cactus species (Janu and Raghuvanshi, 2011). This finding was supported by previous studies that suggested crassulacean acid metabolism (CAM) plants including cactus species had lower stomatal density compared to C3 and C4 plants (Hernández *et al*., 2007; Silva *et al*., 2001; Silva and Acevedo, 1984). In many cases investigated, water availability was perhaps one of the factors that regulate stomata density (Silva *et al*., 1999; 2001; Silva and Acevedo, 1984, Edward and Meidner, 1978) instead of light intensity (Retallk, 2001; Lu *et al*., 1993), temperature (Ciha and Brun, 1978), geographical location (Retallk, 2001) and CO_2 concentration (Bristow and Looi, 1968; Woodward, 1987;

Woodward and Bazzaz, 1988). Studies on stomatal density offered practical assistances to monitor environmental changes since stomatal characteristic could be used as an indicator of physiological response (Case, 2006; Zarinkamar, 2007). In CAM plants, morphological and anatomical changes basically contribute to increasing water use efficiency (WUE). Usually, a high WUE was found associated with a lower growth rate and it might delay dehydration which allowed the plants to grow under low water condition (Silva *et al*., 2010; 2014). On the other hand, details on morphological and anatomical features are very important parameters to detect any abnormalities that occur during *in vitro* plant regeneration for instance vitrification or somaclonal variation.

Vitrification or hyperhydricity is a morphological and physiological disorder whereas somaclonal variation is known as cytological abnormalities, qualitative and quantitative phenotypic mutation, nucleotide sequence changes, and gene activation and silencing (Kaeppler *et al.*, 2000). Hyperhydricity could be observed clearly through the plant appearance which show translucent characteristic or indicated by the shortage of chlorophylls and high water content instead. This might occur due to high relative humidity, low light intensity, concentrations of gelling agent and so on. The observations on ultrastructural studies showed that no morphological and physiological changes were detected in direct *in vitro* regeneration of *Echinocereus cinerascens.* Furthermore, investigations on cytological studies of meristematic root cells revealed that there was no variation of cell organization and behaviour of *Echinocereus cinerascens* regenerated *in vivo, in vitro* and *ex vitro* which will be discussed further in Chapter 9.

Chapter 4 described the effects of plant growth regulators applied singly or in combinations on somatic embryo induction of *Echinocereus cinerascens.* In this study, a protocol for indirect *in vitro* regeneration of *Echinocereus cinerascens* was successfully established as somatic embryogenesis was observed both in solid and

liquid cultures. Studies on somatic embryos are essential to investigate the regulation of embryo development besides acting as a tool for large scale vegetative propagation (Von Arnold *et al*., 2002). A number of reports indicated that somatic embryogenesis is a preferred vegetative propagation technique and considered as the best *in vitro* regeneration for most of the plants species including hardwood and softwood trees (Stasolla and Yeung, 2003; Merkle and Nairn, 2005; Oh *et al*., 2010). Additionally, somatic embryogenesis also represents a unique developmental pathway which involves several events such as dedifferentiation of cells, activation of cell division and reprogramming of their physiology, metabolism and gene expression patterns (Zimmerman, 1993; Schmidt *et al*., 1997; Komamine *et al*., 2005). According to Stasolla and Yeung (2003), somatic embryogenesis integrated with conventional breeding, molecular and cell biology would offer a significant alternative for genetic improvement of commercial crop species.

Embryogenic tissues produced by somatic cells would develop into somatic embryos which were influenced by either the levels of exogenously applied auxins or the ratio of auxin to cytokinin (Baskaran and Van Staden, 2012). Other studies recommended that the optimization of supplementation with plant growth regulators is prerequisite for the establishment of somatic embryogenesis and *in vitro* plant regeneration (Zhao *et al*., 2011; Zhou *et al*., 2012). Meanwhile, several researchers reported the origin of somatic embryos is much dependent on the types of explants used and culture medium conditions (Schwendiman *et al*., 1988; Kanchanapoom and Domyoas, 1999; Verdeil *et al.*, 2001; Sané *et al*., 2006; Sáenz *et al*., 2006). As noticed in the present study, stem explants of *Echinocereus cinerascens* showed direct somatic embryogenesis in solid culture while indirect somatic embryogenesis was observed in liquid culture. In direct somatic embryogenesis of *Echinocereus cinerascens*, somatic embryos could be detected with the formation of globular-shaped after 1 month

whereas, after 2 months, both globular and heart-shaped were observed (Figure 4.1, 4.2). However, the globular and heart-shaped were retained with no further development after 3 and 4 months. Thus, the highest total mean production of somatic embryos (globular and heart-shaped only) in solid culture was 100% observed after 2 months in MS medium supplemented with 1.0 mg/l 2,4-D + 0.1 mg/l thiamine HCl (Figure 4.3). Similarly, the failure of globular stage develops into mature embryos was also detected in the induction of somatic embryogenesis of *Opuntia ficus-indica* as medullar discs and zygotic embryos were used as the explants (Santacruz-Ruvalcaba *et al*., 1998; Da Costa *et al*., 2001). Consequently, somatic embryogenesis of *Opuntia ficus-indica* was achieved directly as shoot apices were used as the explants (Kang *et al*., 2006) and indirectly as immature anthers were used as the explants (Bouamama *et al*., 2011). Both direct and indirect somatic embryogenesis of *Opuntia ficus-indica* presented similar stages of somatic embryos development including globular, heart, torpedo-shaped and cotyledonary stage. On the other hand, successful direct somatic embryogenesis induction of other plant species, *Drimia robusta* showed white globular embryoids developed directly from leaf explants after 4 weeks and further observations revealed that the other different stages were obtained in all media tested after 10 weeks (Baskaran and Van Staden, 20 14).

Incomparable, through indirect somatic embryogenesis of *Echinocereus cinerascens*, somatic embryos could be detected with the formation of proembryo masses after 1 month which further developed into all stages of somatic embryos (globular, heart, torpedo-shaped and cotyledonary stage) after 2 months (Figure 4.7, 4.8). Total mean production of somatic embryos (all stages) in liquid culture after 2 months was 90%, observed in MS medium supplemented with 0.5 mg/l 2,4-D + 0.1 mg/l BAP + 0.5 mg/l thiamine HCl (Figure 4.9). Finally, the production of somatic embryos gradually increased and reached 100% after 4 months, which were observed in

two treatments including MS medium supplemented with 0.5 mg/l 2,4-D + 0.1 mg/l BAP + 0.5 mg/l thiamine HCl and MS medium supplemented with 0.5 mg/l 2,4-D + 0.5 mg/l BAP + 0.5 mg/l thiamine HCl (Figure 4.12). Thus, both treatments were considered as the optimum medium for the induction of somatic embryogenesis of *Echinocereus cinerascens*. Even though the induction of direct somatic embryogenesis of *Echinocereus cinerascens* in solid culture (formation of somatic embryos directly from explants) was faster compared to indirect somatic embryogenesis induced in liquid culture (formation of somatic embryos through intermediate callus phase), however the results verified that all stages of embryo development were studied in indirect somatic embryogenesis as callus of stem explants were cultured in liquid media. Nhut *et al*. (2006) stated that masses of proembryogenic callus grew slowly in liquid compared to solid medium. The evidence indicated that there was a different embryogenic ability of callus cultured in liquid and solid culture where callus cultured in solid medium grew faster than in liquid culture.

Other cactus species which showed successful induction of indirect somatic embryogenesis were *Turbinicarpus pseudomacrochele* (Torres-Muñoz and Rodríguez-Garay, 1996) and *Opuntia ficus-indica* (Bouamama *et al*., 2011). Both studies reported that indirect somatic embryogenesis were obtained in solid culture. Somatic embryogenesis of *Turbinicarpus pseudomacrochele* was achieved as medullar tissue discs were cultured in MS medium supplemented with L2 vitamins + 3.0 mg/l 2,4-D + 2.0 mg/l NAA + 2.0 mg/l KIN + 500 mg/l L-glutamine + 250 mg/l casein hydrolysate. Observations after 4 weeks revealed that the explants produced creamy-yellowish embryogenic callus incorporated with globular and well-defined embryo structures. In contrast, somatic embryogenesis of *Opuntia ficus-indica* was developed when immature anthers were cultured in Chée and Pool medium (Chée and Pool, 1987) containing 2.0

mg/l 2,4-D + 2.5 mg/l TDZ. Primary nodular and greenish callus were observed after 6-8 weeks and development of embryos was detected after 4-5 months.

Several studies indicated that somatic embryogenesis is generally induced by auxins, either alone or in combination with cytokinin (Fei *et al*., 2002; Shahana and Gupta, 2002; Anthony *et al*., 2004; Shu *et al*., 2005). Most of the studies demonstrated that the induction of somatic embryogenesis is normally initiated by the action of auxin (Rao, 1996; Dodeman *et al.*, 1997; Jiménez and Thomas, 2006; Fehér, 2005) whereby 2,4-D is considered as the most effective auxin for the induction (Nomura and Komamine, 1986; Jasrai *et al*., 2003; Jiménez, 2005, Lema-Rumińska and Kulus, 2012). According to Lema-Rumińska (2011), the induction of somatic embryogenesis was also influenced by species or type of explant used, instead of auxin. Several studies in cactus species revealed that high concentrations of auxin with low concentrations of cytokinin induced somatic embryogenesis in *Neomammillaria prolifera* (Minocha and Mehra, 1974), *Aztekium ritteri* (Rodríguez-Garay and Rubluo,1992), *Mediocactus coccineus* (Infante, 1992), *Turbinicarpus pseudomacrochele* (Torrez-Muńoz and Rodríguez-Garay, 1996), and *Mammillaria san-angelensis* (Marín, 1998). Lema-Rumińska and Fijałkowska (2006) reported that somatic embryogenesis was induced as meristematic explants of *Copiapoa tenuissima* Ritt. *f. monstrosa* and *Gymnocalycium mihanovichii f. aurantica* were cultured on MS medium supplemented with 2.0 mg/l 2,4-D. A study by Lema-Rumińska and Kulus (2012) suggested that somatic embryogenesis was successfully induced in *Astrophytum asterias* (Zucc.) Lem. when the explants were cultured on MS medium supplemented with 7.0 mg/l 2,4-D.

