MICROPROPAGATION, PIGMENTS EXTRACTION AND ANTIMICROBIAL ACTIVITY OF *Cucumis melo* L.

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ABSTRACT

In vitro regeneration of *Cucumis melo* L. was successfully achieved in this study. The best seeds germination polarity was horizontal position cultured on MS medium without plant growth regulators. Explants from 7-day-old aseptic seedlings were used as explant sources for subsequent experiments since the explants gave the best response at this age. Different responses were observed when cotyledonary petioles were cultured on MS media supplemented with different concentrations of 2,4-D and coconut water for *in vitro* regeneration study. Results obtained showed the best media was MS supplemented with 1 mg/L 2,4-D with the combination of 25% of coconut water which gave 100% shoots and 66.67% roots. For callus induction, 4 types of explants were tested includes hypocotyl, cotyledon, node, and leaf cultured on MS media supplemented with different concentrations of BAP and 2,4-D. All of the explants induced callus except leaf explants. The highest rate of callus formation was observed from hypocotyl explants, followed by node explants, and the least callus proliferation was observed on cotyledon explant. Node and hypocotyl explants were then used in induction of embryogenic callus. The highest embryogenic callus obtained from node explants cultured on MS media supplemented with 2 mg/L BAP in combination with 1 mg/L 2,4-D (100.00 %) with the average rate of the shoot formation of 13.10 ± 0.36 per explant. Hypocotyl explants induced non embryogenic callus on MS media supplemented with BAP and 2,4-D but on MS media supplemented with different concentrations of IAA and Kin, hypocotyls induced callus with root formation, where the highest root formation (86.67%) with the average roots formation of 4.20 ±0.50 per explant were observed on MS media supplemented with 3 mg/L IAA. However, callus induced on MS media supplemented with 0.1 M of mannitol regenerated high rate of shoots where the highest rate was observed from node explants (76.67%). Shoots obtained were transferred to MS medium without plant growth regulators for roots

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formation (93.33%). After 2 months, the established plantlets were then transferred to 3 types of substrates for acclimatization process where the best substrate medium was black soil, followed by mixture of black soil and red soil (1:1) and cocopeat. For carotenoids and chlorophyll pigments extraction, acetone was identified as the best extraction solvent. For colour stability, the pigments were most stable at pH 9 where the colour was last faded. In the application of pigments in coating technology, carotenoids gave the weakest response compared to chlorophyll where the color faded with increasing temperature. In antimicrobial studies, the non embryogenic callus induced from hypocotyl explant on MS media supplemented with 1 mg/L of BAP and 1 mg/L of 2,4-D (1.58 g fresh weight) and *in vitro* hypocotyl were harvested and extracted with methanol. The extracts were tested against *Escherichia coli, Bacillus subtillis*, and *Staphylococcus aureus, Aspergillus niger*, *Penicillium* sp, and *Fusarium* sp. The strongest antimicrobial activity of callus extract was observed against *Bacillus subtilis* and *Penicillim* sp with inhibition zone of 14 mm and 11 mm, respectively.

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ABSTRAK

Pertumbuhan *Cucumis melo* L. secara *in vitro* telah berjaya diperolehi dalam kajian ini. Polariti terbaik percambahan biji benih adalah secara posisi mendatar didalam media MS tanpa hormon. Eksplan dari anak benih berumur 7 hari dari anak benih aseptik telah digunakan untuk eksperimen yang seterusnya kerana tindak balas yang baik pada umur ini. Reaksi yang berbeza diperhatikan apabila kotiledon dikultur di dalam media MS yang ditambah dengan kepekatan hormon 2,4-D dan air kelapa yang berbeza untuk pertumbuhan *in vitro*. Keputusan yang diperolehi menunjukkan media MS yang ditambah dengan 1mg/L 2,4-D dengan kombinasi 25% air kelapa adalah medium terbaik untuk pembentukan 100% pucuk dan 66.67% akar. Untuk induksi kalus, eksplan yang telah diuji adalah hipokotil, kotiledon, nod, dan batang kotiledon di dalam media MS ditambah dengan kepekatan BAP dan 2,4-D yang berbeza. Semua eksplan menunjukkan kesan dalam induksi kalus kecuali eksplan daun. Kadar tertinggi pembentukan kalus diperhatikan pada eksplan hipoktil diikuti oleh eksplan nod, dan perkembangan kalus yang paling rendah diperhatikan pada eksplan kotiledon. Eksplan nod dan hipokotil kemudiannya digunakan dalam induksi kalus embriogenik. Pembentukan kalus embriogenik (100%) diperolehi daripada eksplan nod yang dikulturkan dalam media MS yang ditambah dengan 2 mg/L BAP dalam kombinasi dengan 1 mg/L 2,4-D dengan kadar purata pembentukan pucuk 13.10 ± 0.36 daripada setiap eksplan. Kalus yang dibentuk daripada eksplan hipokotil menghasilkan kalus bukan embriogenik didalam media MS yang ditambah dengan BAP dan 2,4-D tetapi dalam media MS yang ditambah dengan kepekatan IAA dan Kin yang berbeza, pembentukan kalus dengan akar diperhatikan. Pembentukan kadar akar tertinggi (86.67%) dengan purata pembentukan 4.20 ± 0.50 setiap eksplan diperhatikan dalam media MS yang ditambah dengan 3 mg/L IAA. Walaubagaimanapun, kalus terhasil dalam media MS yang ditambah dengan 0.1 M mannitol menghasilkan kadar

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pembentukan kadar pucuk tertinggi (76.67%) dari eksplan nod. Pucuk yang diperoleh dipindahkan ke media MS tanpa hormon untuk pembentukan akar (93.33%). Selepas 2 bulan, anak pokok yang stabil dipindahkan ke 3 jenis substrat untuk proses penyesuaian di mana substrat terbaik ialah tanah hitam, diikuti dengan campuran tanah hitam dan tanah merah (1: 1) dan serbuk sabut kelapa. Untuk pengekstrakan pigmen karetenoid dan klorofil, aseton telah dikenal pasti sebagai pelarut yang terbaik. Untuk kestabilan warna, pigmen adalah paling stabil pada pH 9 kerana warna lebih tahan lama. Dalam teknologi salutan, karotenoid memberikan tindak balas yang lemah berbanding klorofil, di mana warna pudar dengan peningkatan suhu. Dalam kajian antimikrob, kalus bukan embriogenik yang dihasilkan daripada eksplan dalam media MS yang ditambah dengan 1 mg/L BAP dan 1 mg/L 2,4-D (1.58 g kalus segar) dan hipokotil *in vitro* telah dituai dan diekstrak dengan metanol. Estrak telah diuji terhadap *Escherichia coli*, *Bacillus subtillis, Staphylococcus aureus*, *Aspergillus niger, Penicillium sp.*, dan *Fusarium sp*. Aktiviti antimikrob daripada ekstrak kalus memberi kesan paling kuat terhadap *Bacillus subtilis* dan *Penicillium sp*. dengan zon perencatan masing-masing bersaiz 14 mm dan 11 mm.

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LIST OF SYMBOLS AND ABBREVIATIONS

µL	:	Microliter
µM	:	microMolar
2, 4-D	:	2,4-Dichlorophenoxyacetic acid
ABA	:	Abscisic Acid
ADP	:	Adenosine Phosphate
ANOVA	:	Analysis of variance
BAP	:	Benzylaminopurine
CO_2	:	Carbon dioxide
Cv	:	cultivar
g	:	Gram
g/L	:	Gram per liter
GA3	:	Gibberalic acid
HCl	:	Hydrochloric acid
IAA	:	Indole-3-acetic acid
IBA	:	Indole-3-butyric acid
kcal	:	Kilo Calories
Kinetin	:	6-furfurylaminopurine
mg	:	Miligram
mg/L	:	Miligram per liter
mm	:	Milimeter
mM	:	miliMolar
MS	:	Murashige and Skoog
MSO	:	Murashige and Skoog (without hormone)
NAA	:	Naphthalene acetic acid

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NaCl	:	Sodium chloride
NaOH	:	Sodium hydroxide
NR	:	No response
Picloram	:	PIC 4-Amino-3,5,6-trichloro-2-pyridinecarboxylic acid
PMMA	:	Poly (methyl methacrylate)
SE	:	Standard error
TDZ	:	Thiadiazuron
USDA	:	United State Department of Agriculture
Var	:	Variety

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CHAPTER 1: INTRODUCTION

1.1 General Introduction

1.1.1 Plant tissue culture

Plant tissue culture is an area of plant research that involves the growing of plants under controlled nutritional and aseptic conditions that lead to the production of the clone plants. *In vitro* conditions include proper supply of nutrients, pH medium, adequate temperature and proper gaseous and liquid environment. It is also referred to *in vitro*, axenic or sterile cultures that have wide applications either for studies, applications and commercialization (Thorpe, 1990). The wide application and research of plant tissue cultures includes clonal propagation, cellular behaviour, secondary metabolites production, plant modification and development, production of pathogen free plants and germplasm storage (Hussain *et al*., 2012).

The basic finding of the cell theory, by Schleiden and Schwann in 1838 brought to the visualization of the cell autonomy characteristic that is capable to regenerate into the whole plant. Based on the concept proposed, in 1902, a German physiologist, Gottlieb Haberlandt proposed his *in vitro* culture method where he made his first attempt to culture the isolated single palisade cells from leaves in Knop‘s salt solution enriched with sucrose. The results obtained was that the cell remained alive for up to one month, increased in size, accumulated starch but failed to divide. Even though the experiments were considered as failed, the findings led to the new discoveries and established the theory of totipotency (Haberlandt, 1902). Other studies then led to the culture of isolated root tips where it was discovered that meristematic cells produced successful and indefinite culture.

1940s to 1960s was dubbed as an era for plant tissue culture where the development of this field rapidly increased and the successful stories were made one by one. The first

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true plant culture was obtained by Gautheret from cambial tissue of *Acer pseudoplatanus* (Loyola-Vargas *et al*., 2008). The subsequent years showed that the cultures of young embryos and the formation of meristems from callus were achieved.

The plant growth regulators or also known as hormones were discovered where Skoog and Miller discovered the first cytokinin which was kinetin in 1955. The discoveries provided information about the role of both auxin and cytokinin for *in vitro* plant cultures. Two years later, Skoog and Miller came up with the concept of hormonal control of both auxin and cytokinin in the medium influenced the morphogenic or organ formation. The ratio of auxin and cytokinin also plays the roles that favour the rooting and shooting formation to the callus proliferation. The MS medium is still used until today perhaps considered as the best medium for the *in vitro* culture. Nowadays, the rapid development of plant tissue culture has widened the application of the field to grow in to higher levels and penetrate the agricultural industries.

There are few types of techniques in cell and tissue culture which includes culture of callus, suspension cultures, protoplast anther and ovule cultures, somatic embryos and meristem tissues (Loyola-Vargas *et al*., 2008). The cell cultures also depend on the starting materials, or also known as explants. Explants were chosen based on the species employed and the kind of response desired. Explants can be any part of the plants and the explants generally used are leaf, stems, hypocotyls, roots, petioles and many more. The explants are isolated and cut into desired sizes and then culture on the media. There are three important considerations for culture initiation which are; a) selection of explants, b) selection of suitable culture medium and environmental conditions for its development, c) the isolation and maintenance of callus for subsequent experiments (Loyola-Vargas *et al*., 2008).

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In tissue sulture, callus can be formed. Callus is a largely unorganized, proliferating mass of parenchyma cells found in a wounded tissue which was produced due to the injuries of the explants. It is a slow growing, small and convenient to handle (Loyola-Vargas *et al*., 2008). The growth rate, friability, colours, morphology and structures of the callus are dependent on the types of explants, medium used and the culture conditions. Not all callus cell types appear to be competent to express totipotency or ability of regeneration. For the callus that does not regenerate may be manipulated to be the source for secondary metabolites productions like pigments, antimicrobial and other phytochemicals production. Hormonal controls play important roles that generate the types of callus formation in the culture medium (Loyola-Vargas *et al*., 2008).

Other than callus culture, organ culture has been established since 1934 by Philip White, where he developed the first system that allowed the infinite proliferation of tomato root tips by introducing the vitamin B as the growth supplement. Other than root, shoot culture was also established by Ball in 1946 that raised plant of *Lupinus* (Hussain *et al*., 2012). The root and shoot cultures has provided various tools to be studied like biochemistry and molecular biology of secondary metabolite biosynthetic pathway (Loyola- Vargas *et al*., 2008). The studies can be manipulated and can be used to synthesize secondary metabolites in more effective way with the control of environment, control of nutrients where it can be further applied with the developmental stage of the culture. The results have been shown by the synthesis of hyoscyamine from *Hyosciamu muticus* by Flores and Filner. Hyoscyamine synthesized was equal to or greater than the roots in *Planta* (Loyola-Vargas *et al*., 2008).

Plant morophogenesis can be studied in tissue culture system. Morphogenesis can be defined as the integration of growth and differentiation, mediated by cell division and specialization as a result of a complex spatial and temporal hormonal regulation that

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subsequently occur through regulation and expression of multiple gene systems, correlative action of meristems and their derivatives and environmental condition (De Almeida *et al*., 2015). Morphogenesis *in vitro* can occur in two ways, which are, somatic embryogenesis and organogenesis that subsequently develop into organ like shoots, roots and flowers. Both somatic embryogenesis and organogenesis can take place other directly or indirectly. Direct organogenesis occur when there is no callus form in the intermediate stage of morphogenesis but for indirect pathway, there is callus proliferation before organ formation and it is also known as *de novo* organogenesis.

There are many factors that can contribute to the stimulation of either embryogenesis or organogenesis and the types of morphogenesis. The factors includes; the natural environment, concentrations of plant growth regulators and exposure period of plant growth regulators applied, the status of endogenous phytohormones, and the source and the types of explants that able to respond. The factors mentioned above play important roles in the pathways to be taken in morphogenesis like stimulation, induction and reaction to produce specific pathways of cell differentiation and development (Loyola-Vargas *et al*., 2008). Generally, the somatic embryogenic pathway depends on high concentration of auxin to pass from somatic which is in differentiated stage to determined stage where the cells differentiated and further into morphogenesis which is growth and development but in organogenesis pathway is develop by high ratio of cytokinin : auxin (Loyola-Vargas *et al*., 2008).

Plant tissue culture or also known as micropropagation produces rapid multiplication of plantlets true-to-type to the original material. Several explants can be used in micropropagation which are meristem tissues (terminal/axillary buds) and hypocotyls have great response to culture condition like nitrogen sources, light regime, temperature and atmosphere. Somatic embryogenesis is the process where somatic cells generate

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embryogenic cells under induction condition where it undergoes a series of morphological and biochemical changes resulting in the formation of somatic embryo which will develop into a plant. From totipotency cell, which is a cell that is able to differentiate into any type of body cell, the somatic embryogenesis is detectable, easily controlled and lack of material is not limiting factor for experimentation. The first report of somatic embryogenesis was in the late 1950s of *Daucus carota* and *Oenanthe aquatic*a. The different stages of somatic embryogenesis are globular, heart, torpedo and cotyledonary stage (Kato and Takeuchi, 1963).

The basic procedure for producing somatic embryogenesis involves the use of synthetic medium supplemented with plant growth regulators or plant growth hormones like auxin (2,4-D), cytokinin (Kinetin), abcsicic acid (ABA), or a combination of two or several growth regulators. Reports on the carrot somatic embryogenesis showed the induction of these types of cell required auxin and later the cell must be transferred into a culture medium with low concentration of auxin or without it for further plant growth and development (Loyola-Vargas *et al*., 2008).

In vitro plantlets obtained from tissue culture will be transferred to soil for further development. This is a crucial process that will determine the ability of the plant to survive and grow. This process is called acclimatization process. In the process, plantlets will gradually be introduced to natural environment where they need to adapt to the environmental biotic and abiotic factors like change in temperature, humidity, photoperiod, and pH. Plantlets grown in the natural environment are also known as *ex vitro* plants. The acclimatization process will allow the *ex vitro* plants to maintain performance across a range of environmental conditions.