Although the induction of somatic embryos has been successfully established for many species, however only a few studies on cactus species were reported on germination and subsequent development of embryos into plantlets. In this study, further development of embryos into plantlets (Figure 4.13) were achieved once somatic

embryos of *Echinocereus cinerascens* were transferred into MS basal medium. This result was comparable with previous research reported by Torrez-Muńoz and Rodríguez-Garay (1996) that stated germination of somatic embryos of *Turbinicarpus pseudomacrochele* was evident after 16 weeks on MS basal medium. Meanwhile, Bouamama *et al.* (2011) found that further development of somatic embryos of *Opuntia ficus-indica* was accomplished as they were cultured on ½ MS basal medium containing 1% (w/v) of activated charcoal. Besides, somatic embryos of *Neomammillaria prolifera* (Minocha and Mehra, 1974) and *Ariocarpus retusus* (Olguín, 1994) were also successfully developed into plantlets albeit with low percentage of germination. There are diverse factors that can inhibit or promote germination of embryos. Studies by Litz *et al.* (1998), identified the factors involved in the induction of germination and maturation of embryos which were culture media and types of plant growth regulators applied. Debergh (1987) reported that many dicotyledonous species need a period of dehydration and dormancy before zygotic embryo germination. According to Gray and Purohit (1991), this natural process can be emulated to improve the germination and development of somatic embryos as well.

Recently, somatic embryogenesis has been considered as an essential tool in the improvement of many crop varieties and production of synthetic seeds (Kumar and Thomas, 2012). Somatic embryo is mostly used as propagule in synthetic seed production because it is analogous to the natural seed (Kumar and Thomas, 2012), which possess the radical and plumule that are able to develop into root and shoot (Kitto and Janick, 1982; 1985; Gray *et al*., 1991; Redenbaugh, 1993; McKersie and Bowley, 1993), besides it promotes high efficiency to be formed clonally in bulk (Gantait *et al*., 2015). Therefore, synthetic seeds have been defined as somatic embryos engineered for commercial propagation of plants (Gray and Purohit, 1991; Redenbaugh, 1993).

Synthetic seeds have the possibility of being an alternative planting materials for agriculture sector, especially for highly demanded plant species (Asmah *et al*., 2013) due to ease of handling, storage, shipping, facilitate germplasm conservation of elite plant species, exchange of axenic plant materials between laboratories and pharmaceutical industries (Danso and Ford-Lloyd, 2003; Rai *et al*., 2009) and so on. Hence, synthetic seed technology has been widely studied and discovered to be applicable for various plant species including medicinal plants, crops, ornamentals and others where different types of propagules such as somatic embryos, shoot tips, axillary buds, micro shoots and other meristematic tissues were applied (Table 5.1). Nevertheless, no report has so far established on the production of synthetic seed of *Echinocereus cinerascens*. Thus, the current research aimed to develop a protocol for synthetic seed production of this species.

In Chapter 5, an efficient procedure for the production of synthetic seed of *Echinocereus cinerascens* was established. An ideal synthetic seed of *Echinocereus cinerascens* was produced by encapsulating propagule such as micro shoot and stem in 3% sodium alginate which serves as synthetic endosperm and then, hardened in 100 mM of calcium chloride dehydrate solution for 30 minutes. The synthetic seeds were firm, isodiametric and round shaped. It was evident from the previous researches that most of the optimum results produced spherical beads with 3% sodium alginate which hardened in 100 mM of calcium chloride dehydrate solution. This condition resulted in 80-100% regeneration observed in *Stevia rebaudiana, Ceropegia bulbosa, Withania somnifera, Ochradenus baccatus, Anethum graveolens, Terminalia arjuna, Cassia angustifolia* Vahl., *Phyllanthus fraternus, Balanites aegyptiaca,* and *Vitis trifolia* L. (Ali *et al.*, 2012; Dhir and Shekhawat, 2013; Fatima *et al.*, 2013; Al-Qurainy *et al*., 2014; Dhir *et al.*, 2014; Gupta *et al.*, 2014; Parveen and Shahzad, 2014; Upadhyay *et al.*, 2014; Varshney and Anis, 2014; Shaheen and Shahzad, 2015). Ganapathi *et al*. (1992),

Sarmah *et al*. (2010) and Tabassum *et al*. (2010) suggested that 3% sodium alginate with 100 mM of calcium chloride dehydrate solution was an ideal combination for the production of synthetic seeds. Besides, other aspects that also contribute to obtain desirable shape and size of synthetic seeds include the successful use of pipette and tip while in preparation (Gantait *et al.*, 2012; Mallón *et al.*, 2007).

In the current study, the effects of different types of propagules and encapsulation matrix for the production of synthetic seeds and the optimum medium for synthetic seed germination were investigated. Several factors extensively influenced the success of encapsulation of propagules for synthetic seed production, their storage and regeneration include types of propagules or plant materials and encapsulation agent or matrix (Gantait *et al*., 2015). Gantait *et al*. (2015) reported that a variety of unipolar (nodal segment, shoot tip, micro shoot) and bipolar (somatic embryos) vegetative propagules were successfully exploited for synthetic seed production. Some researchers demonstrated that 100% regeneration of synthetic seeds from encapsulated nodal segments (Lata *et al*., 2009; Singh *et al*., 2010; Germanà *et al*., 2011; Hung and Trueman, 2012a; 2012b; Dhir and Shekhawat, 2013), shoot tips (Tsvetkov *et al*., 2006; Singh *et al*., 2009; Hung and Trueman, 2011; 2012a; 2012b) and somatic embryos (Chithra *et al*., 2005; Cheruvathur *et al*., 2013) while the highest regeneration of synthetic seeds from encapsulated micro shoots (Saha *et al*., 2014) was 98.6% reported in *Ocimum gratissimum*.

Instead of propagules, encapsulation matrix is also vital for achievement of synthetic seed production. It has been proven that synthetic seed coat (encapsulation matrix) is absolutely necessary to protect the explant as it holds the explant firmly and subsequently provides sufficient resistance to exterior mechanical stress, for easy handling (Gantait *et al*., 2015). Besides, it promotes efficiency to supply nutrients for seed germination and growth as reported by Mohanraj *et al*. (2009) that encapsulation

matrix serves as a reservoir and supplies essential nutrients to encase explants. Redenbaugh *et al.* (1991; 1993) stated the factors that responsible for successful production of synthetic seeds including level and type of gel required for encapsulation and the extent exposure of encapsulated seeds to calcium chloride dehydrate solution. There are several types of gels that have been exploited for the encapsulation such as agar, alginate, carboxy methyl cellulose, carrageenan, gelrite etc. (Saiprasad, 2001), however alginate is highly preferred among the others since it aids to ameliorate capsule development (Gantait *et al.*, 2015). In addition, sodium alginate is the most frequently used for encapsulation matrix because of its low cost, good gelling properties and nontoxic nature (Cheruvathur *et al*., 2013). Principally, the hardness of encapsulation matrix is dependent on the number of Na^+ (in sodium alginate solution) exchanged with Ca^{2+} (in calcium chloride dehydrate solution), which resulted in the creation of insoluble calcium alginate (Daud *et al*., 2008).

Present study on the production of synthetic seeds of *Echinocereus cinerascens* is generally consistent with previous research findings by Gantait *et al*. (2015) which clarified that 3% sodium alginate and 100 mM of calcium chloride dehydrate solution assisted in the most advantageous ion exchange of Na^+ and Ca^{2+}, producing compact, transparent and isodiametric beads. Lesser concentrations of sodium alginate (1 and 2%) harden in 25-50 mM $CaCl_2.2H_2O$, displayed asymmetrical shape beads and being exceedingly fragile and squashy to hold, whereas, at elevated levels of sodium alginate (4 and 5 %) with 150 mM $CaCl_2.2H_2O$, the beads formed were effectively firm resulting in considerable impediment in germination of synthetic seeds (Singh *et al.*, 2006). Several findings suggested there is a direct relation between higher frequency of synthetic seed germination and reduce exposure time to calcium chloride dehydrate solution (Castillo *et al.*, 1998; Prewein and Wilhelm, 2002; Malabadi and Van Staden, 2005). Nagesh *et al.* (2009) clarified that low resistance beads exposed for a long period

to $CaCl_2.H_2O$ might cause $CaCl_2$ toxicity. High accumulation of $CaCl_2$ could restrain further development of the synthetic seeds. Thus, resulting in low frequency of germination.

Observations recorded that ideal synthetic seeds of *Echinocereus cinerascens* were germinated in different types of media and encapsulation matrix (Table 5.3, 5.4). Synthetic seeds produced by encapsulating the propagules in different sodium alginate were successfully germinated in MS basal medium and MS medium supplemented with 2.0 mg/l Kinetin + 0.5 mg/l IBA. Among the propagules tested, micro shoot was found to be the most responsive since synthetic seeds produced by encapsulating micro shoots as propagules gave the highest germination rate (100%), after 4 months of culture (Table 5.3). Meanwhile, synthetic seeds produced by encapsulating stems as propagules showed only 50% of germination rate after 4 months of culture (Table 5.4). The results verified that germination of synthetic seeds was influenced by the type of propagule, encapsulation matrix or synthetic endosperm and selected medium for germination. Synthetic seeds germinated and produced single shoot almost in all treatments while the production of multiple shoots was obtained only in several treatments tested.

In MS basal medium, germination of synthetic seeds with synthetic endosperm contained sodium alginate added with 2.0 mg/l Kinetin + 0.5 mg/l IBA was better compared to the others (germination of synthetic seeds with synthetic endosperm contained sodium alginate (control) or sodium alginate without sucrose). Synthetic seeds produced by encapsulating micro shoots in sodium alginate without sucrose showed the highest production of single shoot with 73% (Table 5.3) which was not significantly different from control (70%). The highest production of multiple shoots was 50%, as synthetic seeds produced by encapsulating micro shoots in sodium alginate added with 2.0 mg/l Kinetin + 0.5 mg/l IBA were cultured on MS basal medium. Meanwhile, synthetic seeds produced by encapsulating stems in sodium alginate added

with 2.0 mg/l Kinetin + 0.5 mg/l IBA showed the production of single and multiple shoots with only 30% and 13% (Table 5.4), respectively.

In MS medium supplemented with 2.0 mg/l Kinetin + 0.5 mg/l IBA (optimum medium for direct *in vitro* regeneration of *Echinocereus cinerascens*), both synthetic seeds produced by encapsulating micro shoots and stems in sodium alginate showed higher production of multiple shoots compared to single shoot. Synthetic seeds produced by encapsulating micro shoots in sodium alginate showed the highest production of multiple shoots with 100% (Table 5.3) while synthetic seeds produced by encapsulating stems in sodium alginate gave only 33% production of multiple shoots (Table 5.4). MS basal medium gave higher production of single shoot while the production of multiple shoots was higher in MS medium supplemented with 2.0 mg/l Kinetin + 0.5 mg/l IBA. This indicated that plant growth regulators play important role in the production of multiple shoots. Previous works demonstrated that synthetic seeds grew vigorously when nutrient component and phytohormones were added during encapsulation (Machii, 1992). Soneji *et al.*, (2002) reported that the germination of synthetic seeds to plantlets depending on hormone concentrations in the bead medium or encapsulation matrix. According to Machii (1992), encapsulation matrix added with BAP gave desirable result in synthetic seed germination of *Morus alba*.

Synthetic seeds of *Echinocereus cinerascens* stored at 4 ± 1 ºC for 30 days gave discouraging results as the synthetic seeds became dormant or dead as no germination was observed even until 4 months of culture. This result was unparalleled with previous research finding, which indicated that synthetic seed germination of *Camellia japonica* was significantly increased after storage at 4 ± 1 ºC for 2 months (Janeiro *et al.*, 1995). In addition, low temperature (4 ± 1 ºC) was essential for reasonable post-storage viability that gave promising results for survival and germination of synthetic seeds of *Clitoria ternatea* Linn (Kumar and Thomas, 2012). It was found that synthetic seeds of

Clitoria ternatea Linn storage at 4 ± 1 ºC sprouted even after 5 months. Similarly, encapsulated embryos of *Santalum album* retained their ability to germinate after storage at 4 ± 1 ºC for 45 days (Rao and Bapat, 1993) while synthetic seeds of *Picea glauca engelmannii* complex and *Picea mariana* Mill. storage at 4 ± 1 ºC for a month maintained with no loss of germination capacity (Lulsdorf *et al*., 1993). Ikhlaq *et al*. (2010) noted that synthetic seed germination of olive cv 'Moraiolo' stored at 4 ºC for 15 days increased as compared to control (synthetic seeds culture immediately after encapsulation). Interestingly, the conversion frequency (germination) of olive cv 'Moraiolo' increased with the increasing storage days up to certain extent and after that, the viability of encapsulated beads started to drop.

The decline in conversion frequency observed among encapsulated propagules stored at low temperature may have been a result of inhibited respiration of plant tissues, perhaps due to alginate cover (Redenbaugh *et al*., 1987), related to both oxygen deficiencies in the gel beads and due to rapid drying (Redenbaugh *et al*., 1991) or probably due to an anaerobic environment in the capsule as the embryos are not developmentally arrested and continue their active respiration (De, 1992). Studies on synthetic seed germination of *Echinocereus cinerascens* indicated that after storage for 30 days, synthetic seeds appear desiccated, dehydrated and lost the viability to germinate. The propagules became necrosis and some morphological changes were detected as the propagules turned to reddish and brown colour. Similar results were observed by Gantait et al. (2012) whereby, the synthetic seeds or capsules turned necrotic, shrunken and brown colour after storage at 4 °C for 120 days. In addition, the research demonstrated that storage at 25 °C gave promising results for the germination and conversion of capsules, which is in agreement with results obtained by Hung and Trueman (2011) as the encapsulated propagules survived longer at 25 °C compared to 4 °C. Previous studies by Rai *et al.* (2008) suggested an important feature of the

encapsulated vegetative propagules is the ability to retain viable after storage for a long period.

Shoots or plantlets of *Echinocereus cinerascens* grown *in vitro* were subjected to medium for rooting, before being acclimatized. Even though rooting *in vitro* is considered to be labour-intensive and expensive (Hazarika, 2003) however, it was apparent that this important step promoted well-developed roots which guaranteed a higher survival rate when *in vitro* plantlets were transferred for acclimatization (Sankar-Thomas *et al*., 2008). Sankar-Thomas and others suggested MS medium containing 0.5 mg/l IBA without sucrose was the optimum medium for *in vitro* rooting of *Camptotheca acuminate*. Nevertheless, this report was highly unparalleled with the current study that found MS basal medium was the optimum medium for rooting *in vitro* of *Echinocereus cinerascens* as a high frequency of plantlets exhibited healthy growth with well-developed roots system (complete plantlets). Primary root formation was observed during the first week, while secondary roots developed by the third week.

Other cactus species which exhibited root formation on MS basal medium including *Opuntia ficus-indica* (Angulo-Bejarano and Paredes-López, 2011), *Notocactus magnificus* (De Medeiros *et al*., 2006), *Cephalocereus senilis* (Choreńo-Tapia *et al*., 2002) and *Coryphanta elephantidens* (Bhau, 1999). A few studies indicated that possibly half-strength MS medium was suitable for *in vitro* rooting of *Opuntia* spp. (El Finti *et al*., 2013), *Epithelantha micromeris* (Villavicencio *et al*., 2012) and *Turbinicarpus laui* (Rosas *et al*., 2001). According to Hubstenberger *et al.* (1992), cactus species is known to produce high levels of auxins as accumulation of cytokinin-like substances in the regeneration medium due to repeated culture in media containing cytokinin that contributed to balance out high endogenous levels of auxins. A high level of endogenous auxins seemingly the evidence of spontaneous *in vitro* rooting of cactus species. Practically, this might lead to reduction of the costs and time for rooting or

production of complete plantlets (Clayton *et al*., 1990; Choreńo-Tapia *et al*., 2002; Bhau, 1999; El Finti, *et al*., 2012). Nonetheless, certain findings indicated that *in vitro* rooting of cactus species were favoured by auxin availability. Pérez-Molphe-Balch *et al*. (2002) applied 1.0 mg/l IBA in the medium which resulted in higher rooting frequency in three columnar cacti namely, *Stenocereus thurberi, Carnegiea gigantha* and *Pachycereus pringlei*. Moreover, Pelah *et al*. (2002) added 1.0 mg/l NAA in the medium for root induction of *Selenicerus megalanthus* that commonly known as yellow pitaya.

Complete plantlets with well-developed roots were selected for acclimatization. Acclimatization is required to ensure micropropagated plants survive and grow vigorously when transferred to soil. Chandra *et al.* (2010) explained that micropropagated plants need acclimatization for successful establishment and survival when transferred to *ex vitro* conditions (exposed to extreme changes in temperature, light intensity and water stress conditions). Micropropagated plants transferred directly from *in vitro* condition (heterotrophic) to greenhouse condition (autotrophic) might desiccate or wilt rapidly and finally die. Dhawan and Bhojwani (1986) claimed that tissue cultured plants were susceptible to transplantation shocks leading to high mortality during the final stage of micropropagation. Plantlets grown *in vitro* were exposed to constant temperature, very high air humidity, low irradiance, very low air turbulence, variable and often insufficient CO_2 concentration, water potential dependent on medium composition, sugars as a carbon source, growth regulators in nutrient medium etc. The environment might lead to the production of plantlets with different morphology, anatomy and physiology from naturally grown plants. Therefore, the plantlets would need to be put in shade for some time to correct *in vitro*-induced abnormalities and gradually lowering air humidity that is prerequisite for the successful

establishment of vigorous plants transferred to the greenhouse with low air humidity, high irradiance and so on (Pospíšilová *et al.*, 2007).

Chapter 6 presented acclimatization of *Echinocereus cinerascens* in different types of planting substrate viz. red soil, black/ garden soil and sand. Complete plantlets of *Echinocereus cinerascens* were successfully acclimatized in the planting substrates with different survival rates. The highest survival rate of acclimatized plantlets was 90% observed in sand, followed by red soil, 84% and black/ garden soil, 66% after 9 months being acclimatized in the natural environment. Therefore, sand was identified as the most suitable planting substrate for acclimatization of *Echinocereus cinerascens.* Quiala *et al.* (2009) similarly reported the highest survival rate of *Pilosocereus robinii* was 91.6% obtained in the substrate with 100% of cattle manure covered with 2.0 cm layer of zeolite, while 66% of survival rate was achieved in substrate with a mixture of 85% compost and 15% zeolite. According to the National Botanical Garden in Cuba, a complex mixture composed of 35% of well washed thick sand, 15% of rotten earth, 35% of humus and 15% of charcoal is recommended for sowing of cactus. However, the current study which in agreement with Quiala and others proved that a simple substrate is practical for acclimatization of cactus species.