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1.2 General Description of *Cucucmis melo* L.

1.2.1 Introduction of *Cucumis melo* L.

Taxonomic hierarchy

Kingdom	*Plantae – plantes, Planta, Vegetal, plants*
Subkingdom	*Viridiplantae*
Infrakingdom	*Streptophyta* – land plants
Superdivision	*Embryophyta*
Division	*Tracheophyta* – vascular plants, tracheophytes
Subdivision	*Spermatophytina* – spermatophytes, seed plants, phanérogames
Class	*Magnoliopsida*
Superorder	*Rosanae*
Order	*Cucurbitales*
Family	*Cucurbitaceae* – gourds, squashes, citrouilles, gourdes
Genus	*Cucumis* L. – melon
Species	*Cucumis melo* L. – cantaloupe

Source: *http://www.itis.gov/*

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Cucumis melo L. belongs to *Cucurbitaceae* family. It is classified as the family of *Cucubitoideae*, tribe *Melothriceae*, genus *Cucumis* and species of *melo* by Jeffrey in 1962 (Guis *et al*., 1998). Based on Lim, (2012), *Cucumis melo L.* was originated from India and Africa. It was found that this fruit was first cultivated by the Egyptians and followed by the Greeks and Romans. The fruits' cultivation then expanded to North America by Christopher Columbus.

Previously, the fruits can grow on warm climate , cold and frost sensitive, grown in summer and heated in glass houses during winter. They require a very good internal and surface drainage soil with an optimum pH ranged of 5.8 - 6.2. However, due to research and development in the agricultural field, nowadays, there are many propagation of this fruit all over the world even in the countries with lower temperature.

The fruits of *Cucumis melo L.* are eaten fresh or as dessert fruits and also juice. Matured fruits are canned and used for syrup or jam. The immature fruits benefitted as fresh salads, pickled or cooked in soup. Melon seeds are a dietary source of unsaturated vegetable oil and protein (Lim, 2012).

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1.2.2 Classification and nomenclature of *Cucumis melo* L.

Based on Kirkbride, (1993) in Guis *et al*., (1998) there are 7 groups of *Cucumis melo L.* where the characteristics of the polymorphism in leaf, flower, fruit, and shape have been used in the classification of the melons, groups.

Table 1.1: Seven groups of melons

Group	Characteristics
Cucumis melo L.var. cantaloupensis	The fruits size are medium, round shape, smooth surface, marked ribs, orange flesh, aromatic flavour and sweet.
Cucumis melo L.var. reticulatus	The fruits size are medium, have netted surface, few marked ribs, have green to orange flesh colours.
Cucumis melo L.var. inodorus Naud	This variety have smooth or netted surface with the flesh commonly white or green, however have lacks of typical musky flavour. These varieties are commonly later maturity and can be kept longer than cantaloupensis.
Cucumis melo L.var. saccharinus Naud	This variety have medium fruit size, can be found in oblong or round shape with the surface of the fruit usually smooth with grey tone sometimes with grey spots. This variety has very sweet flesh.
Cucumis melo L.var. flexuosus Naud	This variety has a long and slender shape. The fruits are eaten in an immature state as an alternative cucumber.
Cucumis melo L.var. conomon Mak	This variety has small fruits size, with smooth surface and white flesh. These melons ripen rapidly. The flesh is very sweet since it develops high sugar content in fruits. However,the aromatic characteristics are less.
Cucumis melo L.var. dudaim Naud	This variety has small fruits, yellow rinds with red steak and has white to pink flesh.

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1.2.3 *Cucumis melo* L. as plantation crop

Cucumis melo L. or rockmelon has high demand either in Europe, Asia, and United States or Mediterranean countries. Nowadays, the production of rockmelon is conducted using fertigation system (Ahmad, 2013). Fertigation comes from two words which are fertilizer and irrigation. The concept of fertigation system is the plants are grown in a pot or polybag which are placed under protected structure like transparent shelter and netted surroundings. Their fertilization requirement will be monitored and scheduled at the right time for plant growth and fruiting.

In the field, rockmelon are germinated from seeds that will be sown on the germination tray using peat moss as the substrate medium. After one week of seeds germination or after the second true leaf appeared, the plants will be transferred to bigger polybags with cocopeat as a substrate (Hasan, 2013).

In the new substrate, the fertilization requirement will be supplied. Usually, the concentration of fertilizers will gradually increase weekly. Each plant produces both male and female flowers. Between 5th to 26th day of growth, the flowers will be pollinated. Since the plants were grown under shelter, *Cucumis melo L.* rely on insects (honeybees are ideal) for pollination. If it is done by hand pollinates but time consuming and required right materials. The pollination should be done early in the morning (Hasan, 2013).

After the pollination process, fruits development will take place. In order to harvest the highest quality of the fruit, only one or two fruits will be chosen to continue to grow per plant. The fruits chosen to be kept will depend on the counts of healthy leaves. The rates of the leaves are about 20 leaves per fruit. Healthy leaves marked the ability of the plant to produce higher quality of the fruit. The concentration of fertilizers supplied and

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other plant's growth supplement is continuously supplied. The fruits were harvested at day 70-85 (Hasan, 2013).

The fruits produced usually weighed between 2-3 kg per/ fruit. In Malaysia, the production of rock melon became vigorously important since 2009. The production of rock melon had promised of earning RM 500, 000/ year (Ahmad, 2013).

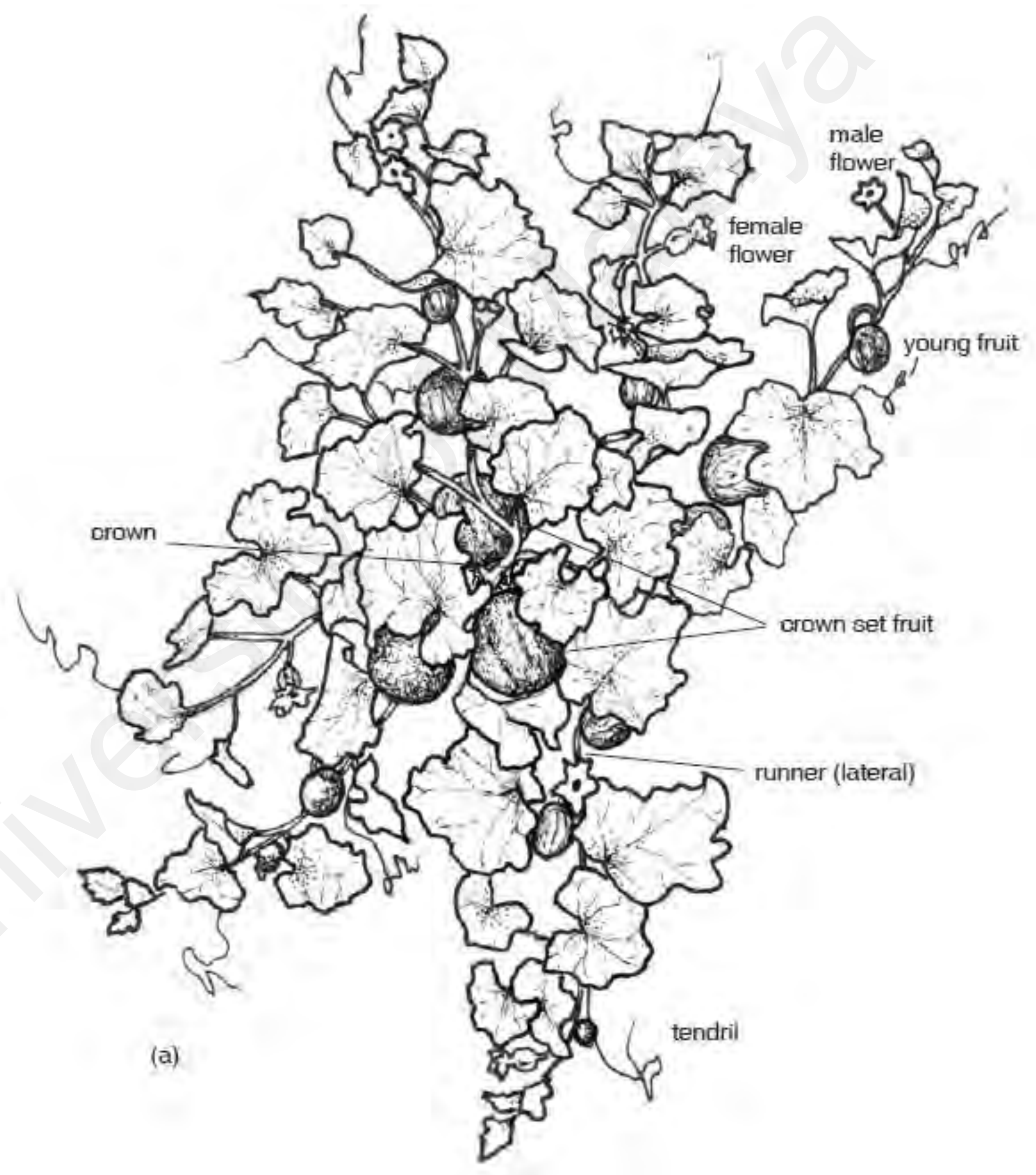

Figure 1.1: Above ground of rockmelon plant parts. (Source: http://www.melonsaustralia.org.au)

Figure1. 2: Close up picture of *Cucumis melo* L. matured fruit showing the netted surface and orange flesh. (Source: http://www.exportersindia.com)

Figure 1.3: *Cucumis melo* L. planted in soiless medium in polybag under greenhouse conditions. (Source: http://www.ipohecho.com.my)

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1.2.4 The nutritional and medicinal properties of *Cucumis melo* L.

There are many advantages of *Cucumis melo L.* in terms of nutritional and medicinal values.

Table 1.2: Nutrients content in 100 g of edible portion of *Cucumis melo* L. fruit (USDA, 2010)

Properties	Value/ 100 g of fruit
Energy	34 kcal
Protein	0.84 g
Fat	0.19 g
Carbohydrate	8.16 g
Dietary fiber	0.9 g
Total sugar	7.86 g
Vitamin A	169 µg
Vitamin C	36.7 mg

Table 1.3: Pharmacological values and benefits of *Cucumis melo* L. (Lim, 2012)

Value	Description
Antioxidant activity	The phenolic content and antioxidant activity of methanol extracts from leaf, stem, skin, seed and flesh of the fruits was reported to have high phenolic and total flavanoid content. This serve as potential source of natural antioxidant for food and nutraceutical industry (Ismail *et al*., 2010).
Antidiabetic activity	The fruit contain oxykine that rich in vegetal superoxide dismutase that can reduce diabetes-induced oxidative stress and kidney cell damage. Oxykine can be a safe and cheap source for the prevention of diabetic nephropathy (Naito *et al*., 2005).
Antiinflammatory activity	The *Cucumis melo L.* extract contain high superoxide dismutase activity that contain Glisodin, promotes the cellular antioxidant status and protects against oxidative stress-induced cell death (Voldoukis *et al*., 2004).
Antiplatelet Aggregation activity	Adenosine isolated from an aqueous of *Cucumis melo* L. was shown to inhibit human platelet aggregation induced by epinephrine, ADP (adenosine phospahate, collagen, and thrombin). This showed that the potential of using the compound as blood thinning agent in patient with heart disease (Altman *et al*., 1985)

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Anticancer Activity	Cucurbitacin A and cucurbitacin B extracted from stem of explant displayed significant effect of cytotoxic activity against the proliferation of human lung adenocarcinoma BEL7402 cells *in vitro* (Chen *et al*., 2009)
Dyslipidemia, Hypothyroidism Activity	The peel extracts of *Cucumis melo L.*was found to have the ability to inhibit lipid peroxidation in liver (Parmar and Kar, 2009).
Traditional Medicinal Uses	The root was reported to be emetic and the peduncle has been used medicinally for general anasarca and digestion by the Chinese and arrest vomiting in Indo-China. Other than served as a cooling drink, the flesh is also beneficial as a lotion for chronic and acute eczema (Lim, 2012).

1.2.5 The limitations of *Cucumis melo* L. production.

Melons production became important when the conventional breeding methods have variety of efforts in the varietal improvement of the crops. In 1990, the improvement of melon production was focused on obtaining a uniform melon with high organoleptic traits, good yield and disease resistance. The obstacles of having the desired interest traits occurred due to strong barriers that incompatible either at the level of interspecific or intergeneric. The barriers were identified to be one of the factors that limit the utilization of genetic potential through the conventional breeding methods. Thus, the progress of plant biotechnology in plant and agricultural field provided wide opportunities to study, develop and overcome the natural barrier (Guis *et al*., 1998).

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CHAPTER 2: LITERATURE REVIEW

2.1 Micropropagation of *Cucumis melo* L.

Micropropagation is the *in vitro* clonal propagation of plants from any parts of the plant known as explants that have the ability to proliferate and regenerate during subcultures (Hussain *et al.*, 2012). *Cucumis melo L.* var reticulatus listed as one of the varieties that have high frequencies of embryogenic when compared to inodorus varieties (Oridate *et al*., 1992, Gray *et al*., 1993). However, based on the reports by Ficadenti and Rotino (1995), *inodorus* varieties have higher rate of uniform regeneration while the reticulatus varieties showed wide difference in their organogenic response. The embryogenic property of the melon is under genetic control of individual genotype within species. Due to the property, embryogenic regeneration potential have the ability to be transfered from superior to inferior by sexual crossing.

2.1.1 Direct regeneration of *Cucumis melo* L.

Previous studies by Ficcadenti and Rotino, (1994) showed that MS basal medium, with BAP at 2.8 g/L and combination of 1.0 g/L of abscisic acid (ABA) gave the highest shoot regeneration frequencies from cotyledon explants of *Cucumis melo L.* var. reticulatus and inodorus. The findings also suggested that different genotypes examined gave different morphogenic response.

Mali and Chavan (2016) have reported on the successful of *in vitro* plant regeneration via direct organogenesis from axenic seedlings of *Cucumis trigonus* Roxb. using nodal explants as the starting materials. The auxins and cytokinins used were BAP and IBA either singly or in combinations. Results obtained showed the highest shoot induced was achieved from MS medium supplemented with 2.0 mg/L 6-benzylaminopurine (BAP) with an average of 4.90 ± 0.25 shoots per node explant. Half

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strength MS medium exhibited the best rooting induction medium when incorporated with 1.0 mg/L indole-3-butyric acid (IBA) by producing the highest frequency of root formation (100%) with number of roots of 21.6 ± 0.88 per explant.

Another achievement of direct regeneration in *Cucumis melo L.* was marked by Parvin *et al*., (2015) whereby an efficient protocol of *in vitro* from shoot tips, nodal segments and cotyledonary nodes explants were developed. Plant growth regulators used in the study includes BAP, NAA, GA_3 and IBA aimed for multiple shoot regeneration. The best results exhibited with MS medium supplemented with plant growth regulators of 2.0 mg/L BAP, where 90% and 70% of cultures induced multiple shoots from nodal segments and shoot tip explants respectively, whereas, 70% cultures of cotyledonary nodes were found to form shoots on MS medium with 1.5 mg/L BAP and 0.1 mg/L GA_3. Half strength MS medium incorporated with 1 mg/L IBA showed effective results for rooting where up to 70% of the cultures formed roots.

Based on the direct organogenesis studied by Vankateshwarlu (2012), on *in vitro* plantlet regeneration of *Cucumis melo* L*.,* results showed that from 20-25 days old seedlings using shoot tip segments as the best explant that formed multiple shoots. It was found that, semi-solid medium of MS supplemented with 2.0 mg/L BAP and 0.5 mg/L IAA was the best medium for multiple shoots proliferation and rooting induction was best obtained on MS medium supplemented with 1.0 mg/L IAA.