Studies on acclimatization of *Echinocereus cinerascens* suggested that the survival rate was influenced by types of planting substrates applied which varies in nutrient supplies that are very important for the establishment of plant growth. Investigations on major and trace elements in red soil, black/ garden soil and sand were performed by XRF. Through XRF the element's total compositions in each of the planting substrates could be analysed. Application of the XRF offers the possibility to realize quick multi element soil analysis and speciation analysis (Baranowski *et al*., 2002). Observations revealed that the highest major elements detected in sand and red soils were similar, SiO_2 which showed 80% and 49%, respectively. Then, followed by

Al_2O_3 with 13% in sand while 42% in red soil. In contrast, the highest major elements obtained in black soil was CaO with 72%, whereas for SiO_2 and Al_2O_3 were correspond to 5% and 2%. Higher percentage of CaO in the sample examined might be influenced by a presence of clay and high content of organic matter (Baranowski *et al*., 2002). In general, the present study showed that most of the fractions examined were elements bound with oxides whereby, 2 major elements that were identified viz. SiO_2 and Al_2O_3 play important roles for growth of *Echinocereus cinerascens*.

Previous studies showed several cactus species that were successfully transferred to soil were *Echinocactus platyacanthus, Opuntia ellisiana,* and *Opuntia ficus-indica* (Pérez-Molphe-Balch *et al*., 1998; Juarez and Passera, 2002; El Finti *et al*., 2012). Studies by Wakhlu and Bhau (2000) and Bhau (1999) proved that *Coryphantha elephantidens* plantlets with good development of roots grown healthily with 100% of survival rate after being acclimatized. Overall, the regenerated plants showed no morphological difference as compared to the mother plant. In addition, higher survival rate (80-100%) of plants regenerated *in vitro* were also observed in *Mammillaria carmenae, Mammillaria prolifera, Astrophytum myriostigma, Trichocereus spachianus, Cereus peruvianus* and *Hylocereus purpusii* (Vyskot and Jára, 1984; De Olivera *et al*., 1995; Feria *et al*., 2012). Meanwhile, several cactus species which were more susceptible promoted 50-60% of survival rate (Pérez-Molphe-Balch *et al*., 1998). Nonetheless, the present study clarified that plantlets of *Echinocereus cinerascens* grown vigorously and healthily after successfully acclimatized and transferred to the natural environment with morphological similar to naturally grown plants, which is in agreement with the previous studies.

Relatively, little work has been reported on the induction of coloured callus. Yet, the induction of coloured callus is typically related to the production of secondary metabolites and valuable compounds. Nowadays, production of secondary metabolites

and valuable compounds from callus is preferred as an alternative to prevent devastation and extinction of plant species besides offering the enhancement of desirable compounds through modification of medium for callus induction. Fujita (1990) suggested that the callus culture can be an alternative method for synthesis of secondary metabolites which are commercially essential for the production of drugs, flavours, perfumes and pigments that are often difficult to synthesize chemically. Moreover, studies by De Oliveira and da Silva Machodo (2003) clarified that the production of callus of *Cereus peruvianus* was established and have been used for alkaloid production. Callus of *Cereus peruvianus* produced higher level of alkaloids in medium supplemented with tyrosine. Further investigation verified that the level of alkaloid production was two-fold higher in callus compared to mature plant shoots.

Recent studies in molecular biology, enzymology, fermentation technology and physiology of plant cell cultures recommended that callus induction has a high potential in the production of valuable natural products. Therefore, current study was carried out on the induction of callus of *Echinocereus cinerascens*. Chapter 7 presented the induction of coloured callus of *Echinocereus cinerascens* in MS medium supplemented with various plant growth regulators. Observations showed that production of coloured callus was affected by concentrations and combinations of plant growth regulators/ hormones and vitamin applied. The results revealed that the highest production of green callus, 100% was detected in media treated with single hormone (Table 7.1, Table 7.3), combination of hormones (Table 7.2, Table 7.4), and hormone/ hormones in the addition of vitamin (thiamine HCl) (Table 7.4, Table 7.5). It was found that stem explants of *Echinocereus cinerascens* produced 100% of green callus in MS medium supplemented with hormones applied singly such as 4CPA (1 treatment), BAP (2 treatments), 2,4,5-T (2 treatments), NAA (3 treatments), and 2,4-D (4 treatments).

Besides, 100% of green callus were observed also in media with the combination of 2,4-D + thiamine HCl (4 treatments) and 2,4-D + BAP + thiamine HCl (17 treatments).

Similarly, the highest production of yellow callus, 100% was also detected in media treated with single hormone (Table 7.3), combination of hormones (Table 7.2, Table 7.4), and hormones in the addition of vitamin (thiamine HCl) (Table 7.5). Stem explants of *Echinocereus cinerascens* produced 100% of yellow callus in MS medium supplemented with hormones applied singly such as 4CPA (1 treatment) and others in media with combination of 2,4-D + BAP (1 treatment), 2,4-D + BAP + thiamine HCl (1 treatment) and NAA + BAP (4 treatments). Conversely, the stem explants of *Echinocereus cinerascens* treated with single hormone, combination of hormones, and hormone/ hormones in the addition of vitamin (thiamine HCl) produced lesser pink callus compared to green and yellow callus. It was found that the highest production of pink callus, 100% was detected only in a medium with combination of hormones and in the addition of vitamin (thiamine HCl) (Table 7.5) viz. MS medium supplemented with 2,4-D + BAP + thiamine HCl applied in combinations (2 treatments) whereas the highest production of pink callus obtained in medium supplemented with hormone applied singly, NAA (1 treatment) (Table 7.1) was 63% only. Consequently, MS medium supplemented with 2,4-D + BAP + thiamine HCl applied in combination was identified as the optimum medium for the production of coloured callus since 100% production of green, yellow and pink callus of *Echinocereus cinerascens* were found in several treatments tested with different concentrations of the combination.

Previous studies reported by Angulo-Bejarano and Paredes-López (2011) noted different morphology of callus (colours and shapes) produced from cactus species, *Opuntia ficus-indica* cultured on MS medium with the addition of various concentrations and combinations of 2,4-D and BA. Most of the callus identified were compact and display in a range of green to yellow colours. The observation was

comparable with the results of current study on the production of coloured callus of *Echinocereus cinerascens,* which indicated that treatments with different concentrations of 2,4-D + BAP applied in combinations stimulated the production of coloured callus (green and yellow). In contrast, studies reported by Caetano Nunez *et al.* (2014) suggested that the explants of *Selenicereus megalanthus* treated with 2,4-D and 2,4-D + BAP applied in combinations showed production of light green to white with a spongy structure of callus. However, compact green purple with white fluffy callus was observed in this yellow pitahaya when the explants were treated with TDZ and TDZ + BAP applied in combinations. In addition, Wakhlu and Bhau (2000) obtained two types of callus viz. translucent friable white callus and green compact callus as the explants of *Coryphantha elephantidens*, commonly known as elephant tusk cactus were cultured in MS medium supplemented with 2,4-D alone and 2,4-D + kinetin, respectively. Finding by Moebius-Goldammer *et al*. (2003) indicated that Mexican cactus, *Ariocarpus kotschoubeyanus* produced callus in medium with approximately the same proportion of BA and NAA, which differed from *Echinocereus cinerascens,* as the callus was induced in all treatments tested. The finding did not support the supposition that callus formation was achieved when the level of auxin and cytokinin are equal, as reported by a few researchers such as Nuñez-Palenius and Ochoa-Alejo (1999), Moebius-Goldammer *et al*. (2003), Zhao *et al*. (2005), Angulo-Bejarano and Paredes-López (2011). Besides, De Medeiros *et al*. (2006) also attained production of callus from *Notocactus magnificus* in MS medium supplemented with 2,4-D + BA + thiamine HCl + i-inositol applied in combination as auxin to cytokinin ratio of almost 1:8.

In general, callus developed and formed on the cut surfaces of the stem explants of *Echinocereus cinerascens* in all treatments and initiation of coloured callus was noticed as early as the first week of culture. Previous study by Wakhlu and Bhau (2000) reported that callus was observed over the entire surface of explants within two weeks

of culture. According to Viñas *et al*. (2012), callus was frequently produced at the base of the explants in all MS medium fortified with BAP applied singly while Zhang *et al*. (2013) and Muñoz-Concha (2010) stated that MS was the best medium selection for callus induction and auxin such as 2,4-D or NAA was necessary for higher callus production in Agave hybrid. Other studies in some cactus species clarified that 2,4-D is an herbicide that acts as auxin which induced callus formation when applied with other growth regulators in different combinations and concentrations (Bhau, 1999; Llamoca-Zárate *et al*., 1999; De Medeiros *et al.*, 2006; Angulo-Bejarano and Paredes-Lopez, 2011). The findings were also supported by George and Sherrington (1984), Bonga and Von Aderkas (1992) that claimed auxin was generally required for callus induction and being a strong promoter, 2,4-D was frequently applied to initiate callus growth. Callus formation is probably resulted from the activation of the plant's own cytokinin biosynthesis genes by wound derived compounds (Green *et al*., 1987). Theoretically, the role of wound is triggering signal for callus induction and it promotes transfer of endogenous hormone to the wounding area (Bhatia *et al*., 2005). In summary, previous studies indicated that the induction of callus was influenced by several factors such as genotype and type of explant (Mori *et al.*, 2005), medium composition and concentration of plant growth regulator applied (Jiménez, 2005). Nevertheless, Muñoz-Concha *et al*. (2012) suggested that the callus induction was more affected by plant genotype rather than the culture condition.

Generally, the colour of callus represents the explant colour. However, in certain cases, the colour of callus induced was totally different from the explant as it could display dramatic changes in response to the treatment applied. Yet, the colour of callus mostly remains for a certain period before undergoing necrosis afterwards. Dramatic changes of coloured callus were clearly observed in the conversion of green to pink callus of *Echinocereus cinerascens* within 2 months of culture. Subsequently, the pink

callus was faded by time and eventually appears green after 4 months. Unfortunately, observations recorded no changes of green and yellow callus which produced after 2 months of culture. In nature, plants illustrate a diverse range of colours and certain plants can surprisingly exhibit colour changes. Alkema and Seager (1982) suggested the phenomena occurred due to the production, interaction and breakdown of natural chemical substances such as porphyrins, carotenoids and flavonoids. Porphyrin or chlorophyll pigments consist of chlorophyll a and b that slightly differ in chemical structures whereas carotenoid pigments are isoprenoids which comprise compounds such as carotenes (α, β, γ and lycopene) and xanthophylls. In contrast, flavonoid pigments belong to phenylpropanoids that give widest colour range to plants due to compounds like flavone, flavonol or anthocyanin. In the current study of *Echinocereus cinerascens*, the conversion of coloured callus from pink to green colour occurred due to changes in chlorophyll levels as a result of the reaction between endogenous and exogenous hormones, explants sources, culture conditions such as exposure to light, temperature and so on. The result indicates the chlorophyll intensify by time.

Currently, various methods were applied to isolate, identify and purify natural pigments since numerous researchers shown a great interest in these valuable compounds due to high potential in health benefits. These natural phytochemicals which synthesized by plants and microorganisms are known to have important role in the prevention of various diseases in human health, reported as powerful antioxidants and so on. Previous studies have demonstrated the health benefits of chlorophyll derivatives especially for digestion system, absorption and metabolism. According to Ferruzzi *et al.* (2001), the uptake of chlorophyll derivatives stimulate both the gastric and small intestinal phases of process. It was found that the native chlorophylls were converted to Mg-free pheophytin derivatives during digestion. The study explained the uptake of chlorophyll derivatives by human intestinal cell and this support the potential of

chlorophylls as health-promoting phytochemicals. There are high correlation between chlorophyll and carotenoid pigments. Generally, the carotenoid is masked by the chlorophyll as the dominant pigment. A number of researches have been done on carotenoids due to their roles in photobiology, photochemistry and photomedicine (Dutta *et al.*, 2005). Zaripheh and Erdman Jr. (2002) reported two nutritional important plant-derived carotenoids viz. β-carotene and lutein. β-carotene plays an important role as the principle precursor of vitamin A that involved in vision, reproduction, growth and development of bones (Wolf, 1980), and acts as a suppressor of the Human Immunodeficiency Virus (Garewal *et al.*, 1982). Studies by Mortensen et al. (2001) indicated the intake of lutein and β-carotene could decreased risks of cancer and age-related macular degeneration. Moreover, Le Marchand *et al.* (1993) suggested that dietary intake of lutein, β-carotene and α-carotene associated with reduced risk of lung cancer. Besides, dietary intake of foods consist of rich carotenoids could decreased risk of chronic eye diseases (Johnson *et al.*, 2000), Alzheimer's disease (Zaman *et al.*, 1992), etc. Carotenoid and chlorophyll are secondary plant compounds which have attracted the interest of researchers mainly from medicine, pharmacy, nutrition, food science and technology as these natural pigments offer crucial profits and potential application in medicine, food, textile and cosmetic industry. In response to the matters, studies on pigment extraction from *in vitro* plantlets and callus of *Echinocereus cinerascens* were carried out. Chapter 8 described the studies, where pigments were analysed using UV-VIS spectroscopy (a frequently used method) and HPLC (an accurate method), as the most useful tools for photosynthetic pigments analysis.

UV-VIS spectroscopy is a very simple and useful method for quick estimation amount of pigments (Jodłowska and Latała, 2011). Hence, it became popular among researchers and widely used in pigment analysis. Nevertheless, there are some limitations that need further clarifications and more advanced studies. As a result, an

expensive and sophisticated apparatus viz. reverse-phase HPLC was applied which enables detection and quantification of pigments from the mixture separately (Riaux-Gobin *et al*., 1987; Millie *et al*., 1993). Besides, HPLC offers high reproducibility and low detection limit, therefore it has been considered as a method of choice in pigment analysis (Gatti *et al*., 2001). Even though HPLC method allows for detailed carotenoid separation and quantification, however, it is relatively time and cost intensive. Thus, a spectrophotometric method is still commonly applied for various purposes and food matrices analysis (Lichtenthaler, 1987; Hornero-Méndez and Mínguez-Mosquera, 2001; Peng *et al*., 2005; Davey *et al*., 2006; Bunea *et al*., 2008; Kimura *et al*., 2007).

In this study, samples of *in vitro* plantlets and callus of *Echinocereus cinerascens* were prepared before being subjected to UV-VIS spectrophotometer and HPLC analysis. As recommended in previous research, the experiment was carried out in dim or subdued light (Melendez-Martinez *et al*., 2007; Boyer, 1990) and under controlled ambient temperature due to pigments are both light and heat sensitive (Rodriguez, 2001; Ferruzzi and Schwartz, 2001). Thus, the extracts should be wrapped with aluminium foil for storage and also during analysis (Lichtenthaler and Buschmann, 2001). Present study involved pigment extraction of fresh samples for UV-VIS spectrophotometer analysis while dried samples for HPLC analysis. Pigments were extracted with various solvent extractions and it is advisable to proceed with pigment detection immediately to avoid evaporation of solvents and pigment degradation. In UV-VIS spectrophotometer analysis, fresh samples were preferred as the absorption in the red and blue maxima is highest in freshly isolated pigments. A small amount of $MgCO_3$ was added during extraction to neutralize plant acids that might cause the formation of pheophytin a from chlorophyll a (Lichtenthaler and Buschmann, 2001). In this experiment, results showed the absorption spectra which depicted two types of pigments namely, chlorophylls and carotenoids. Normally, absorption maxima of

chlorophylls were detected in two regions such as in blue region near 412 and 436 nm wavelengths, whereas in red region near 642 and 665 nm wavelengths. Absorption maxima of carotenoids are only in blue region with three shoulders, between 400 to 500 nm wavelengths. The pigment content includes chlorophyll a, chlorophyll b, total chlorophyll (chlorophyll a + chlorophyll b), total carotenoid (xanthophyll + carotenes) were calculated to identify the most efficient solvent extraction for both of *in vitro* plantlets and callus of *Echinocereus cinerascens* (Table 8.1, Table 8.3). In *in vitro* plantlets, the highest total pigment extracted was 64.10 mg/g which obtained in 100% Methanol (Table 8.1) whereas in callus, the highest total pigment extracted was 39.84 mg/g observed in 80% Acetone (Table 8.3). Therefore, the most efficient solvent extraction for *in vitro* plantlets was 100% Methanol while for callus was 80% Acetone.

Dere *et al*. (1998) stated that types of solvents were important in pigment extraction, and methanol was selected as the best solvent extraction for some algae including *Chladophora glomerata, Ulva rigita, Codium tomentosum and Chladostephus verticillatus* which is in agreement with the present study. However, Ritchie (2006) presented inconsistent result with the present study, where acetone which probably the most used solvent produces sharper absorption peaks is considered to be less efficient in pigment extraction. Furthermore, studies conducted by Sartory and Grobbelaar (1984) suggested that ethanol was the best solvent extraction among the others (ethanol, methanol and acetone) which were used to extract pigments from freshwater phytoplankton. Indeed, previous studies on pigment extraction of higher plant leaves using various solvent extractions such as acetone, chloroform, diethyl ether, dimethylformamide and methanol demonstrated results with different extraction rate in every solvent assessed (Wellburn, 1994). The differences in extraction rate and determination of the best solvent extraction might be influenced by different species or samples used (Dere *et al*., 1998).

Diverse solvent polarities (100% Acetone, 80% Acetone, 95% Ethanol, 100% Methanol, 90% Methanol) were assessed in the present study and it was proven that the absorption maxima shifted to a longer wavelength as the polarity of solvent increased (Figure 8.1, Figure 8.2). Increase in polarity caused absorption maxima to be shifted from 660 to 665 nm wavelength, 428 to 432 nm wavelength for Chl a whereas for Chl b from 642 to 652 nm wavelength, 452 to 469 nm wavelength (Lichtenthaler and Buschmann, 2001). Absorption maxima shifted to longer wavelength due the present of high water content in the sample (Boyer, 1990). This evidence clarified that the absorption maxima reliable on solvent polarity.

Although methanol was considered as a good solvent extraction, however, it was toxic. Therefore, further studies on the stability of pigments extracted from coloured callus of *Echinocereus cinerascens* (green, yellow and pink) were done where 95% ethanol was selected as a solvent extraction. Analysis of this study showed the pigment content of coloured callus (green, yellow and pink) and their stability, before and after 3 months storage at – 20 ºC (Table 8.5). The highest pigment content was found in green callus (83.28 mg/g) before the extracts were stored while the lowest pigment content was found in both pink (10.55 mg/g) and yellow callus (12.23 mg/g) after the extracts were stored. Comparison of the results indicated that *Echinocereus cinerascens* consists of chlorophyll as the major pigment and carotenoid as the minor pigment. However, it was found that carotenoid is more stable compared to chlorophyll whereby higher degradation rate of pigments was observed in green callus. Ratio of chlorophyll a to chlorophyll b evaluated after storage was higher than before the extracts were stored (Table 8.6). In contrast, the ratio of chlorophyll to carotenoid analysed before was higher than after the extracts were stored (Table 8.6).