Study on *Momordica charantia* or also known as bitter melon by Wang *et al*., (2001), which is another family member of Cucurbitaceae showed *in vitro* flowering formation, whereby, shoot tips used as the explants cultured on MS media supplemented with 90 mM sucrose, 0.05 mM Fe^{2+} and 4 μM N6-benzyladenine (BA). Function of Fe^{2+} in the medium was to prevent chlorosis of the explant and promote normal flowering. Wang *et al*., (2001) also showed that cytokinin gave significant effect on the

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explants morphogenesis. The highest flower formation was obtained from Kinetin or BAP at 15 to 20 days of cultures. Specifically, the function of Kinetin and BAP was different whereby Kinetin promoted female flower formation, but inhibited branch bud formation. Conversely, BAP promoted male flower formation and branch bud formation at lower concentration of the hormones (1–2 µM).

Ahmad and Anis, (2005) working on another member of Cucurbit family (*Cucumis sativus* L. or also known as cucumber) showed an efficient reproducible protocol for the *in vitro* multiplication developed from nodal explants. In their study, it was found that the addition of casein hydrolysate to the shoot induction medium, MS media supplemented with BAP, significantly induced multiple shoots or growth of the regenerants. MS media supplemented with 1 mg/L of BAP incorporated with 200 mg/L of casein hydrolysate induced the optimization shoot regeneration. For rooting of the *in vitro* shoots, half strength MS medium supplemented with 1 mg/L of NAA was found to be the best medium.

2.1.2 Callus Induction and somatic embryogenesis of *Cucumis melo* L.

Callus is an unorganized proliferated mass of undifferentiated tissues that develops around the injured area or cut plant surface. In tissue culture, callus formation is an advantage because it can be a source of many wide applications includes plant regeneration and commercially important secondary metabolites.

One of the most spectacular achievements in plant tissue culture has been the discovery of the induction of somatic embryogenesis in cell culture by which somatic cells develop into differentiated plants through characteristic embryological stages without fusion of gametes. These steps are regulated by many factors, including plant genotype, level of sugar in the medium, type and concentration of growth regulators,

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photoperiod, gelling agents, time exposure and induction and maturation medium which stimulates somatic embryogenesis (Elmeer, 2013). Zhang *et al*., (2014), added other factors that contribute to somatic embryogenesis including explant type, seedling age, basal culture medium, carbohydrate, nitrogen, growth regulators, additives and illumination.

In melon tissue culture studies, the first report on somatic embryos was by Blackmond *et al*., (1981) where cantaloupe hypocotyl was used as the explant in the study. In 1986, the first regenerated plants from somatic embryos were obtained by Oridate and Oosawa (1986). Two phases of somatic embryogenesis of *Cucumis melo* L. are induction phase and developmental phase. The induction phase suggested that the callus was induced for a period of time on MS media supplemented with auxin and cytokinins (usually BAP and 2,4-D). During the second phase, the callus induced will be subcultured for somatic embryo development on MS media supplemented with lower auxin, or cytokinins or without plant growth regulators.

Study by Rhimi, (2006) showed that somatic embryogenesis can be induced from hypocotyl, cotyledon and zygotic embryo explants from two Tunisian *Cucumis melo L.* cultivars Beji and Maazoun, cultured on the MS medium added with (0.25–1 mg/L) 2,4-D and (0.10–0.50 mg/L) BAP. Based on the results observed, different explants and different cultivars exhibited different rate of embryos induction. Rhimi, (2006) showed that callus with somatic embryos formed after 3 weeks of culture. The difference of embryogenesis ability based on cultivars and types of explants was shown when Beji cultivar gave the highest percentage (62.50%) of somatic embryogenesis from cotyledons followed by embryogenesis induction from zygotic embryos which reached 33.50% and lastly with ability of the hypocotyls did not exceed 12.50%. in contrast,. Maazoun explants showed less efficiency on induction of somatic embryogenesis when

embryos formation was observed at the percentage of 29% for cotyledons and 25% for zygotic embryos explants.

The study by Comlekcioglu *et al*., (2009) described the effects of medium, explant type and photoperiod on somatic embryogenesis of snake melon (*Cucumis melo* var. flexuosus). Using two different types of explants (cotyledon and hypocotyl) obtained from 3-day-old snake melon seedlings and culture conditions were investigated for somatic embryogenesis. MS medium supplemented with NAA and 2,4-D alone and combinations with cytokinin induced embryogenic callus from cotyledon explants. The highest somatic embryo formation was achieved from cotyledon explant which was 20.00 ± 7.94 somatic embryos per culture dish on MS medium supplemented with 0.5 mg/L BAP and 4 mg/L 2,4-D. The cotyledon and hypocotyl explants cultured on the media with 0.5 mg/L BAP with 2 mg/L 2,4-D showed embryogenic callus formation but with less count of somatic embryos which were 17.33 ± 5.51 and 16.33±3.06 per culture dish, respectively.

2.1.3 Regeneration through somatic embryogenesis of *Cucumis melo* L.

Research on *in vitro* regeneration of hypocotyl and cotyledonary explants by Rasheed *et al*., (2013) on watermelon (Cucurbitaceae family) on MS media supplemented with plant growth regulators of BAP and NAA showed the successful regeneration of shoots from embryogenic callus. The successful shoot regeneraton was observed on MS media supplemented with 1.0 mg/L BAP + 0.2 mg/L NAA with 6.3 shoots per explant and roots were best induced on MS media supplemented with 0.1 mg/L NAA.

Results obtained by Zhang *et al*., (2012) showed an efficient and rapid regeneration system of thick-skinned muskmelon had been set up using cultivars 'Queen' cultured in

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MS medium supplemented with 1 mg/L 6-BA for the best adventitious buds induction. Then, the regenerated buds were further elongated on MS medium supplemented with 0.1 mg/L 6-BA and 0.1 mg/L GA_3. The roots of the regenerated shoots showed the ability to be formed on MS medium without plant growth regulators with 99% success rate.

2.1.4 Explants source

The explants types play an important role as the main source of the somatic embryogenesis induction. Guis *et al*., (1998) stated that the cotyledon explants were the best tissue for induction of somatic embryogenesis with the particular type was from cotyledons of quiescent seed or from 1-day-old seedlings. There were more studies conducted and various explants were successfully used to induce somatic embryogenic callus. The explants that have been used in the somatic embryogenesis studies include hypocotyls explants (Naderi *et al*., 2013; Bairwa *et al*., 2010; Rhimi *et al*., 2006), cotyledons (Naderi et la., 2011, Moradmand *et al*., 2011, Rhimi *et al*., 2006), zygotic embryo (Rhimi *et al*., 2006; Moradmand *et al*., 2011), cotyledonary nodes explants (Zhang *et al*., 2012, Naderi *et al*., 2011) and leaves explants (Stipp *et al*., 2001).

The age of the explants also plays an important role when the successful culture of the root explants of *Cucumis melo L.* cv. Pusa sharbati shows there are difference in callus growth and nodule formation (Kathal *et al*., 1994). In the same study, results obtained also showed that the callus induction and nodule formation also were influenced by part or segment of the roots used as explants (basal, sub-basal, sub-apical and apical) and position of explants. Results showed the basal root segment from 21-day-old seedlings responded best for callus growth and nodule formation. However, the least regeneration frequencies were found the least at the part of apical of the roots.

2.1.5 Media composition

Murashige and Skoog (MS) medium was commonly used as the source of the nutrients. About 2-3% of sucrose was commonly utilized as the source of carbon in the basal medium. The amount of sugar gave effect to the production of somatic embryos as Lou and Kako, (1995) showed that high sugar concentration contributed to higher number of somatic embryos induced from cucumber cotyledon explants.

The application of combination of different types of carbon sources or sugars like sucrose, fructose, galactose can increase the rate of embryogenesis as reported by Oridate and Yasawa, (1990). Guis *et al*., (1997) showed that the use of glucose enhanced the somatic embryogenesis rate by two fold in the culture of *Cucumis melo* var Vendrantais. Besides carbon sources and MS as the basal medium, the first phase of somatic embryogenesis induction required the supplementation of plant growth regulators or hormones. For induction medium, there are many types of auxin, cytokinin and combination of both have been used for *Cucumis melo L.* somatic embryogenesis induction. Among the plant growth regulators used includes 2,4-D, IAA, BAP, Kinetin, and NAA.

Somatic embryo maturation was commonly achieved on hormone free medium (Naderi *et al*., 2013), lower concentration of BAP, 2,4-D, NAA, Kinetin (Bairwa *et al*., 2010), lower concentration of BAP and IAA (Moradmand *et al*., 2011). For regeneration medium, usually MS media without hormone was used. Other than MS media without hormone, half strength MS media was also used for regeneration of the plants, especially for root formation.

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2.1.6 Physical cultural factors

Light is one of the environmental factors that give significant effect on the induction of somatic embryogenesis of melon. Blanchard and Chateau, (1988) and Tabei *et al*., (1991) stated that somatic embryos were able to be induced in light conditions. However, reports form Guis *et al*., (1997), showed that the embryogenic callus induced when incubated under dark condition for a short period at the early period of culture. Other than Guis *et al*., (1990), Bairwa *et al*., (2010) also found that embryogenic callus was induced in the dark for 1 week after cultured.

Other physical state of the media also influenced the embryo quality. For melon, embryogenic callus induced on solid media, was found to have better quality (Blanchard and Chateaul, 1988). For cell suspension, it was found that embryogenic callus induced was successfully obtained, however the somatic embryos developed exhibited abnormal development (Oridate and Oosawa,1986; Moreno *et al*., 1986). Other than exhibited abnormal development the regenerated plantlets also showed vitrification effect.

2.1.7 Acclimatization of regenerated plantlets

The success of micropropagation depends on the survival of the acclimatized plantlets. It is marked by the ability of the plantlets to adapt to the natural environment at low cost with high survival rate (Bhojwani and Dhawan, 1989). Sterile and control condition of tissue culture promotes rapid growth and multiplication of shoots often result in lesser capability of the plantlets to survive under the natural environment because of low qualitative mechanism reaction towards the environment (Hussain *et al*., 2012). With acclimatization, the plantlets will be gradually exposed to the natural environment and will minimize the transplantation shock.

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Cucumis melo L. cv. glamour belongs to Cucurbitaceae family and commonly known as muskmelon has been micropropagated using *in vitro* culture technique in MS medium with different concentrations of cytokinin by Sidik *et al*.,(2012). The study on acclimatization showed vermiculite growth medium gave the highest survival rate of 81 % followed by mix of top soil and vermiculite of 69 % and top soil of 13.3 % gave the least survival rate. Survived plantlets on vermiculite medium grew up vigorously with large and green leaves. Plantlets were transferred to sandy soil, where 70 % plantlets were successfully survived the natural condition (Parvin *et al*., 2013).

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2.2 Secondary Metabolites Studies of *Cucumis melo L.*

Plants produce a various and diverse organic compounds, where the great majority of which do not appear to participate directly in growth and development. These substances, traditionally referred to as secondary metabolites, often are differentially distributed among limited taxonomic groups within the plant kingdom. Their functions, many of which remain unknown, are being elucidated with increasing frequency (Croteau *et al*., 2000). Nowadays, the disease preventing potential of naturally occurring substances in the diets is a major area of scientific interest. Antioxidants and secondary metabolites have attracted a great deal of attention for their effect in preventing disease due to oxidative stress, which always leads to degeneration of cell membranes and many pathological diseases including cancer (Ahmed and Beigh, 2009). There are many researches have been done on every part of the *Cucumis melo L.* plants. All of these researches indicated that every part of the plant has their own advantages and benefits. All of the findings contribute additional information and act as value added to various industries thus will support the economic growth.

Hayet *et al*., (2015) reported there are valuable compound of anticandidal, antiviral and free radical scavenging effects of aerial part and flesh extracts of *Cucumis melo* L. it was found that the anticandidal activity from the ariel parts of extractants of Tunisian *Cucumis melo* showed the strongest inhibition using ethanolic extracts with the minimum inhibitory concentration (MIC) of 0.256 mg/ml and minimum fungal concentration (MFC) of 2.5 mg/L and moderate inhibition was found in flesh extracts. This is due to high polyphenol compound that contributes to the efficiency of of antimicrobial properties activity (Ediziri *et al*., 2011). The aerial part and flesh extracts parts also tested for antiviral and antiradical scavenging activities. The results turned out

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to show aerial parts extractants of the plants exhibited highest antiviral and antiradical activities.

Sasi *et al*., (2015), reported that the ethanolic extract of fruit from *Cucumis melo* L. exhibited 10 compounds where the major proportion of the compounds was 5-hydroxy-6,7,8-trimethoxy-2,3-dimethyl-4H-chromen-4-one (21.04%). The fresh fruits extract also showed a remarkable effect activities against the microorganisms tested which are *Salmonela typhi, Escherichia coli, Klebsiella pneumonia, Bacillus cereus,* and *Staphylococcus aureus.* The studies also present the results of the ability of extractants to exhibit the greatest antioxidant activity through the DPPH radicals scavenging activity.

Investigation on dried seeds of *Cucumis melo* or musk melon and *Citrullus lanatus* water melon by Mehra *et al*., (2015), marked a finding on large potential advantages of the seeds. The phytochemical analysis exhibited the seeds as good source of flavonoids, phenolics, saponins, alkaloids and other secondary metabolites. The essential oil extracted from the seeds by cold pressing method exhibited high content of iodine value. Other than that, the oil of also contain polyunsaturated fatty acids such as omega-6 (linoleic acid), monounsaturated fatty acids such as omega-9 (oleic acid). These provide an additional information and natural value as dietary supplements and medicines that will bring various advantages especially in medicinal and pharmaceutical industries.

β-carotene extracts from *Cucumis melo L.* have high antioxidative and cytotoxic activities potencies. The research done by Widowati *et al*., (2015) examined the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity assay. The cytotoxic potency was determined by the MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay on HeLa, HepG2 and

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NIH3T3 cell lines. Results observed showed that β-carotene exhibited more active DPPH free radical scavenging activity compared with *Cucumis melo* extract. *Cucumis melo* extract showed more active anti-cancer both in HeLa (IC50: 23.649 μg/mL) and HepG2 (IC50: 110.403 μg/mL) cancer cells. *Cucumis melo* extract (IC50: 16,670.404 μg/mL) and β-carotene (IC50:50,645.994 μg/mL) had low cytotoxicity in NIH3T3 fibroblast. *Cucumis melo* extract has lower antioxidant activity, but higher cytotoxic potency compared with β-carotene.

2.2.1 Different solvent extraction effects on *Cucumis melo* L. compound

The secondary metabolites of the plants were extracted using different solvents to examine the active biological compound of interest. The extraction solvent of the compound will react with the compound of interest. The solvents selection is an important factor to be considered because it determines the degree of affinity towards the compounds to be extracted (Henriques *et al*., 2007). Other than the dissolution ability factor of solvents toward the compound to be extracted, the solvent also plays an important role in cell lysis (Henriques *et al*., 2007). Jamur and Oliver, (2010) stated in the experiment to detect intracellular antigen, the organic solvent dissolved lipids from the cell membranes making them permeable to antibodies and because the organic solvents coagulate proteins, they can be used to fix and permeabilize cells at the same time. This would tend to suggest that the solvents used in this study has increased the permeability of the cells membranes and disrupted the protein in the phospholipid bilayer.

Other than types of solvent to be used, the other factor to be considered is the ratio of the solvents to be used. The ratios of the samples and solvents extraction also act as significant factor in order to get the highest content of the compound. The extraction using different chemical solvents play an important role because different chemical

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solvents have different polarities, extraction time temperature and sample to solvent ratio. Different samples or different plant materials may yield varying compounds (Dai and Mumper, 2010). Therefore, the extraction procedure must be determined in order to gain the highest yield of compounds of interest. Besides, natural pigments extraction with organic solvents generates a large amount of waste (Ribeiro and Olivera, 2013). This suggests that the best solvent and the best sample to solvent ratio are to be used that will minimize the amount of waste.

2.2.2 Pigments extraction of *Cucumis melo* L.

Pigments are chemical compounds that absorb light in the visible region wavelength. The visible colour produced is due to chromophore structure that captures energy. Once energy captured, the electron was excited and form external orbital to a higher orbital. The non-absorbed energy then will reflect the colour that captured by eye. The neural impulse generated then transmitted to the brain where subsequently the colour interpretation occurred (Hari *et al*, 1994).