Quantification of chlorophyll and carotenoid concentrations are essential as the indicators of plant responsiveness to light intensity (Strauss-Debenedetti and Bazzaz, 1991; Vieira, 1996). Meanwhile, the relationship between chlorophyll and carotenoid might be used as a potential indicator of photooxidative damages caused by strong irradiation (Hendry and Price, 1993). It was found that the ratio of Chl a to Chl b, (Chl a/b) is very important as an indicator of the functional pigment equipment and light adaptation of the photosynthetic apparatus (Lichtenthaler *et al*., 1981). The ratio can vary considerably depending on light exposure and other factors. Studies revealed that shade plants possess Chl a/b ratios lower than in sun-exposed plants (Lichtenthaler *et al*., 1982; 1984). Generally, light harvesting pigment protein LHC-I of PS I has a Chl a/b ratio of ~3 whereas LHC-II of PS II exhibits a Chl a/b ratio of 1.1 to 1.3. A decrease in the ratios may be interpreted as an enlargement of the antenna system of PS II. Additionally, further research suggested the ratio of Chl a and b to total carotenoid, Ca+Cb/C(x+c) is an indicator of the greenness of plants (Lichtenthaler and Buschmann, 2001). Normally, Ca+Cb/C(x+c) ratios are between 4.2 to 5.0 for sun leaves and sun-exposed plants, 5.5 to 7.0 for shade leaves or shade-exposed plants, 3.5 for yellowish-green leaves, and lower than 3.0 indicated of senescence, stress, or damage to the plant and the photosynthetic apparatus, which is expressed by a faster breakdown of chlorophylls than carotenoids.

Based on the information stated, analysis on Ca/Cb and Ca+Cb/C(x+c) ratio of the present study (Table 8.6) showed that differences of the ratios before and after storage were due to the breakdown of pigments. Observations recorded Ca/Cb ratio after storage was higher than before storage as a result of chlorophyll b degradation. Chlorophyll b decreased significantly after 3 months storage at – 20 ºC. Conversely, Ca+Cb/C(x+c) ratio before storage was higher than after storage in respond to the chlorophyll a and chlorophyll b degradation since the result showed a remarkable

decline of chlorophyll content after 3 months storage at – 20 ºC. According to Masarovičová and Eliás (1987), chlorophyll concentrations vary with plant age. Besides, a light condition also plays important role in chlorophyll concentrations of plant species (Martin and Churchill, 1982). Pigments are typically related to the physiological function of leaves. Hence, variations in pigment content may provide evidence concerning the physiological state of the leaves. Several researchers reported variations in leaf chlorophyll content detectable by spectral reflectance related to leaf development and senescence (Carter and Knapp, 2001; Gamon and Surfus, 1999). Studies by Willstätter and Stol (1918) concluded chlorophyll a and chlorophyll b disappeared at the same rate. However, studies on chlorophylls degradation using more refined techniques indicated that chlorophyll a destroyed faster than chlorophyll b (Rudolph, 1933; Nagel, 1939; Egle, 1944; Seybold, 1943; Wolf, 1956). Moreover, it was found that chlorophylls tend to decline more rapidly than carotenoids when the plants are under stress or during leaf senescence (Gitelson and Merzlyak, 1994a; 1994b; Merzlyak *et al*., 1999).

According to Louda and Monghkonsri (2004), excellent results for chlorophyll a determination were obtained by spectrophotometric compared to HPLC analysis. Nevertheless, a few studies reported spectrophotometric method tend to overestimate carotenoid content as minor compounds and degradation products were also detected (Kimura *et al*., 2007; Almela *et al*., 2000). Hence, reversed-phase HPLC become the most commonly used method for carotenoid analysis as separation of the pigments can be successfully accomplished (Craft, 2001). These indicated that election of the analytical method depends on the particular interest of the analysis and the kind of product analysed. For a simple and quick alternative to pigment analysis, spectrophotometry can be applied whereas for particular pigment analysis especially carotenoid content, HPLC should be applied (Olives *et al*., 2006).

In the present study, freeze-dried samples of *in vitro* plantlets and callus of *Echinocereus cinerascens* were extracted, subjected to saponification before analysed by HPLC for carotenoids detection and quantification. The samples (*in vitro* plantlets and callus) were freeze-dried to remove the moisture content and in the meantime, phytonutrients, proteins and other bioactive compounds that present naturally in the plants were preserved. HPLC chromatograms revealed several identified carotenoids while the others were detected but unable to be identified due to the absence of commercial standards (Figure 8.5, Figure 8.6). Carotenoids which were detected and identified in *in vitro* plantlets including neoxanthin (5.34 mg/g), violaxanthin (0.44 mg/g), lutein (1.28 mg/g) and β-carotene (2.16 mg/g) whereas in callus were lutein (1.09 mg/g) and β-carotene (1.41 mg/g) only (Table 8.8). The highest carotenoid content in *in vitro* plantlets was neoxanthin, followed by β-carotene, lutein and lastly violaxanthin. Meanwhile, the highest carotenoid content in callus was β-carotene and then followed by lutein.

To the best of our knowledge, present study on the extraction, detection and quantification of pigments from *in vitro* plantlets and callus of *Echinocereus cinerascens* is the only reported to date. Yet, a few studies published on carotenoid content analysed by HPLC suggested that the most commonly detected carotenoid is β-carotene. Interestingly, studies on carotenoid content including β-carotene, xanthophyll, lutein and zeaxanthin in 20 selected plant species clarified that β-carotene was the highest carotenoid content for all species (Tharasena and Lawan, 2012). They reported several species that obviously showed higher β-carotene content including *Centella asiatica* (L.) Urb., *Piper sarmentosum* Roxb., *Alpinia nigra* (Gaertn.) B. L. Burtt and *Momordica charantin* Linn. However, only two species promoted results in line with the present study viz. *Alpinia nigra* (Gaertn.) B. L. Burtt and *Momordica charantin* Linn that showed β-carotene content correspond to 1.44 mg/g and 1.70 mg/g. Moreover,

they also recorded lutein content of 0.03 mg/g observed only in *Ipomcea aquatica* Forsk. This result was significantly different with the present study that promoted lutein content which in the range of 1.00 – 1.30 mg/g for both of *in vitro* plantlets and callus of *Echinocereus cinerascens*.

Carotenoids are found naturally in esterified form or being in complex with proteins or lipids (Andersen and Francis, 2004). Therefore before HPLC analysis, the extracts were subjected to saponification to separate lipid from carotenoids and to remove chlorophylls that also present in the samples tested (Craft, 2001). Saponification is commonly included during extraction to prevent overestimation of carotenoid by the presence of chlorophyll though it may lead to carotenoid losses (Lietz and Henry, 1997). Previous studies recommended saponification (alkaline hydrolysis) step for carotenoid mainly to simplify chromatographic profiles by removing interfere compounds such as chlorophyll degradation products, chlorophyll esters and unwanted lipids (Granado *et al*., 2001; Schierle *et al*., 2004). Besides, the addition of methanolic KOH during extraction is practical to assist in degradation of chlorophyll (Biehler *et al*., 2010). Nonetheless, once carotenoids have been extracted and isolated, they tend to be very unstable. Consequently, antioxidants such as BHT, a tocopherol, vitamin E or others should be added to the extracts or during extraction (Rodriguez, 2001).

As noted previously, observations on ultrastructural studies verified that there is no morphological and physiological changes were detected in direct *in vitro* regeneration of *Echinocereus cinerascens* even after transferred to the natural environment (Chapter 3). In addition, cytological studies also confirmed that there is no variation of cell organization and behaviour of root cells of *Echinocereus cinerascens in vitro* and *ex vitro*. Chapter 9 clarified the cytological analysis and detail explanation of this study which involves the detection of somaclonal variation at the cellular level. Some of the factors that determine the frequency of variation *in vitro* viz. propagation

methods, genotypes, a source of explants, types and concentrations of plant growth regulators, number and duration of subcultures (Pierik, 1987). In fact, one of the factors detected which leads to the occurrence of somaclonal variation is the presence of disorganised growth phase in tissue culture (Rani and Raina, 2000; Sivanesan, 2007). Several studies reported that chromosomal instability known as a frequent somaclonal variation is found associated with regenerants in long-term cultures or cultures that initiated from intermediate callus phase or high rates of multiplication treatments (Roux *et al*., 2004; Bairu *et al*., 2006; Bairu *et al.*, 2011).

Several studies stated somaclonal variation *in vitro* were observed in calamondin (Siragusa *et al*., 2007), soybean (Radhakrishnan and Ranjitha Kumari, 2008), cotton (Jin *et al*., 2008), arabica coffee (Etienne and Bertrand, 2003), banana (Sahijram *et al*., 2003; Bairu *et al*., 2006; Martin *et al*., 2006), wheat (Mehta and Angra, 2000), strawberry (Popescu *et al*., 1997) and others. Therefore, the present study was conducted to verify if any somaclonal variant present in direct *in vitro* regeneration of *Echinocereus cinerascens*, and subsequently after the plants were transferred to the natural environment. Various strategies which could be applied for the detection of somaclonal variation including morphological, physiological/ biochemical studies, molecular detection and cytological analysis (Bairu *et al*., 2011; Vujović *et al*., 2012). Morphological and physiological studies involve observations based on characters like differences in morphology and also the use of physiological responses for detection of somaclonal variant are known as easier and faster compared to cytological analysis. However, cytological analysis offers direct and strong evidence of changes in genetic composition presented by variation in ploidy level, chromosome number and structures, thus it is preferable and has been widely used (Bogdanova, 2003; Nakano *et al*., 2006; Fiuk *et al*., 2010).

In the present study, cytological analysis involved measurement of cellular parameters such as mitotic index (MI), chromosome counts (CC), nuclear areas (NA), cell areas (CA) and ratio of nuclear to cell areas (NA/CA) for *in vivo, in vitro* and *ex vitro* primary root tips of *Echinocereus cinerascens* which harvested before secondary root emergence. In order to proceed with the measurements, it is essential to study the standard growth of *Echinocereus cinerascens* beforehand to determine the rate of primary root growth that is applicable to standardize the size of all samples for subsequent cytological analysis. The rate of primary root growth is a complex parameter which associated with cell division and cell growth. Previous study by Dubrovsky *et al.* (1998) indicated that rapidly growing roots had a shorter duration of the cell division cycle while roots growing slowly had a longer duration of the cell cycle. Finding by these researchers clarified that a relatively short cell division cycle in the apical meristem is essential for the primary and lateral root growth and also for the rapid seedling establishment in the desert whereas a relatively short rate of root growth is essential for rapid root-system development. *Cactus* spp. promoted slow growth in the natural environment with relatively short cell division cycle, has an important adaptive significance for a drastic pressure of a stressful environment and short optimal periods for growth.

In the present study, it was found that the rate of primary root elongation of *Echinocereus cinerascens* was 0.0768 cm per day as clarified by the standard growth graph in linear regression line of $y = 0.0768x – 0.1984$, with the correlation coefficient, $R^2 = 0.9437$ (Figure 9.2). Meanwhile, the standard root length was obtained on day 9, with mean primary root length of 0.53 cm (Table 9.1) where the optimum primary root growth was detected. Subsequently, the primary roots with the length of 0.53 cm were harvested to collect the root tips for preparation of temporary slides using squash technique. These procedures were applied to all the samples tested including the

primary root of seedlings grown *in vivo*, plantlets grown *in vitro* and plants grown *ex vitro* to study the similarities and differences of their cellular behaviour.

Observations on temporary slides prepared which were viewed under a light microscope found that all the phases such as interphase, prophase, metaphase, anaphase and telophase were comparable for all the samples tested. Nevertheless, analysis of cellular parameters showed that several differences in mitotic index, chromosome counts, nuclear and cell areas and ratio of nuclear to cell areas. Generally, the mitotic index is applied to examine cell proliferation in primary root. Besides, it can also be used to quantify the differences in cell division when the environment changed. Investigations revealed that mitotic index of primary root tip (Table 9.2) decreased in aged plants as the highest mitotic index was observed in primary root of seedlings grown *in vivo* (38.80) which was significantly different with mitotic index measured in primary root of plantlets grown *in vitro* (26.40) and plants grown *ex vitro* (19.00). The evidence clarified that the primary root cells of seedlings grown *in vivo* divided rapidly which indicated that high proportion of cells undergo mitosis. Indeed, the cells gradually lose the capacity to divide as they mature. In addition, statistical analysis also indicated that there was no significant difference between the mitotic index of *in vitro* and *ex vitro* primary roots which suggests that the mitotic index remained stable though the plantlets grown *in vitro* were transferred to *ex vitro* condition. Further studies on cellular parameters showed that the majority chromosome counts of *Echinocereus cinerascens* calculated were in the range of 20-22, since the mean chromosome counts for *in vivo*, *in vitro* and *ex vitro* primary root tips harvested before secondary root emergence were slightly different with 20.33, 21.93 and 21.40 (Table 9.2), respectively.

According to Pinkava and Parfitt (1982), the first report of chromosome counts derived from flower bud cells of *Echinocereus cinerascens* grown in native habitats or in cultivation, Mexico was n=11. Other species in Mexico which gave similar

chromosome counts of n=11 was *Echinocereus pectinatus* while *Echinocereus triglochidiatus* had chromosome counts n=22 that showed comparable results with other species found in Arizona such as *Echinocereus engelmannii* (n=22) and *Echinocereus fasciculatus* (n=22). Present study strongly supports the first report by Pinkava and Parfitt (1982) which revealed that chromosome counts derived from *in vivo*, *in vitro* and *ex vitro* root meristematic cells of *Echinocereus cinerascens* was 2n=22 (Figure 9.9). Analysis on chromosome reported earlier by Katagiri (1953) mainly dealt with mitotic studies in various *Gymnocalycium* of the family Cactaceae viz. *G. anisitsii*, *G. gibbosum* and *G. platense* showed similar chromosome counts of 2n=22. Subsequent report by Das and Das (1998) described meiotic studies on flower bud (pollen mother cells) of *G. brucbii, G. denudatum, G. queblianum, G. borridispinum, G. mibanovichii* and *G. saglionis* which suggested chromosome counts for all *Gymnocalycium* tested was n=11. This evidence obviously clarified that meiosis reduces chromosome number from 2n=22 to n=11.

Moreover, the present study concerning other cellular parameters such as nuclear and cell areas of *in vivo*, *in vitro* and *ex vitro* primary root harvested before secondary root emergence showed incomparable results. Similarly, the mean nuclear and cell areas of primary root increased accordingly from *in vivo* to *in vitro* and *ex vitro* samples (Table 9.3). Observations recorded that the mean nuclear (286.71 μm^2) and cell area (845.11 μm^2) of *ex vitro* primary root tip was the highest. Nevertheless, there was no significant difference with the mean nuclear (256.39 μm^2) and cell areas (747.81 μm^2) of *in vitro* primary root tip. However, statistical analysis confirmed that there was a significant difference with the mean nuclear (184.20 μm^2) and cell areas (523.70 μm^2) of *in vivo* primary root tip. Interestingly, all the samples promoted almost equal ratio of nuclear to cell area (Table 9.3). The results verified that both nuclear and cell areas of

primary root tip of *Echinocereus cinerascens* increased with age. Yet, the nuclear to cell area ratios were retained, with no different statistically.

Cytological studies on primary root tips of *Echinocereus cinerascens* harvested before secondary root emergence showed incomparable results with the previous study reported by Taha and Othman (2001) which indicated that the mean mitotic index of *in vitro* root sample of *Zinnia elegans* was higher than *in vivo* root sample. Conversely, the mean nuclear and cell areas of *in vitro* sample were lower than *in vivo* sample, though the chromosome counts remained stable. Nonetheless, the results of the current study were in accordance with findings by Yaacob *et al*. (2013), which stated that the mitotic index of *in vitro* sample of *Dianthus caryophyllus* was lower than *in vivo* sample. Meanwhile, the mean nuclear and cell areas of *in vitro* sample were higher compared to *in vivo* sample. In addition, observations recorded no changes in the chromosome counts. Hence, analysis of the results discovered that no somaclonal variation occurred in both *in vitro* and *ex vitro* root samples of *Dianthus caryophyllus* which are in agreement with the present study as both *in vitro* and *ex vitro* samples of *Echinocereus cinerascens* presented similar results for chromosome counts, structures and ratio nuclear to cell areas.

Cytological analysis based on conventionally stained, condensed chromosomes in root or shoot tip meristem cells is mostly applied as it is a convenience method for rapid determination of numerical/ structural variation in chromosomes of *in vitro* plants including *Rubus fruticosus* L. (Vujović *et al*., 2012), *Vitis* spp. (Kuksova *et al*., 1997), *Centaurea ultreiae* (Mallón *et al*., 2010), *Picea mariana* and *P. glauca* (Trembley *et al*., 1999), *Paulownia tomentosa* (Tang *et al.*, 2010), *Solanum tuberosum* L. (Vargas *et al*., 2008). However, sometimes it is not possible to accurately count the chromosomes as they tend to stick together or overlapped due to poor slide preparation which leads to the great variation in the counts. Additionally, this cytological analysis technique is time

consuming and often tedious especially when the chromosomes are too small and difficult to be observed. Hence, flow cytometry is now widely used as a more rapid, accurate and convenience method for ploidy level estimation mainly for species that have important advantages in chromosome counts (Doležel, 1997). Seemingly, flow cytometry is mostly applied in plant biotechnology for ploidy screening (Pinto *et al*., 2004; Loureiro *et al*., 2005; Ghimire *et al.*, 2012), detection of polyploidy, mixoploidy and aneuploidy (Elmaghrabi and Ochatt, 2006; Tang *et al*., 2010) assessment of the degree of polysomaty (Iantcheva *et al*., 2001) and also the occurrence of endoreduplication (Lema-Rumińska, 2011) in *in vitro* cultures.

CHAPTER 11

CONCLUSION

In vitro plant regeneration technique could produce a great number of plantlets de novo from dedifferentiated cell cultures that would offer remarkable chances in the achievement of genetic transformation. Successful *in vitro* plant regeneration and genetic engineering approaches promoted high possibilities in producing superior plants such as pathogen-free or disease-resistant, plants with higher content of valuable compounds and possess desirable characteristics. Nowadays, advanced research in plant biotechnology leads to the discoveries of efficient procedures for massive production of these valuable compounds. Hence, the present study involved investigation mainly concerning rapid *in vitro* regeneration of *Echinocereus cinerascens*, an endangered cactus species besides detection of their valuable compounds especially for potential applications in the future and health benefits.

The present study revealed that direct *in vitro* regeneration of *Echinocereus cinerascens* was affected by plant growth regulators, as the parameter that varied and evaluated regardless of the other factors. Results in the present study demonstrated that stem explants formed the highest mean number of shoots (4.37) in MS medium supplemented with 2.0 mg/l Kinetin + 1.0 mg/l IBA, which promoted the highest production of shoots after 4 months, 131 shoots in total. Meanwhile, morphological and physiological studies on ultrastructural of *in vivo, in vitro* and *ex vitro* samples revealed similarities in the parameters examined which clarified that no visible alterations especially on the apical shoots, vascular bundles, starch grains, stomata, areoles, ribs and spines due to *in vitro* manipulation. Thus, direct *in vitro* regeneration of *Echinocereus cinerascens* was successfully established with the production of true-to-type plants which highly useful for commercial micropropagation and conservation of the species.