The main sources of natural colorants are presence in plants (Saxena and Raja, 2014). As the principle producers of the pigments, each of the plant parts plays important roles. The pigment can be obtained from various plant parts including roots, leaves, twigs, stems, heartwood, bark, wood shavings, flowers, fruits, rinds, hulls, and husks (Saxena and Raja, 2014). Due to characteristic of natural colorants that are low in concentration and their instability of the natural colorants due to pH changes and temperature changes become the obstacles for the compound to be used as the main source of colorants. Thus, an adequate understanding of the actual source of the pigments to be used as the colours are contributing to better production and optimizing usage.

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There are four major pigments found in the plants which are carotenoids, chlorophyll, anthocyanin and betalains (Kaimainen, 2014). This study is focus on the carotenoids and chlorophylls pigments. The orange flesh of *Cucumis melo L.* fruits consists of carotenoid compound while the chlorophyll pigment was found on leaves of the plant. The pigments were extracted with various solvent for production of natural colorants. The extractions with different solvents exhibit different quality and quantity of the desired pigments. Production and application of synthetic colorants showing threats to environment and life cycle on earth. Due to the reason “synthetic pigments” and “illness” association, the increasing awareness in utilisation of natural colorants become vigorously important. Natural colorants are not something new in the world today. The productions of natural colorants have been known in many decades ago. Natural colorants are derived from naturally occurring sources such as plants, insects, microbes and minerals (Kaimainen, 2014).

Delgado-Vargas *et al*.,(2000) define carotenoids as compounds consist of eight isoprenoid units (ip) and located in inverted order at the molecule center. Caretoniods can be obtained from all photosyntethic plant tissue. β-carotene can be found in yellow, orange and red colored fruits and vegetables. Carotenoids functions includes provide colors in flowers, seeds and fruits that will further assist in pollen, seeds or spores dispersion by animals in order to sustain their production (Delgado-Vargas *et al*,2000). Naturally, β-carotene is mostly found as all-trans isomers and lesser as cis-isomers (Khoo *et al*., 2011)

Carotenoids are lipid-soluble, yellow–orange–red pigments found in all higher plants and some animals (Mortensen, 2006). Carotenoids found in fruits and vegetables have also attracted great attention for their functional properties, health benefits and prevention of several major chronic diseases (Khoo *et al*., 2011).The fresh fruit of rock

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melon have high beta carotene with 242.8 μg/g dry weight (Fleshman *et al*., 2011) and Khoo *et al*., (2008) reported that orange colored underutilized fruits contained high amount of β-carotene.

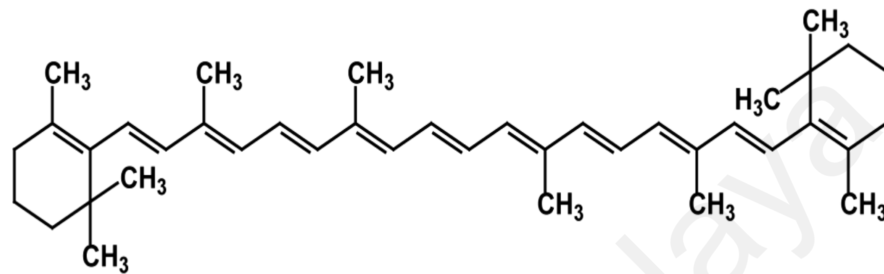

Figure 2.1: Basic structure of Beta carotene.

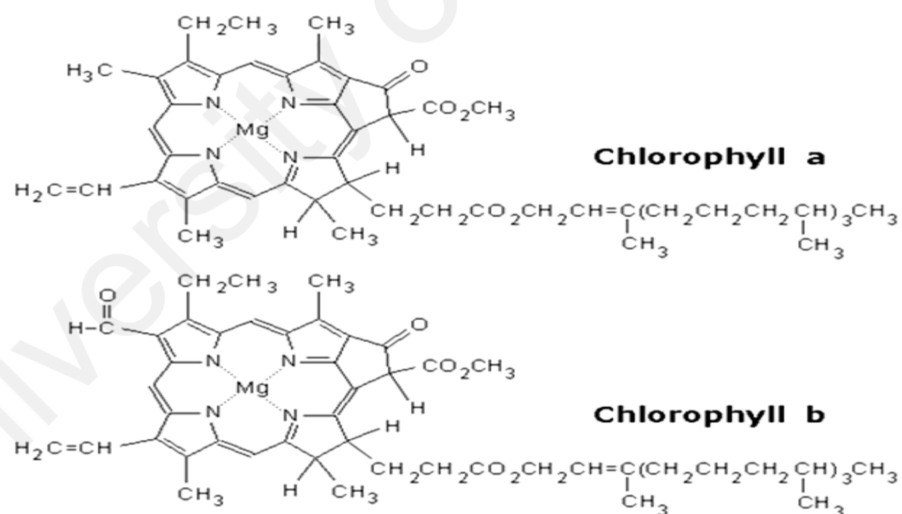

Figure 2.2: Basic structure of chlorophyll a and chlorphyll b.

Chlorophyll is the green pigment utilized by all higher plants for photosynthesis and lipid-soluble compound. The chlorophyll is extracted with using organic solvents. There are two types of chlorophyll which are chlorophylls *a* and *b*. They are found in an approximate ratio of 3:1 respectively. The difference in the structures of chlorophylls a

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and chlorophylls b is the only one substituents which shown in Figure 2.2 (Scheer, 1991, Schwartz *et al*. 2008).

In this study, *Cucumis melo L.* or rock melon fruit flesh and leaves were used as sources for carotenoids and chlorophyll pigment respectively. Sources of chlorophyll were obtained from the big leaves in size of 244.5±1.84 cm^2 (Sidik *et al*., 2013) that were trimmed in order to maintain the quality of the fruit. Thus, the green leaves have potential to be used as one of the sources of chlorophyll for natural colour production.

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2.3 Research Objectives

Cucumis melo L. is a plant that has high commercial value with high benefits of secondary metabolites compound. Through plant tissue culture, this plant can be mass propagated and grown in many places. Thus, the objectives of the present works are:

1. To determine the best seed position for *in vitro* seeds germination and direct regeneration using cotyledonary petiole as explant of *Cucumis melo* L.
2. To determine the best explant for callus induction and somatic embryogenesis in *Cucumis melo* L.
3. To investigate regeneration from somatic embryogenesis obtained from the best explants of *Cucumis melo* L. and to induce *in vitro* rooting and establish acclimatization of *Cucumis melo* L. plantlets.
4. To study the pigments extraction and antimicrobial activity of *Cucumis melo* L.

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CHAPTER 3: ASEPTIC SEEDLINGS GERMINATION AND *IN VITRO* REGENERATION OF *Cucumis melo* L.

3.1 Experimental Aims

One of the most important factors governing the growth and morphogenesis of plant tissues in culture is the composition of the culture medium, plant growth regulators and the explants used based on objectives of the study. The basic nutrient requirements of cultured plant cells are very similar to those of intact plants. Murashige and Skoog (MS) salts compositions are the most commonly used especially in plant regeneration protocols. The culture medium in the study consists of MS supplemented with vitamins and different types and concentration of plant growth regulators (PGRs).The functions of PGRs to induce adventitious shoots and roots of the explants tested, so that the comparison between MS without PGRs and media supplemented with PGRs can be observed.

In this chapter, i*n vitro* regeneration of *Cucumis melo* L. was examined. The studies include the effects of polarity of the seeds, the effects of cotyledonary petioles used as explants and the effects of MS media supplemented with different PGRs for direct regeneration of the plants. The polarity determination was conducted primarily to identify the polarity of seeds either placed in horizontal or vertical positions for optimum seed germination whereby the germinated seeds were used as the source of explants. The cotyledonary petioles were used in this study as explants to regenerate the best response to form multiple shoots and rooting *in vitro*. The optimum plant growth regulators and conditions for regeneration will also be identified.

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3.2 Materials And Methods

3.2.1 Source of seeds

Seeds of *Cucumis melo* L. were bought form Nine Top Trading, Rawang a Rockmelon Seed distributor in Malaysia. The sterilized seeds were germinated in Murashige and Skoog basal medium. Seven to 10 days old of aseptical seeds were used as explant sources.

3.2.2 Sterilization of seeds

The seeds were soaked in distilled water for 2 hours. Then, the seed were uncoated using scalpel blade. The uncoated seeds were soaked in 70% sodium hypocholrite mixed with 1mL of Dettol and 3 drops of Tween 20. The soaked seeds were agitated on a shaker for 20 minutes. After the agitation, the seeds were rinsed with distilled water for three times. The rinsed seeds were transferred into laminar flow chamber to prevent air contamination and maintained the aseptic conditions. In the laminar flow, the seeds were soaked again in 70% alcohol for 1 minute. Prior to inoculation, the seeds were rinsed again with sterile distilled water. The rinsed seeds were blotted dry with sterilized tissue.

Sterilized seeds were cultured onto MS basal medium containing 30g/L glucose, 4.4g/L MS media, 2.8 g/L gelrite. The pH of the media was adjusted to pH 5.8 ±0.01 using sodium hydroxide (NaOH) and hydrochloric acid (HCL).

3.2.3 Germination of seeds on MS basal medium

The seeds were cultured on MS media either in vertical or horizontal positions on the media. The cultured seeds were incubated in the dark for three to four days. After day 4,

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the germinated seeds were maintained in the culture room at 24± 1°C with 16 hours light and 8 hours dark with light intensity of 1000 lux for 14 days.

3.2.4 Identification of the best position for seeds germination

Sterilized seeds were cultured on MS basal medium in vertical and horizontal position form in order to determine the best polarity of the seeds. The seeds were then incubated in the dark condition for 3-4 days at room temperature for faster germination. After 3-4 days, the cultures were transferred to the culture room at 24± 1°C with 16 hours light and 8 hours dark with light intensity of 1000 lux.

The aim of the experiment was to identify the most suitable polarity of the seeds for the germination and the most responsive position of seeds for faster germination. Thirty replicates were used in this experiment and observations were made for 2 weeks.

3.2.5 Preparation of explants

Explants preparation was a crucial step in tissue culture. So, there is a need for explants to be fully sterile to ensure the cultures will not be contaminated. However, in this study, explants were obtained from aseptic seedlings in order to minimize contamination. A germinated seed consists of hypocotyl, cotyledon, cotyledonary petiole, petiole and leaf. The explant used for direct regeneration studies is cotyledonary petioles from 7- day-old aseptic seedling.

3.2.6 Direct regeneration of *Cucumis melo* L. using cotyledonary petioles as explant

In the laminar flow, explants of cotyledonary petioles were cut using a scalpel into a small size of 10 mm x 10 mm in size. All explants were cultured on the MS medium supplemented with PGRs mentioned (Section 3.2.8). The formation of shoots, roots and

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callus on explants were observed. From the results obtained in this experiment the most responsive explant and the most optimum medium for regeneration of shoots and formation of roots were identified.

Thirty replicates of explants were used in each treatment. All cultures were maintained in the culture room at at 24 ±1 °C with 16 hours light and 8 hours dark for 8 weeks. Callus induction frequencies and scale of callus production were obtained and recorded.

3.2.7 Preparation of basal culture media

In this study, the basic nutrient requirement was obtained from Murashige and Skoog (MS) salts composition. This media have been used widely especially in plant regeneration procedures. The MS medium consists of macronutrients that include high levels of nitrate and organic additives such as agar, sugars, vitamins and growth regulators. The additional of other vitamins and plant growth regulators can help in production of the whole plants. The basic media was prepared as follows.

Preparation of culture media was done by preparing 800 ml distilled water in 1000 ml conical flask. 30 g of sugar was added to distilled water and stirred with magnetic stirrer. Then, 4.4 g of MS including vitamin was added and stirred until the solution become evenly dissolve .The pH of medium then adjusted to 5.8 using 1.0 N Hydrochloric acid (HCl) or 1.0 Sodium hydroxide (NaOH). After that, gelrite that was weighed at 2.8 g then added to the media. The gel-rite act as a solidify agent to provide surface and support for the plants growth. Lastly, the volume of the medium then was made up with distilled water to 1 L using graduated cylinder. Mixture was stirred and heated up to dissolve the medium completely. The medium was heated until the colour of the medium become transparent.

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The medium was autoclaved at 121 °C (15 psi) for 20 minutes. After autoclaved, MS medium was allowed to cool to about 50°C and then were dispensed into 60ml sterile containers in the laminar flow in order to maintain the aseptic condition.

3.2.8 Preparation of culture media with hormones

Preparation of culture media was done as mentioned previously (3.2.7). However, the difference of the way it was prepared is the addition of the PGRs. Prior autoclave, hormones were added at desired concentration into the media. Then, the mixture were stirred and dissolved completely.

There are two types of hormone which are heat sensitive and non-heat sensitive hormone. In this chapter, hormone used are non-heat sensitive hormone like 2,4-D, and coconut. So, the hormones used were added after adjusting the pH and can be autoclaved. However, for hormones that heat sensitive like $GA_{3,}$ the need to be filtered sterilized before being added to the culture media. These hormones were then filtered with 0.22µm sterile membrane filters (Milipore filter). Desired concentrations of sterile hormones were then added to the culture media while the media was at molten phase (50 °C)..

In the present study, there were 10 treatments used which were:

1. MS media
2. MS media supplemented with 1 mg/L 2,4-D + 25% of Coconut water
3. MS media supplemented with 2 mg/L 2,4-D + 25% of Coconut water
4. MS media supplemented with 3 mg/L 2,4-D + 25% of Coconut water
5. MS media supplemented with 1 mg/L 2,4-D + 35 % of Coconut water
6. MS media supplemented with 2 mg/L 2,4-D + 35 % of Coconut water
7. MS media supplemented with 3 mg/L 2,4-D + 35 % of Coconut water

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8. MS media supplemented with 1 mg/L 2,4-D + 45 % of Coconut water
9. MS media supplemented with 2 mg/L 2,4-D + 45 % of Coconut water
10. MS media supplemented with 3 mg/L 2,4-D + 45 % of Coconut water

The medium was autoclaved at 121 °C (15 psi) for 20 minutes. After autoclaved, MS medium was allowed to cool to about 50°C and then were dispensed into 60 ml sterile containers in the laminar flow in order to maintain the aseptic condition.

3.2.9 Conditions of culture

All of the equipment and apparatus used must be cleaned and sterilized before being used. They were washed with Dettol and Clorox under running tap water. This includes forceps, scalpel, petri dishes, jam jars, conical flasks, test tubes, tissues and many others. These culture apparatus were wrapped in aluminium foil and autoclaved for 30 minutes. Forceps and scalpel were dipped in 70% alcohol, sterile distilled water, dried and dipped into hot bead sterilizer to ensure the sterility of culture apparatus. Hot bead sterilizer will reach a temperature of 250°C. Forceps and scalpel need to be cooled by dipping them into them into sterile distilled water before being used to excise plant tissues.

All cultures were incubated in the culture room at the temperature of 24 ± 1°C with 16 hours light and 8 hours. The intensity of light in the culture room was set at 1000 lux. This is suitable to allow cultures to respond and grow.

3.2.10 Subculture

All cultures were sub cultured every 4 weeks on the same medium. This is to provide fresh nutrients for further growth and development. It also prevents the

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3.2.11 Data analysis

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

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3.3 Results

3.3.1 Germination of seeds on MS basal medium with different position of seeds

Explants of *Cucumis melo* L. were obtained from the seeds that were germinated on the MS basal media. In order to get the optimum germination and regeneration rate, the seeds were cultured in different polarity form of the seeds. The seeds were cultured on MS basal media either in horizontal or vertical positions.

Seeds germination on MS basal media was successfully achieved for both vertical and horizontal positions. Based on the observation recorded during day time at 7 days interval, the horizontal cultured seeds were found to be more effective compared to vertical form of cultured seeds. Both seeds treatment were incubated under dark condition at room temperature for the first 3-5 days or until the seeds germinated. Generally, the seeds cultured in horizontal form germinated earlier which is on day 3 onwards while the seeds cultured on vertical form germinated later which is after day 14 onward.

Based on examination of 30 replicates of culture for each treatment, horizontal cultured seeds gave 100% seeds germination on the first observation on the 7th day where the earliest seeds germinated on day 3. The average height of the germinated seeds was 10.12 ± 0.38 cm with no leaf observed except 2 cotyledons. The white fibrous roots formed with an average of 10.70±0.38 cm per plant. The heights of the germinated seeds increased on the 14th day of observation to 17.16 ± 0.36 cm. The leaf formation was observed on day 14 with the average of 1.73 ± 0.08 and the formation off roots are more than 20 per plant. The last observation showed that, the highest height rate of the seeds germinated was 20.28 ± 0.35 cm and there were 3.73 ±0.14 leaves observed per plant. The rate of root formation also increased greatly.

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Vertically cultured seeds showed slower germination rate. The seeds started to germinate after day 7. The highest percentage of seeds germination were 50% after 3 weeks of observation. The rate of the germinated seeds was lower when compared to horizontal cultured seeds, whereby the height observed was about 9.26 ± 0.65 cm. the leaf per explant is 1.46 ±0.380 cm and the roots formed at average of 9.40 ± 0.380 cm per plant.

Based on the results obtained, the 12 weeks of seeds germinated on MS media without plant growth regulators have the ability to induce *in vitro* flowering. The flowering of the *in vitro* plants germinated form the seeds are about 30% of the culture. Yellow flowers with 5 petals in normal condition were obtained. However, the size of the flowers produced were smaller when compared to the *ex vitro* plants. Figure 3.1 below shows the *in vitro* flowering of seeds germinated on MS media without plant growth hormone.

Thus, for subsequent experiments, the seeds were germinated horizontally in order to optimize the rate of germination.

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Table 3.1: Germination of aseptic seedlings of *Cucumis melo* L.

Positions of culture	Days	Percentage of seed germination	Height of plant (Mean±SE)	No. of leaf per explant (Mean±SE)	No. of root per explant (Mean±SE)	Observations
Horizontal	0	0	0	0	0	No growth
	7	100±0.00	10.12±0.38	0	10.70±0.32	2 cotyledons formed
	14	100±0.00	17.16±0.32	1.73±0.08	>20	Leaves formation observed
	21	100±0.00	20.28±0.35	3.73±0.14	>25	Leaves formation observed
Vertical	0	0	0	0	0	No growth
	7	0	0	0	0	No growth
	14	33.33±0.00	5.81±0.22	0	7.00±0.34	2 cotyledons formed
	21	50.00 ±0.00	9.26±0.65	1.46±0.65	9.40±0.80	Leaves formation observed

Figure 3.1: *In vitro* flowering of *Cucumis melo* L. after 12 weeks of seeds germination on MS media without hormone.

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3.3.2 Direct regeneration of *Cucumis melo* L. using cotyledonary petioles as explant

In this study, for the plant regeneration, the explants were obtained from aseptic germinated seeds which were grown on MS basal medium. Subsequently, the cotyledonary petioles of the plants were used as the explant source for *in vitro* direct regeneration studies. Based on the results obtained, the highest shoot regeneration frequency was obtained from MS media supplemented with no hormone where all of the cultures showed 100% of shoots and roots regeneration frequencies. The regenerated shoots and roots were observed in normal conditions. For the treatments tested, the best shoot and roots regeneration frequencies were observed on MS media supplemented with 1mg/L 2,4-D with the combination of 25% of coconut water followed by 1mg/L 2,4-D with combination of 35 % of coconut water. The least responsive for *in vitro* regeneration was observed on MS media supplemented with 3 mg/L 2,4-D with 35 % of coconut water.

On MS media supplemented with 1mg/L 2,4-D with the combination of 25% of coconut water and MS media supplemented with 1mg/L 2,4-D with combination of 45 % of coconut water normal shoots and roots formation observed. Normal shoots exhibited by thin stems formation with the growth of leaves and normal roots exhibited with thin, fibrous and white colour. It was observed, when higher concentration of 2,4-D and coconut water supplied, the abnormal growth of shoots and roots obtained. The shoots formation with big stem and the roots formed was less fibrous, short and thicker from usual.

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Table 3.2: The effects of coconut water and 2,4-D on direct regeneration of *Cucumis melo* L.

Treatment	Shoot regeneration percentage (%)	Root regeneration percentag e (%)	Observations
MS media	100.00	100.00	Normal shoots and roots formation
1 mg/L 2,4-D + 25% of Coconut water	100.00	66.67	Normal shoots formation Normal roots formation observed.
2 mg/L 2,4-D + 25% of Coconut water	50.00	0.00	Normal shoots formation No roots formation
3 mg/L 2,4-D + 25% of Coconut water	33.00	0.00	Shoots formation with big stem No roots formation
1 mg/L 2,4-D + 35 % of Coconut water	50.00	26.67	Normal shoots formation Normal roots formation
2 mg/L 2,4-D + 35 % of Coconut water	50.00	0.00	Abnormal shoots formation Less fibrous and thick roots formation
3 mg/L 2,4-D + 35 % of Coconut water	26.67	0.00	Shoots formation with big stem No root formation
1 mg/L 2,4-D + 45 % of Coconut water	50.00	50.00	Normal shoot formation Normal roots with white fibrous root formation
2 mg/L 2,4-D + 45 % of Coconut water	50.00	23.33	Normal shoot formation Less fibrous, short and thick roots
3 mg/L 2,4-D + 45 % of Coconut water	50.00	0.00	Abnormal shoot with swollen stem No root formation observed

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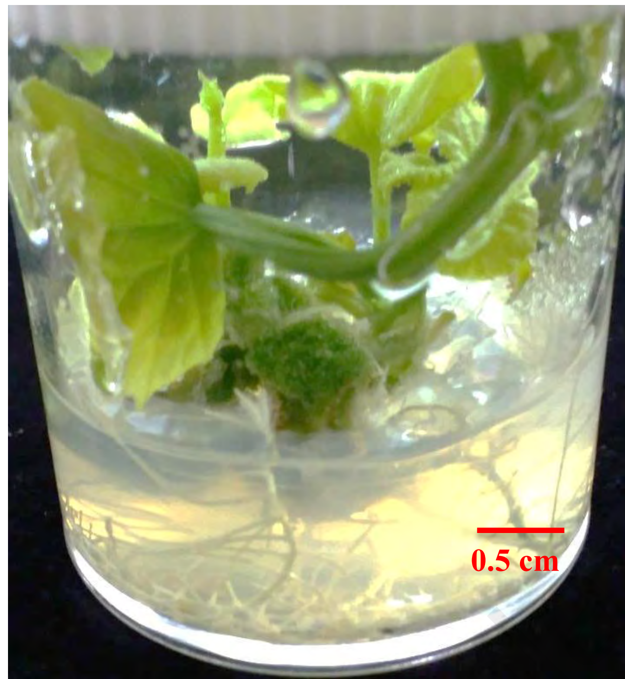

Figure 3.2: Normal *in vitro* regeneration of *Cucumis melo* L. from cotyledonary petiole explant on MS medium supplemented with 1 mg/L 2,4-D with 25% of Coconut water

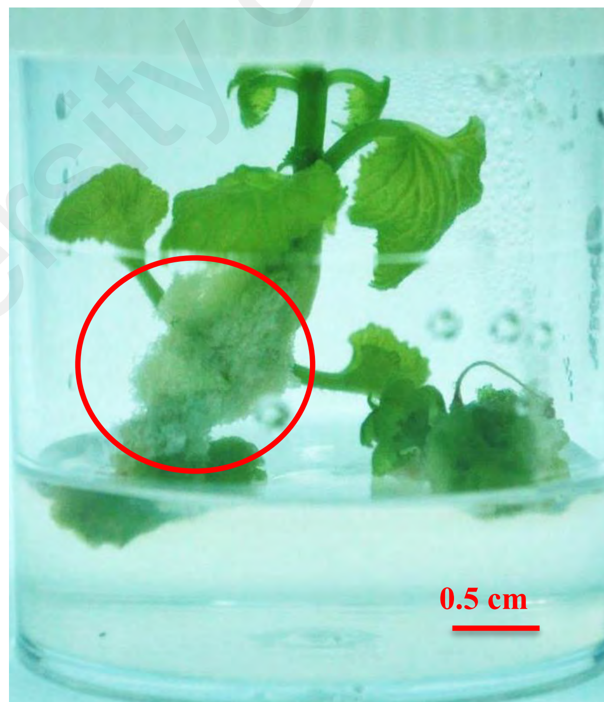

Figure 3.3: Abnormal shoots formation with swollen stem and no root formation observed from cotyledonary petiole on MS medium supplemented with 3 mg/L 2,4-D with 45% of Coconut water.

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3.4 Summary of Results

1. Seeds of *Cucumis melo* L. cultured horizontally on MS basal media were found able to be germinated in shorter time (3 days) when compared with vertical position of the seeds (8 days).
2. The germination rate of *Cucumis melo* L. seeds on MS basal medium was 100%.
3. Normal *in vitro* regeneration of *Cucumis melo* L. from cotyledonary petioles explant was successfully obtained from MS medium supplemented with 1mg/L 2,4-D with the combination of 25% of coconut water.
4. At higher concentrations of 2,4-D (2-3 mg/L) and coconut water (35-45%), abnormal growth of *in vitro* regeneration whereby the stem became swollen and the roots were less fibrous.

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CHAPTER 4: CALLUS INDUCTION FROM VARIOUS EXPLANTS OF *Cucumis melo* L.

4.1 Experimental Aims

The term ―callus" originated from the Latin word collum which means hard and in medicine, it refers to thickening of dermal tissues (Ikeuchi *et al*., 2013). Callus is an unorganized mass of undifferentiated plant cells (Hussain *et al*., 2012). Production of callus is a natural response of the plants cells towards various biotic and abiotic stimulations. The reaction of callus towards the wounds, infestation and grafting applied to the area will produce a single undifferentiated cell and callus have totipotent characteristic which means it is able to regenerate the whole plant body (Nagata and Takave, 1971).

Callus can be divided into few types, based on their morphological and macroscopic appeareance. As example, friable or compact, coloured callus, callus with no organ regeneration observed or callus with regeneration organ like rooty, shooty, or also known as embryogenic callus.

In tissue culture, callus induction can be generated artificially using balanced plant hormones, like cytokinin and auxin. All types of explants can be used for callus induction. Types of explants include leaves, stems, shoots, roots and many more. However, different types of explants give different reactions. There are many studies been done using various parts of explants as starting materials.

The use of hormones also gives significant reaction in callus induction. There are five main classes of hormones which are auxins, cytokinins, gibberalins, abscicic acid and ethylene. Auxins main function is to promote cell division and cell growth.

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Example of auxin are, IAA, and 2,4-D. Cytokinin plays a role in promoting cell division and examples of most commonly used cytokinins are BAP and Kinetin.

When explants cultured on medium with combination of auxin and cytokinin, it can give rise to an unorganized growing and dividing mass cell called callus. In order to develop the shoots and roots from embryogenic callus, the manipulation of auxin and cytokinin ratio is required.

In this chapter, the main aim of the experiment was to investigate the potential of different explants to form callus when different plant growth regulators were used.

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4.2 Materials and Methods

4.2.1 Source and types of explants

Source of explants were obtained from aseptic seedlings germinated on MS basal media. The best seed germinated was on MS media without plant growth regulators placed in horizontal position. Seven to 10-day-old plants germinated from seeds were used. The explants used were hypocotyls, cotyledons, nodes and leaf.

4.2.2 Germination of seeds on MS basal medium

The seeds were sterilized (Section 3.2.2) and germinated on MS basal medium with no plant growth regulators. The seeds were cultured in horizontal position in order to obtain the best seed germination. The cultures were incubated in the dark for 3-4 days. After day 4, the germinated seeds were maintained in the culture room at 25 ± 1°C with 16 hours light and 8 hours dark with light intensity of 1000 lux.

4.2.3 Preparation of explants

In the present study, explants were obtained from aseptic seedlings in order to minimize contamination. Young aseptic seedlings were utilized as explants source after 7-10 days of culture. The explants used were hypocotyls, nodes, cotyledons, and leaves from *in vitro* germinated seeds. Hypocotyls and nodes were cut into 10 mm long and cotyledons and leaves were cut into 10 mm x 10 mm square.

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4.2.4 Preparation of culture media with hormones for callus induction

Preparation of basic culture media was done as mentioned previously (Section 3.2.7). However, the difference of the way it was prepared was the addition of the PGRs. Prior to autoclave, hormones were added at desired concentration into the media. Then, the mixture were stirred and dissolved completely.

The explants prepared were cultured on MS medium with 3% sucrose and 2.8g gel-rite. Different concentration of BAP, 2,4-D, and combination of BAP and 2,4-D at the range of concentration of 1-3 mg/L were added in the culture medium. In this experiment, the concentrations and combinations of BAP, 2,4-D used were as follows:

11. MS media without hormones
12. MS supplemented with 1 mg/L BAP
13. MS supplemented with 2 mg/L BAP
14. MS supplemented with 3 mg/L BAP
15. MS supplemented with 1 mg/L 2,4-D
16. MS supplemented with 2 mg/L 2,4-D
17. MS supplemented with 3 mg/L 2,4-D
18. MS supplemented with 1 mg/L BAP + 1 mg/L 2,4-D
19. MS supplemented with 1 mg/L BAP + 2 mg/L 2,4-D
20. MS supplemented with 1 mg/L BAP + 3 mg/L 2,4-D
21. MS supplemented with 2 mg/L BAP + 1 mg/L 2,4-D
22. MS supplemented with 2 mg/L BAP + 2 mg/L 2,4-D
23. MS supplemented with 2 mg/L BAP + 3 mg/L 2,4-D
24. MS supplemented with 3 mg/L BAP + 1 mg/L 2,4-D
25. MS supplemented with 3 mg/L BAP + 2 mg/L 2,4-D
26. MS supplemented with 3 mg/L BAP + 3 mg/L 2,4-D

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Thirty replicates of explants were used in each treatment. All cultures were maintained in the culture room at 25 ±1 °C and 16 hours light and 8 hours dark with light intensity of 1000 lux. Callus induction percentage and rate of callus proliferation were obtained and recorded.

4.2.5 Determination of callus proliferation rate.

The callus proliferation rate was determined by number form 0, 1, 2, 3 and 4. Table below shows the rates of callus induced based on the numbers indicated.

Table 4.1: Numbers indicated the proliferation rate of callus

Numbers	Callus proliferation response
0	No callus induced at all
1	Little callus induced
2	Callus induced at the margin and the injured areas of the explants
3	Callus induced covering the explants
4	Callus induced covering the surface of media

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4.2.6 Sterilization of media and apparatus

All of the media and apparatus to be used in the tissue culture must be sterile. All of the apparatus like scalpel, forceps, tissues, jam jars, conical flasks, blades and others were washed with soap, Dettol and Clorox. The clean apparatus were dried off and then wrapped in aluminium foil and sealed with tape and sterilized. The wrapped apparatus were autoclaved at 121 °C (15 psi) for 20 minutes.

4.2.7 Data analysis

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

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4.3 Results

4.3.1 Callus induction from different explants.

Callus inductions from various explants were examined in order to achieve the optimum source for callus formation for subsequent experiments. There are four types of explants namely hypocotyls, cotyledons, leaf and nodes in the present study. The explants were cut into desired size (10 mm x 10 mm) and cultured on MS basal media supplemented with BAP and 2,4-D at various concentrations as stated in section 4.2.4. The screening of the best explants were done in order to obtain the best explants and the most responsive for callus induction.