Besides, indirect *in vitro* regeneration was also studied as an alternative of direct *in vitro* regeneration of *Echinocereus cinerascens* that involves the induction of somatic embryogenesis. The protocol for induction of somatic embryogenesis of *Echinocereus cinerascens* was successfully developed in two treatments of liquid medium including MS medium supplemented with 0.5 mg/l 2,4-D + 0.1 mg/l BAP + 0.5 mg/l thiamine HCl and MS supplemented with 0.5 mg/l 2,4-D + 0.5 mg/l BAP + 0.5 mg/l thiamine HCl, since both media promoted 100% total mean production of somatic embryos (all stages detected) after 4 months. Attempts have been made to induce somatic embryogenesis in solid medium which initially showed the ability to produce somatic embryos directly from the stem explants. Nevertheless, the results obtain eventually unsatisfactory. Hence, somatic embryogenesis of *Echinocereus cinerascens* was induced in liquid medium, where all stages of somatic embryos (globular, heart, torpedo-shaped and cotyledonary stage) were found developed from callus explants. Subsequently, the somatic embryos were cultured on MS basal medium for further development into plantlets. Theoretically, the study was quite tedious and time consuming. However, it was likely more preferred nowadays as it offers significant regeneration efficiency with the production of somatic embryos in industrial scales. In fact, high multiplication rate of somatic embryos opens a new vista in plant genetic improvement through transformation or genetic engineering.

Recent advances in plant biotechnology proposed the application of synthetic seeds technology as a rapid tool of plant regeneration which extensively used in conservation and delivery of tissue cultured plant directly to the field. Synthetic seeds technology becoming popular nowadays which offers easy of handling cell and tissues (explants), protecting the explants against strong exterior mechanical stress, serves as an efficient delivery system and so on. Additionally, available literature indicated numerous benefits of synthetic seeds technology that expected to give utmost profits in

the agriculture industry. Based on the theory, studies on the production of synthetic seeds are applicable for the conservation of *Echinocereus cinerascens* to overcome seed germination problems which lead to extinction as synthetic seeds could supply unlimited planting materials throughout the year.

Production of ideal synthetic seeds of *Echinocereus cinerascens* was successfully established by encapsulating propagules such as micro shoots and stems in 3% sodium alginate and then, hardened in 100 mM of calcium chloride dehydrate for 30 minutes. Indeed, the production of a firm, isodiametric and round shaped of synthetic seeds is significantly important to provide the best condition or protection to propagules and simultaneously accelerate breakage during germination. Further studies on germination of synthetic seeds of *Echinocereus cinerascens* revealed that micro shoot was found to be the most responsive propagule, which gave 100% of germination rate after 4 months of culture. Observations in MS basal medium indicated that synthetic seeds produced by encapsulating micro shoots in sodium alginate without sucrose showed the highest production of single shoot (73%). Meanwhile, the highest production of multiple shoots (100%) was observed as synthetic seeds produced by encapsulating micro shoots in sodium alginate which germinated in MS medium supplemented with 2.0 mg/l Kinetin + 0.5 mg/l IBA. The evidence clarified that germination of synthetic seed was influenced by several factors including the type of propagule, encapsulation matrix or synthetic endosperm and selected medium for germination. However, more detail research is needed to improve storage and prolong life or viability of synthetic seeds of *Echinocereus cinerascens* since current study presented insufficient findings for commercial exploitation of synthetic seeds technology.

Shoots or plantlets of *Echinocereus cinerascens* grown *in vitro* were transferred to MS basal medium for rooting, before acclimatization which considered as the final step of *in vitro* regeneration. Present study clarified that *in vitro* rooting was crucial for the production of healthy growth of complete plantlets with well-developed roots system. Basically, this requirement is applicable to enhance chances of plantlets survive during acclimatization. Acclimatization is mainly practiced to ensure the survival of plantlets due to environmental changes that might cause high mortality of plantlets. Complete plantlets of *Echinocereus cinerascens* were successfully acclimatized with the highest survival rate of 90% observed in sand. Thus, sand was identified as the most suitable planting substrate for acclimatization of *Echinocereus cinerascens.* Analysis using XRF confirmed the sand possessed 2 major elements such as SiO_2 and Al_2O_3 that play important roles to support the growth of *Echinocereus cinerascens.*

Beside *in vitro* regeneration, the present study demonstrated production of coloured callus which was detected in different treatments tested. Investigations revealed that various coloured callus of *Echinocereus cinerascens* were influenced by the plant growth regulators and also in addition of the vitamin. MS medium supplemented with 2,4-D + BAP + thiamine HCl applied in combination was identified as the optimum medium for the production of coloured callus since 100% production of green, yellow and pink callus of *Echinocereus cinerascens* were found in several treatments tested with different concentrations of the combination as observed after 2 months. Interestingly, further studies discovered dramatic changes of coloured callus which clearly observed in the conversion of green to pink callus within 2 months of culture. Subsequently, the pink callus faded by time and eventually appears green after 4 months.

A new prospect in studies of natural pigments revealed the importance applications of chemical compounds derived from natural resources in health benefits. Nonetheless, there are some limitations as the supplies of natural resource normally inadequate. In addition, over collection of plants in their habitat would lead to extinction. Therefore, the plants or callus produced *in vitro* were the most recommended which offers unlimited supplies of natural resources throughout the years. Based on the evidence, studies on pigment extraction from *in vitro* plantlets and callus of *Echinocereus cinerascens* were performed and the results were then analyzed by UV-VIS spectroscopy and HPLC. The present study indicated that the most efficient solvent extraction for *in vitro* plantlets was 100% Methanol while for callus was 80% Acetone. Moreover, UV-VIS spectrophotometer analysis showed that both samples of *in vitro* plantlets and callus of *Echinocereus cinerascens* possessed chlorophyll (chlorophyll a and chlorophyll b) as the major pigment while carotenoid (carotenes mixed with xanthophylls) as the minor pigment. However, pigment stability test indicated remarkable declined of chlorophylls content after 3 months storage at – 20 ºC which clarified that the chlorophylls tend to degrade more rapidly than carotenoids. Since UV-VIS spectrophotometer analysis incapable of presenting carotenoids separately, hence HPLC was the right option instead. HPLC chromatograms presented several identified carotenoids including neoxanthin, β-carotene, lutein and violaxanthin which detected in *in vitro* plantlets whereas in callus samples were β-carotene and lutein only. As this preliminary study on pigment extraction from *in vitro* plantlets and callus of *Echinocereus cinerascens* showed indispensable results, related studies should be explored further to discover their potential applications in agriculture, medicine, cosmetic, dye, textile, food industries and so on.

Awareness concerning somaclonal variation derived from *in vitro* system leads to additional analysis on cytological studies of *Echinocereus cinerascens* which involve the detection of variation at cellular level. Examinations on cellular parameters such as mitotic index (MI), chromosome counts (CC), nuclear areas (NA), cell areas (CA) and ratio of nuclear to cell areas (NA/CA) of primary root of seedlings grown *in vivo*, plantlets grown *in vitro* and plants grown *ex vitro* suggested that the parameters were influenced by growth condition and age of the samples. Irrespective of growth condition, the mitotic index, nuclear and cell areas of the samples varied with age, however, the chromosome counts and ratio of nuclear to cell areas were still constant. Thus, the observations clarified that there was no variation of cell organization and behaviour of primary root of *in vitro* plantlets and *ex vitro* plants of *Echinocereus cinerascens*. The evidence verified that the plants grew normally *in vitro*. Nevertheless, further studies especially on nuclear DNA C value, ploidy level and others are also required to provide strong evidence besides deeper understanding on karyological studies of *Echinocereus cinerascens.* Indeed, the findings of karyological studies might offer significant improvement of basic knowledge of the species which applicable for more advanced studies, especially in molecular cytogenetics.

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1. **H. Elias**, R.M. Taha, N.A. Hasbullah, R. Othman, N. Mahmad, A. Saleh and S. Abdullah (2017). Detection and quantification of pigments extracted from callus of *Echinocereus cinerascens*. Pigment & Resin Technology. Accepted (ISI-Indexed Journal).

2. **Hashimah Elias**, Rosna Mat Taha, Nor Azlina Hasbullah, Normadiha Mohamed, Aziemah Abdul Manan, Noraini Mahmad and Sadegh Mohajer. (2015). The effects of plant growth regulators on shoot formation, regeneration and coloured callus production in *Echinocereus cinerascens in vitro*. Plant Cell, Tissue and Organ Culture 120(2):729-739 (ISI-Indexed Journal).

Presentations in conferences

1. Potential of Natural Pigments Extracted from Callus of *Echinocereus cinerascens*. 2015 5th International Conference on Environmental and Agriculture Engineering (ICEAE 2015), Paris, France, 5-6th August 2015. Asia-Pacific Chemical, Biological & Environmental Engineering Society (APCBEES).

2. Comparison on Direct Micropropagation of Cactus (*Echinocereus* sp.) in Two Different Hormones Combinations. The International Symposium on Orchids and Ornamental Plants, Chiangmai, Thailand, 9-12th January 2012.

3. Detection of Pigments from Callus Cultures of Cactus (*Echinocereus* sp.). 3rd International Conference on Functional Materials and Devices (ICFMD), Kuala Terengganu, Malaysia, 14-17th June 2010.