Based on the present observation, all explants tested produced callus except for leaf explant and explants cultured on MS basal media The highest rate and the fastest of callus induction was achieved by hypocotyls explants with 100% of callus induction percentage at scale of 4 (started induced after day 7-9), followed by nodes explants which also showed 100 % of callus induction at scale of 3-4 (started to induced after day 9-12) and the cotyledons showed lower callus induction percentage which is 76.67% at the scale of 2-3. For leaf explants, it was observed that there was no callus induced and the response of the explants only showed that the explants were swollen after few days of cultured. The explants cultured on MS basal media showed no active growth and become necrotic after 7-8 weeks of cultured.

Different explant exhibited different types of callus. For hypocotyls and nodes, the callus induced was bright green and friable callus. The callus induced from cotyledon explants was yellow and green callus, friable and dry callus depending on the plant growth regulators supplied. Based on the results obtained, it showed that the best explant to be used for the subsequent experiments were hypocotyls and nodes.

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Table 4.2: Callus induction of *Cucumis melo* L. from different explants cultured on MS medium supplemented with different concentrations of BAP and 2,4-D at 25 ±1 °C under 16 hours light and 8 hours dark for 8 weeks.

Hormone (mg/L)		Explants	Percentage of callus induced (%)	Callus proliferation rate	Observations
BAP	2,4-D				
0	0	Hypocotyl	0	0	No response
		Cotyledon	0	0	No response
		Leaf	0	0	No response
		Node	0	0	No response
1	0	Hypocotyl	100	4	Bright green and friable callus were observed after day 7-9 of culture
		Cotyledon	100	2	Yellow and friable callus were observed after day 15 of culture
		Leaf	0	0	The explants were swollen, no callus induced
		Node	100	3	Bright green and friable callus were observed after day 9-12 of cultured
2	0	Hypocotyl	100	4	Bright green and friable callus were observed after day 7-9 of culture
		Cotyledon	100	2	Yellow callus, friable, non-embryogenic
		Leaf	0	0	The explants were swollen, no callus induced
		Node	100	3	Bright green and friable callus were observed after day 9-12 of culture
3	0	Hypocotyl	100	4	Bright green and friable callus were observed after day 7-9 of culture
		Cotyledon	100	2	Yellow callus, friable, non-embryogenic

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		Leaf	0	0	The explants were swollen, no callus induced
		Node	100	3	Bright green and friable callus were observed after day 9-12 of culture
0	1	Hypocotyl	100	3	Bright green and friable callus were observed after day 7-9 of culture
		Cotyledon	30	1	Bright green callus, friable, non- embryogenic
		Leaf	0	0	No response
		Node	100	3	Bright green and friable callus were observed after day 9-12 of culture
0	2	Hypocotyl	100	3	Bright green and friable callus were observed after day 7-9 of culture
		Cotyledon	50	1	Bright green callus, friable, non- embryogenic
		Leaf	0	0	No response
		Node	100	3	Bright green and friable callus were observed after day 9-12 of culture
0	3	Hypocotyl	100	3	Bright green and friable callus were observed after day 7-9 of culture
		Cotyledon	50	1	Bright green callus, friable, non- embryogenic
		Leaf	0	0	No response
		Node	100	3	Bright green and friable callus were observed after day 9-12 of culture
1	1	Hypocotyl	100	4	Bright green and friable callus were observed after day 7-9 of culture
		Cotyledon	26.67	2	Bright green callus, friable,non-embryogenic

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		Leaf	0	0	The explants were swollen, no callus induced
		Node	100	4	Bright green and friable callus were observed after day 9-12 of culture
1	2	Hypocotyl	100	4	Bright green and friable callus were observed after day 7-9 of culture
		Cotyledon	43.33	2	Bright green callus, friable,non-embryogenic
		Leaf	0	0	The explants were swollen, no callus induced
		Node	100	4	Bright green and friable callus were observed after day 9-12 of culture
1	3	Hypocotyl	100	4	Bright green and friable callus were observed after day 7-9 of culture
		Cotyledon	50	2	Bright green callus, friable,non-embryogenic
		Leaf	0	0	The explants were swollen, no callus induced
		Node	100	4	Bright green and friable callus were observed after day 9-12 of culture
2	1	Hypocotyl	100	4	Bright green and friable callus were observed after day 7-9 of culture
		Cotyledon	60	3	Bright green callus, friable,non-embryogenic
		Leaf	0	0	The explants were swollen, no callus induced
		Node	100	4	Bright green and friable callus were observed after day 9-12 of culture
2	2	Hypocotyl	100	4	Bright green and friable callus were observed after day 7-9 of culture

		Cotyledon	60	3	Bright green callus, friable, non- embryogenic
		Leaf	0	0	The explants were swollen, no callus induced
		Nodes	100	4	Bright green and friable callus were observed after day 9-12 of culture
2	3	Hypocotyls	100	4	Bright green and friable callus were observed after day 7-9 of culture
		Cotyledon	60	3	Bright green callus, friable,non-embryogenic
		Leaf	0	0	The explants were swollen, no callus induced
		Node	100	4	Bright green and friable callus were observed after day 9-12 of culture
3	1	Hypocotyl	100	4	Bright green and friable callus were observed after day 7-9 of culture
		Cotyledon	60	3	Bright green callus, friable, non- embryogenic
		Leaf	0	0	The explants were swollen, no callus induced
		Node	100	4	Bright green and friable callus were observed after day 9-12 of culture
3	2	Hypocotyl	100	4	Bright green and friable callus were observed after day 7-9 of culture
		Cotyledon	50	3	Bright green callus, friable, non- embryogenic
		Leaf	0	0	The explants were swollen, no callus induced
		Node	100	4	Bright green and friable callus were observed after day 9-12 of culture

3	3	Hypocotyl	100	4	Bright green and friable callus were observed after day 7-9 of culture
		Cotyledon	46.67	3	Bright green callus, friable, non- embryogenic
		Leaf	0	0	The explants were swollen, no callus induced
		Node	100	3	Bright green and friable callus were observed after day 9-12 of culture

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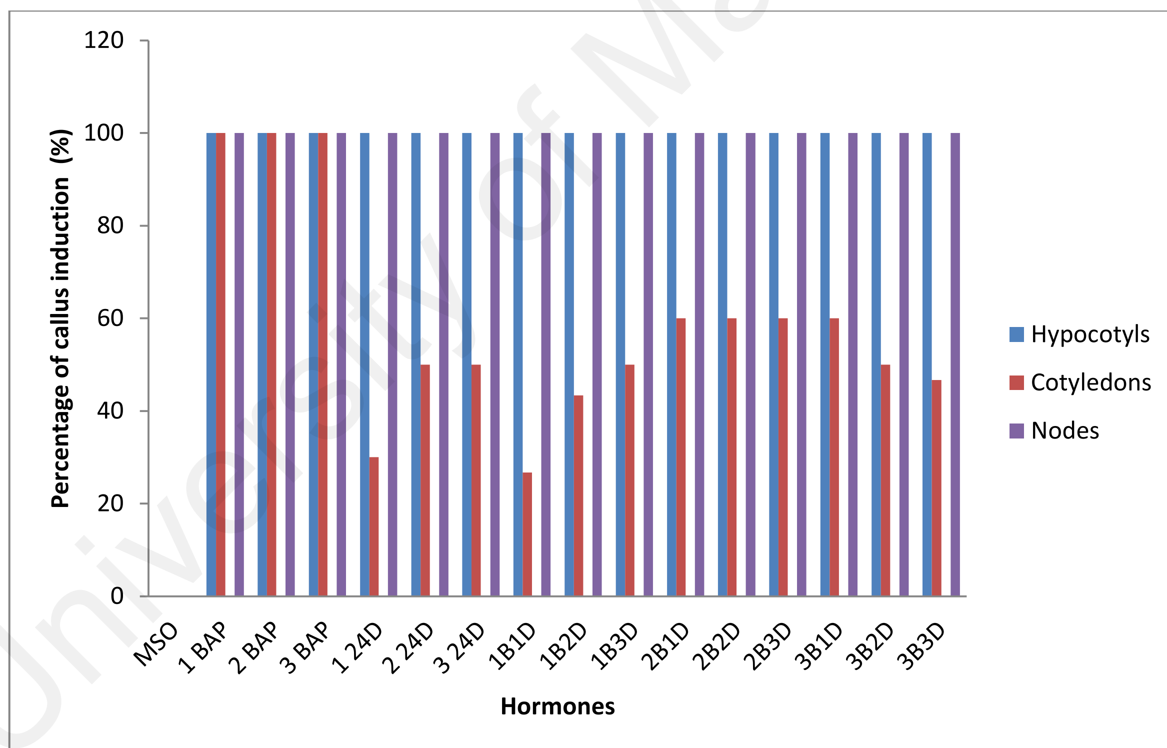

Figure 4.1: Effects of BAP and 2,4-D on callus induction from hypocotyls, cotyledons, and nodes explants.

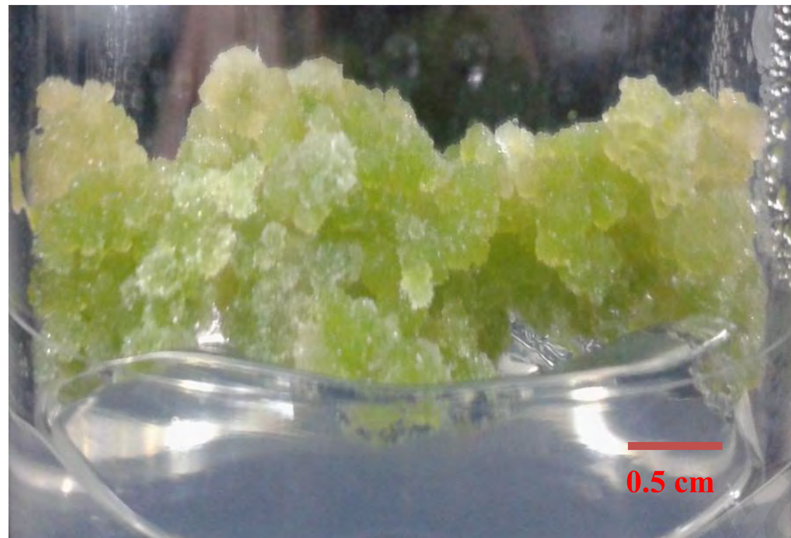

Figure 4.2: Bright green callus derived from hypocotyl explant cultured on MS media supplemented with 1mg/L BAP showing callus induced covering the media surface.

Figure 4.3: Yellow callus derived from cotyledon explant cultured on MS media supplemented with 1mg/L BAP showing callus induced at the margin of the injured area.

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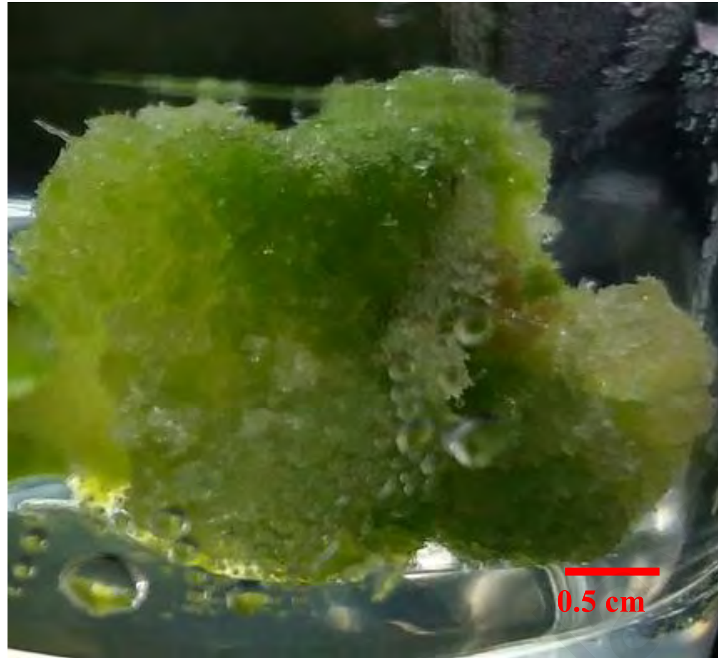

Figure 4.4: Callus derived from node explant cultured on MS media supplemented with 1 mg/L BAP showing the callus induced covering the explant.

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4.4 Summary of Results

5. All types of explants were able to induce callus except leaf explants.
6. The highest callus induction frequencies were observed from hypocotyls explants followed by nodes explants and the least was cotyledon.
7. The callus induced from hypocotyls and nodes were highly proliferated at scale of 4, where callus covering the surface of media.
8. Leaf explants induced no callus. The response of the explants showed that the explants were only swollen.
9. There was no callus induction observed on MS basal media.
10. The earliest callus induced from hypocotyls explant (7-9 days) followed by nodes explants (9-12 days).

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CHAPTER 5: EFFECTS OF DIFFERENT HORMONES ON CALLUS INDUCTION, SOMATIC EMBRYOGENESIS AND REGENERATION OF *Cucumis melo* L.

5.1 Experimental Aims

Somatic embryogenesis is a process resembling a zygotic embryo formation, whereby the speciality of this process is the cells that develop and differentiate into somatic embryos are from somatic cells. The somatic embryos developed have non zygotic bipolar structures obtained from somatic cells (Hussain *et al*., 2012). Somatic embryogenesis occured due to evolution of plants that required them to fulfil the needs to adapt and overcome the environment and genetic factors that prevent conventional fertilization (Arnold *et al*., 2002). There are many advantages of somatic embryo because they are playing roles in the practical applications in large scale of propagation, capable of cryopreservation for gene bank establishment, and also a method for the study and improving the genetic modification.

There are few factors that influence the induction and the formation of somatic embryogenesis which are the types of plant growth regulators supplemented in MS basal media, types of explants used, environmental factors and many others. In plant tissue culture studies, the early focus on inducing somatic embryogenic callus was done on the addition of auxin or cytokinins or combination of both types of plant growth regulators in MS media other than types of explants.

In this experiment, the effects of different plant growth regulators or hormones on somatic embryogenesis from hypocotyl and node explants were determined. In order to determine the embryogenic and non-embryogenic callus, double staining method was applied.

5.2 Materials and Methods

5.2.1 Germination of seeds on MS basal medium

The seeds were sterilized (section3.2.2) and germinated on MS basal medium with no plant growth regulators. The seeds were cultured in horizontal position in order to obtain the best seed germination. The cultures were incubated in the dark for three to four days. After day 4, the germinated seeds were mainatained in the culture room at 24 ± 1°C with 16 hours light and 8 hours dark and light intensity of 1000 lux.

5.2.2 Preparation of explants

In the current study, the highly responsive explants from the screening of explants in the previous chapter were used. The hypocotyls and nodes explants were used in this study. The young aseptic seedlings from seeds germinated on MS without hormone (4.2.1) were utilized as explant source after 7-10 days in culture. The hypocotyls and nodes were cut into 10 mm long and the cotyledons and leaves were cut into 10 mm x 10 mm square and cultured horizontally on the media.

5.2.3 Preparation of culture media with hormones for callus induction

In this study, solid MS media was used for callus induction. Solid media was prepared by adding 800 ml distilled water in 1000 ml conical flask. Sugar (30 g) was added to distilled water and stirred with magnetic stirrer. Then, 4.4 g of MS including vitamin was added and stirred until the solution became evenly dissolve .The pH of the medium then adjusted to 5.8 using 1.0 N Hydrochloric acid (HCl) or 1.0 N Sodium hydroxide (NaOH). After that, gelrite that was weighed at 2.8 g then added to the media. The gel-rite acts as a solidifying agent to provide surface and support for the plants growth. Lastly, the volume of the medium was filled up with distilled water to 1 L using graduated cylinder. Mixture was stirred and heat was applied to dissolve the

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medium completely. The medium was heated until the colour of the medium became transparent. Various concentrations of BAP, 2,4-D, IAA and Kinetin were pipetted into the culture medium. Below is the list of media used in this study.

27. MS media without hormones
28. MS media supplemented with 1 mg/L BAP
29. MS media supplemented with 2 mg/L BAP
30. MS media supplemented with 3 mg/L BAP
31. MS media supplemented with 1 mg/L 2,4-D
32. MS media supplemented with 2 mg/L 2,4-D
33. MS media supplemented with 3 mg/L 2,4-D
34. MS media supplemented with 1 mg/L BAP + 1 mg/L 2,4-D
35. MS media supplemented with 1 mg/L BAP + 2 mg/L 2,4-D
36. MS media supplemented with 1 mg/L BAP + 3 mg/L 2,4-D
37. MS media supplemented with 2 mg/L BAP + 1 mg/L 2,4-D
38. MS media supplemented with 2 mg/L BAP + 2 mg/L 2,4-D
39. MS media supplemented with 2 mg/L BAP + 3 mg/L 2,4-D
40. MS media supplemented with 3 mg/L BAP + 1 mg/L 2,4-D
41. MS media supplemented with 3 mg/L BAP + 2 mg/L 2,4-D
42. MS media supplemented with 3 mg/L BAP + 3 mg/L 2,4-D
43. MS media supplemented with 1 mg/L IAA
44. MS media supplemented with 2 mg/L IAA
45. MS media supplemented with 3 mg/L IAA
46. MS media supplemented with 1 mg/L Kinetin
47. MS media supplemented with 2 mg/L Kinetin
48. MS media supplemented with 3 mg/L Kinetin
49. MS media supplemented with 1 mg/L IAA + 1 mg/L Kinetin

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50. MS media supplemented with 1 mg/L IAA + 2 mg/L Kinetin
51. MS media supplemented with 1 mg/L IAA + 3 mg/L Kinetin
52. MS media supplemented with 2 mg/L IAA + 1 mg/L Kinetin
53. MS media supplemented with 2 mg/L IAA + 2 mg/L Kinetin
54. MS media supplemented with 2 mg/L IAA + 3 mg/L Kinetin
55. MS media supplemented with 3 mg/L IAA + 1 mg/L Kinetin
56. MS media supplemented with 3 mg/L IAA + 2 mg/L Kinetin
57. MS media supplemented with 3 mg/L IAA + 3 mg/L Kinetin
58. MS media + 0.1M of mannitol
59. MS media + 0.2M of mannitol
60. MS media + 0.3M of mannitol
61. MS media + 0.1M of mannitol supplemented with 1 mg/L BAP
62. MS media + 0.1M of mannitol supplemented with 2 mg/L BAP
63. MS media + 0.1M of mannitol supplemented with 3 mg/L BAP
64. MS media + 0.2 M of mannitol supplemented with 1 mg/L BAP
65. MS media + 0.2 M of mannitol supplemented with 2 mg/L BAP
66. MS media + 0.2 M of mannitol supplemented with 3 mg/L BAP
67. MS media + 0.3 M of mannitol supplemented with 1 mg/L BAP
68. MS media + 0.3 M of mannitol supplemented with 2 mg/L BAP
69. MS media + 0.3 M of mannitol supplemented with 3 mg/L BAP

The media were autoclaved at 121 °C (15 psi) for 20 minutes. After autoclaved, MS media were allowed to cool over to 50°C and then were dispensed into 60 ml sterile containers at optimum volume, in the laminar flow in order to maintain the aseptic condition.

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Thirty replicates of explants were used in each treatment. All cultures were maintained in the culture room at at 24 ±1 °C and 16 hours light and 8 hours dark with light intensity of 1000 lux. Callus induction frequencies and scale of callus production were obtained and recorded.

5.2.4 Identification of embryogenic callus

There are two types of callus which are non embryogenic and embryogenic callus. Non embryogenic callus are the callus induced but give no regeneration to new plantlets while embryogenic callus developed embryos and has the ability to mature, germinate and regenerate into new plantlets. The non embryogenic and embryogenic calluses were identified by using ―double staining" method as established by Gupta and Durzan, (1987). The concept of the double staining is the first dye which is acetocarmine will stain the embryogenic cells with dense cytoplasm and large nuclei that develop embryos. The Evan's blue will stained the suspensor cells. Then, the stained callus were observed under microscope with different magnification. The double staining preparation was done as follows.

5.2.4.1 Preparation of 2% Acetocarmine.

Approximately 55 ml of distilled water was measured and poured into a beaker containing a stirrer bar. Using a pipette, 45 ml of glacial acetic acid was pipetted into the beaker containing the distilled water. This produced 45% of acid solution. The solution was boiled gently for 5 minutes on highest setting , stirred evenly with the stirrer bar , cooled down and then filtered using Whatman filter paper. The prepared solution was stored at room temperature.

5.2.4.2 Preparation of 0.5% Evan's Blue.

Distilled water (100 ml) was measured and poured into 250 ml flask containing stirrer bar and 0.5 g of Evan's Blue was added to the 100 ml of distilled water measure previously. The solution was stirred to mix properly. After the solution mixed well, the solution was stored at room temperature.

5.2.5 Double staining method

A small piece of callus about (1-2 mm) was placed on a clean glass slide. A few drops of acetocarmine was added until all callus was submerged. The callus was gently divided with forceps and needle into very small pieces in the acetocarmine solution. The specimens then heated gently on a hot plate without boiling it. The specimens were heated until the acetocarmine dried and the callus on the specimen sticked to the glass slide. The specimen then washed for 2-3 times with slow distilled water to remove all of the excess acetocarmine liquid. Then, 2 drops of 0.5% of Evan's Blue were added to acetocarmine stained cells. After 30 seconds, the slides were gently washed 2-3 times with slow running distilled water to wash the excess Evan's Blue solution. The excess water was removed by blotted dry the specimen. One to two drops of glycerol were added to the specimen and in order to prevent the stained cells from drying. The slides were observed under microscope and the embryogenic and non embryogenic callus were identified by observing the cells, weather blue or red.

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5.2.6 Regeneration of plantlets from somatic embryos

Shoots produced from the somatic embryos were transferred to MS basal medium for further shoot and root development. All cultures were incubated in the culture room at 24 ± 1°C with 16 hours light and 8 hours dark.

5.2.7 Data analysis

Data obtained were analyzed using Duncan‘s Multiple Range test (DMRT). Mean with different letters in the same column differ significantly at p= 0.05

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5.3 Results

5.3.1 Callus induction and identification of embryogenic callus

All MS media supplemented with the plant growth regulators mentioned above induced callus. After 7-10 days, callus induction was observed at the margin and the wound area of the explants. The callus proliferated very well in week 3 and 4. Based on the results observed, all of the media induced callus except for MS basal media where no callus induction was observed. Generally, the rate of callus proliferation response on MS media supplemented with BAP, 24-D and combinations of BAP and 2,4-D, IAA, Kinetin and IAA and combinations of IAA and Kinetin indicated with number 4 which means the callus proliferation in high rate and covering the surface of media. In MS media supplemented with 2,4-D, the rate of callus proliferation observed were lower which was considered number 3. Number 3 indicated the callus covering the explants, without covering the media surface.

Different MS media supplemented with different plant growth regulators induced different types of callus. In MS media supplemented with BAP, 2,4-D and combinations of both induced bright green, friable and watery callus for both explants. MS media supplemented with IAA, Kinetin and combinations of both plant growth regulators induced green, compact and drier callus for both explants when compared to the callus induced on MS media supplemented with BAP and 2,4-D.

Node and hypocotyl explants induced callus on all MS media supplemented with plant growth regulators tested. Both explants exhibited somatic embryos with morphological characteristics showing some at globular, heart and torpedo shaped of somatic embryogenesis.

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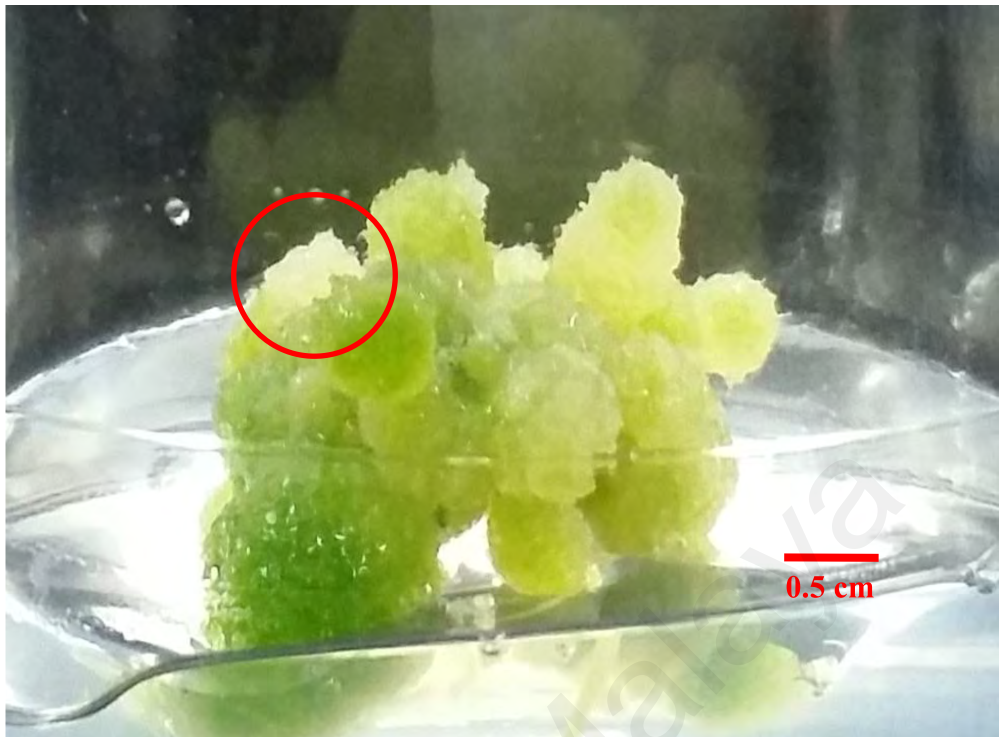

Figure 5.1: Callus with globular stage of somatic embryos.

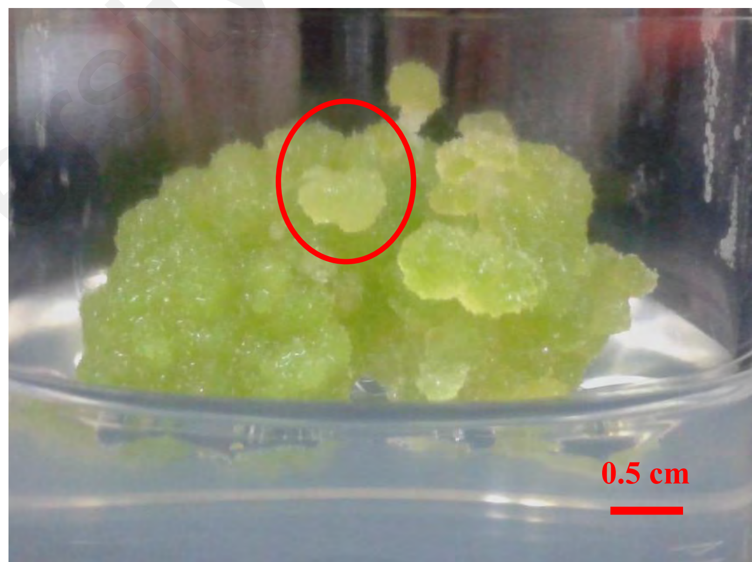

Figure 5.2: Callus with heart shaped stage of somatic embryos.

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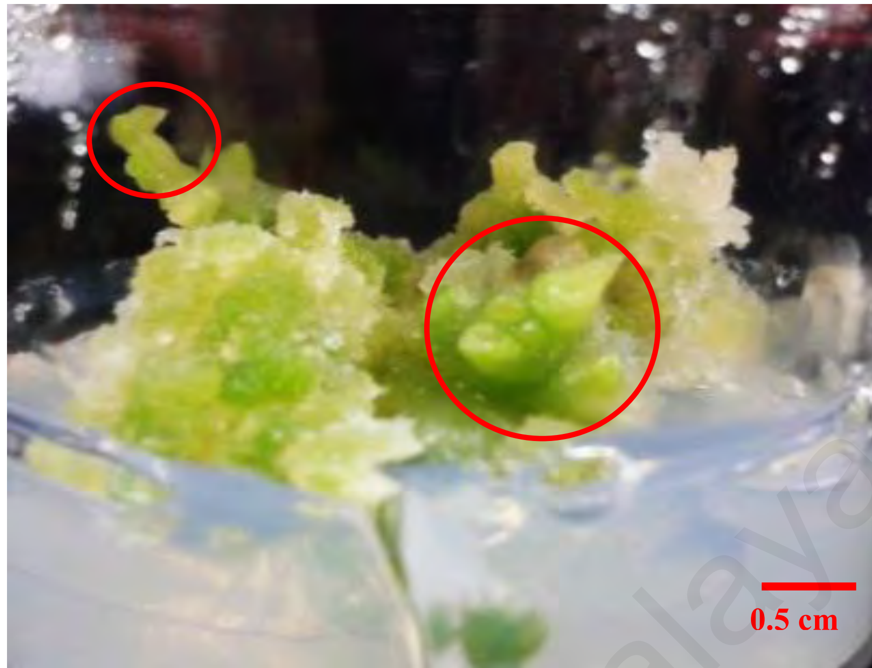

Figure 5.3: Callus with torpedo-shape and cotyledonary stage of somatic embryos

Other than morphological shape characteristic of the callus, the double staining also showed that callus induced on MS media supplemented with all of the plant growth regulators from node explants were embryogenic callus. The double staining method showed the oval cells were stained in red. However, for callus induced from hypocotyls explants on MS media supplemented with BAP, 2,4-D and combinations of both gave negative results from double staining method. The cells were stained with blue colour indicated that there were no embryogenic cells observed. Figures (5.5 nd 5.6) shows the cell of callus stained by double staining method.

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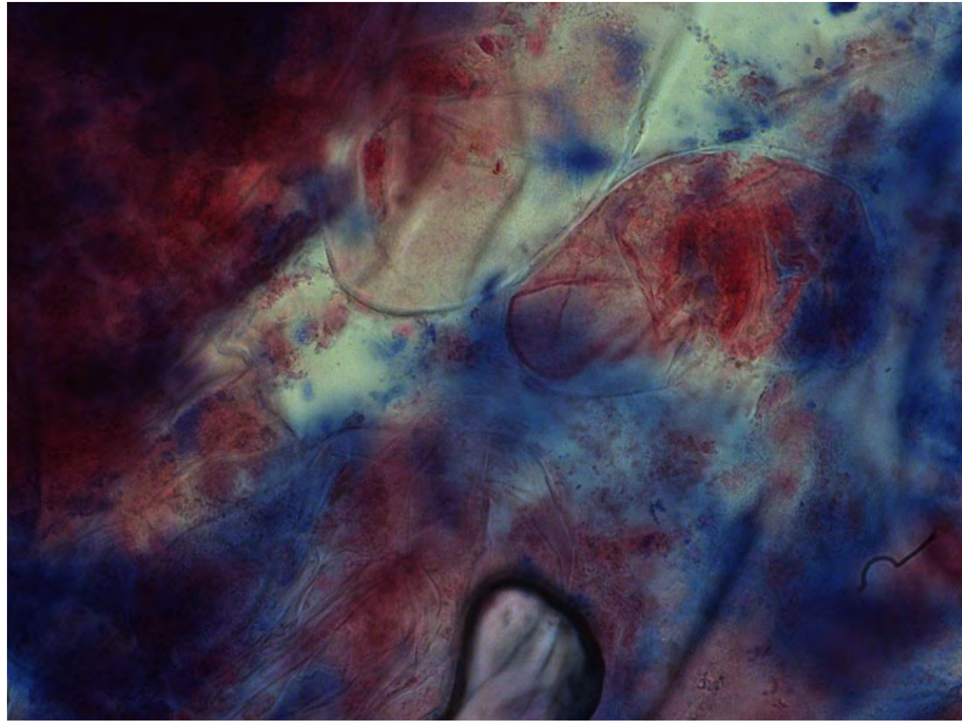

Figure 5.4: Non embryogenic callus stained in blue and embryogenic callus stained in red under 40 x magnification

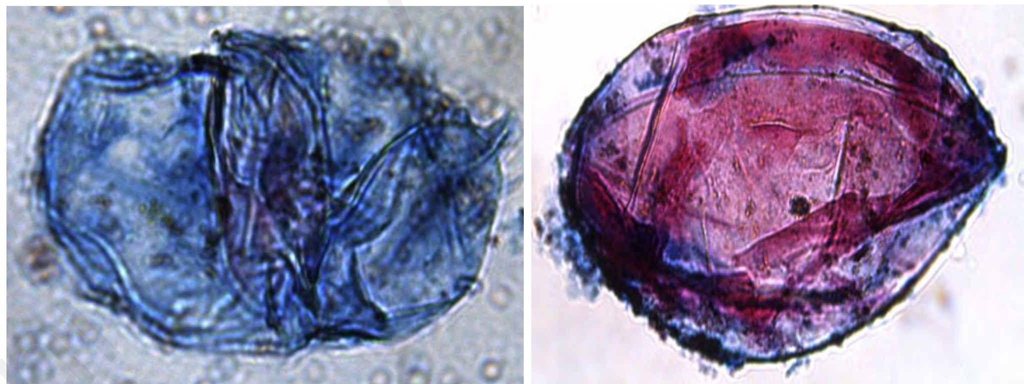

Figure 5.5: Single cell stained in blue and single cell stained in red under 100 x magnification

For hypocotyl explants, MS media supplemented with BAP, 2,4-D and combinations of both induced callus with no shoot or root formation. The highest fresh weight of the callus was observed from MS media supplemented with 1 mg/L of BAP in combination with 1mg/L 2,4-D, where callus fresh weight was 7.53 ± 0.18 g. This was followed by MS media supplemented with1 mg/L BAP where the callus fresh weight was 5.93 ±

0.47 g. The lowest callus fresh weight was from MS media supplemented with 3.0 mg/L of 2,4-D where the callus weight was 0.34 ± 0. 07 g.

Figure 5.6: Green callus on MS media supplemented with 1 mg/L BAP and 1mg/L 2,4-D

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However, hypocotyl explants cultured on MS media supplemented with IAA, Kinetin and combinations of both induced callus with roots. There was no shoot formation observed from hypocotyl explants in this media. The white fibrous and soft roots were induced from the callus. The highest percentage of root formation (86.67%) was observed from the callus induced on MS media supplemented with 3.0 mg/L IAA. This was followed by MS media supplemented with 1.0 mg/L IAA with the percentage of root formation of 80.00%. The lowest root production was observed from the callus induced on MS media with 1.0 mg/L IAA with combination of 3.0 mg/L Kinetin with the percentage of callus inducing roots were only 10.00%.

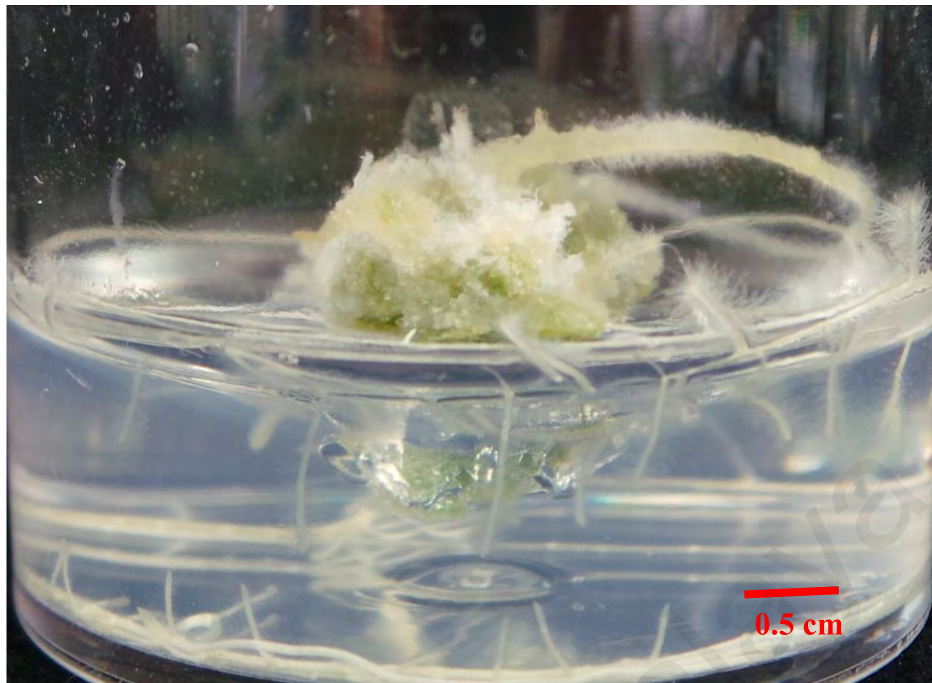

Figure 5.7: Callus with root formation on MS media supplemented with 3 mg/L IAA

After 8 to 10 weeks of culture, shoots formation was observed from node explants. Based on the results obtained, the highest shoot formation was from MS media supplemented with 2 mg/L BAP in combination with 1 mg/L 2,4-D (100.00 % with rate of 4 callus proliferation), with the average rate of the shoot formation of 13.10 ± 0.36 per explant, followed by MS media supplemented with 1 mg/L of BAP in combination with 3 mg/L of 2,4-D (100.00 % with rate of 4 callus proliferation), with the average rate of the shoot formation of 9.57 ±0.22 per explant. The lowest shoot formation was observed on the MS media supplemented with solely 3 mg/L of 2,4-D (100.00 % of callus formation with rate of 3 callus proliferation), with the average rate of 0.34 ± 0.07 per explant.

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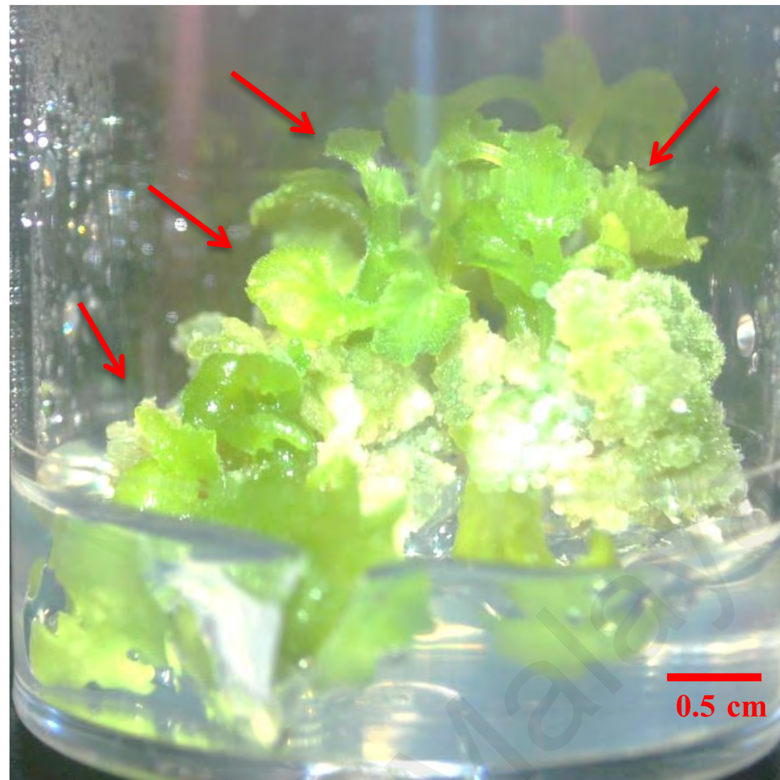

Figure 5.8: Multiple shoots formation on node explants cultured on MS media supplemented with 2mg/L BAP with 1 mg/L 2,4-D

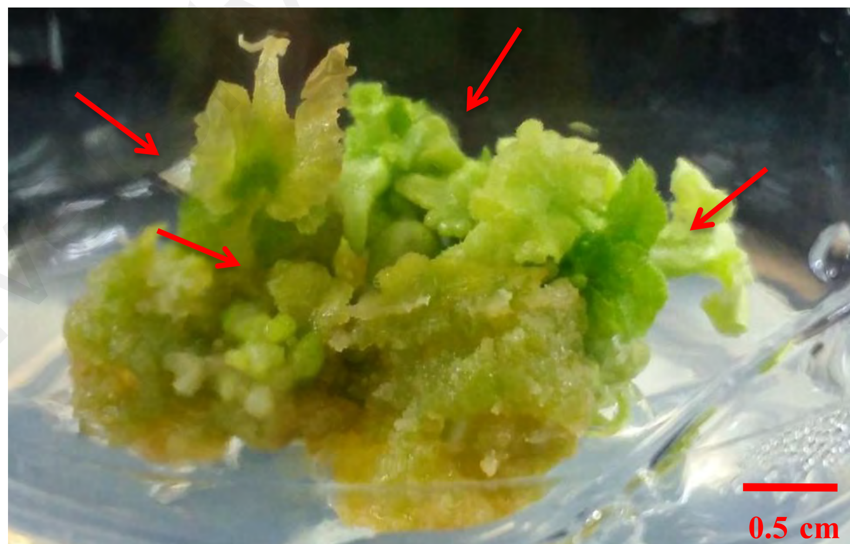

Figure 5.9: Multiple shoots formation on node explants cultured on MS media supplemented with 1 mg/L BAP with 3 mg/L 2,4-D

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Hypocotyls and nodes cultured on MS media supplemented with Mannitol (0.1 - 0.3 M) successfully induced callus. The callus induced was dark green, very compact and dry callus. Callus induction in MS media supplemented with Mannitol induced later which means, it required longer induction time. Based on the observation, the callus induction in MS media supplemented with Mannitol were very slow. It took about 8-10 weeks to be induced. The callus induced showed there were shoot formation after 14-16 weeks of culture. The highest shoot formation was observed on MS media supplemented with 0.1M mannitol with 76.67 % of callus inducing shoot with nodes as explants, followed by MS media supplemented with 0.1 M mannitol with 60.00 % of callus inducing shoots with hypocotyls as explants. MS media supplemented with mannitol in combination with BAP induced callus with no shoots formation. The subcultures were done at 8 weeks interval. There was no root formation observed from the callus induced.

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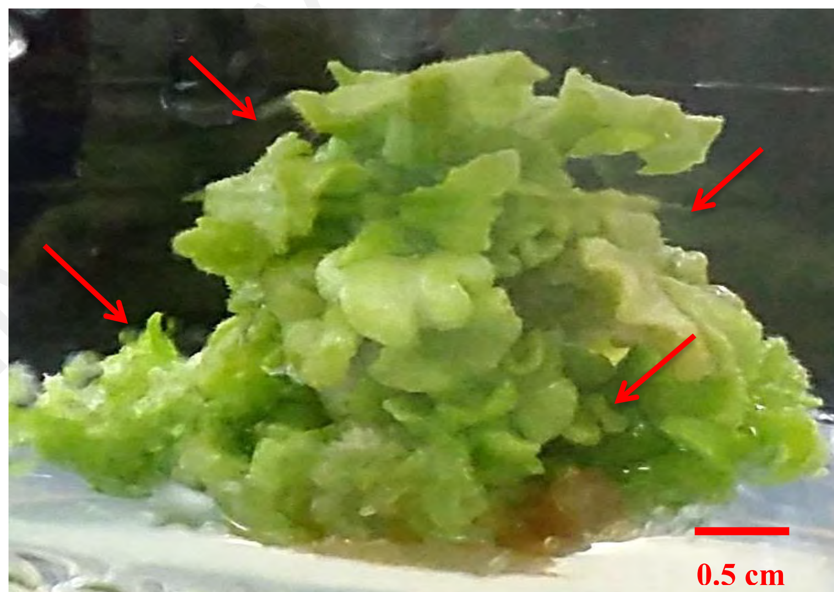

Figure 5.10: Multiple shoots from node explant cultured on MS media supplemented with 0.1 M Mannitol as additional carbon source.

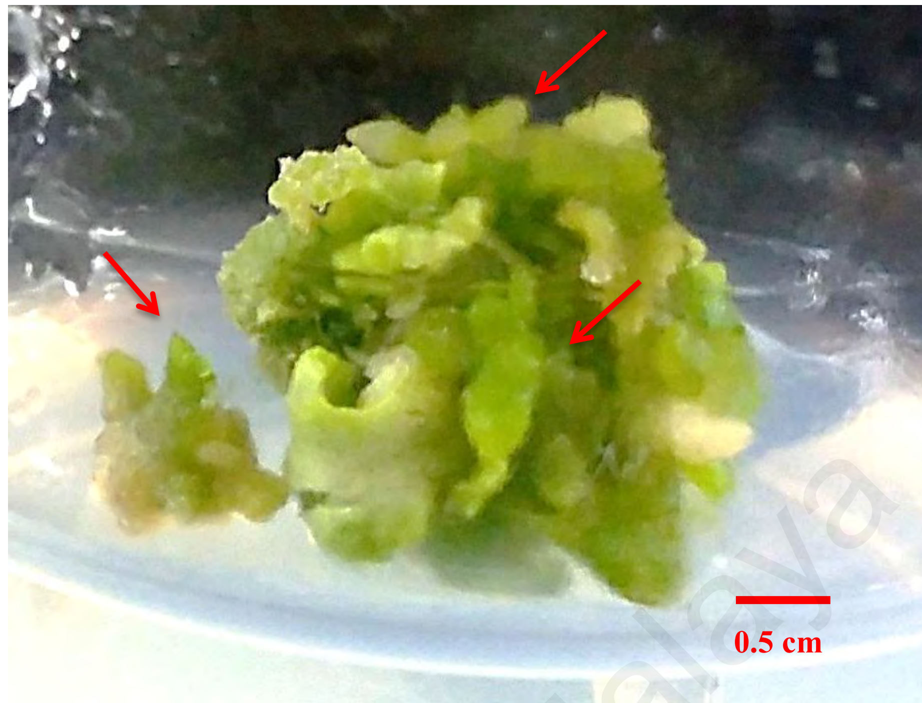

Figure 5.11: Multiple shoots from hypocotyl explant cultured on MS media supplemented with 0.1 M Mannitol as additional carbon source.

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Table 5.1: Callus induction of *Cucumis melo* L. from hypocotyl explants on MS media supplemented with BAP (0-3 mg/L) and 2,4-D (0-3 mg/L) under 16 hours light for 4 weeks.

Hormone (mg/L)		Percentage of callus induced (%)	Callus proliferation score	Callus fresh weight (g) (Mean ± SE)	Observations
BAP	2,4-D				
0	0	NR	NR	0.00 ±0.00	There is no root or shoot induced from the hormones treatment. Friable, light green and soft callus were observed.
1	0	100.00	4	5.93 ± 0.47 [b]	
2		100.00	4	5.55 ± 0.29 [bc]	
3		100.00	4	4.53 ± 0.32 [e]	
0	1	100.00	3	1.22 ± 0.12 [g]	
	2	100.00	3	0.68 ± 0.04 [gh]	
	3	100.00	3	0.34 ± 0.07 [h]	
1	1	100.00	4	7.53 ± 0.18 [a]	
	2	100.00	4	5.20 ±0.34 [cd]	
	3	100.00	4	1.13 ± 0.12 [g]	
2	1	100.00	4	4.75 ± 0.15 [de]	
	2	100.00	4	3.54 ± 0.20 [f]	
	3	100.00	4	4.21 ± 0.19 [e]	
3	1	100.00	4	0.90 ±0.05 [gh]	
	2	100.00	4	0.94 ± 0.09 [gh]	
	3	100.00	4	0.62 ± 0.04 [gh]	

Mean ± SE, n=30. Mean with different letters in the same column differ significantly at p=0.05

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Table 5.2: Callus induction of *Cucumis melo* L. from hypocotyl explants on MS media supplemented with IAA (0-3 mg/L) and Kinetin (0-3 mg/L) under 16 hours light for 3 weeks.

Hormone (mg/L)		**Percentage of callus induced (%)**	**Callus proliferation score**	**Percentage of callus inducing shoots and roots (%)**		**Mean of roots induced per explant (Mean ±SE)**	**Observations**
IAA	**Kin**			**S**	**R**		
0	0	0.00	NR	NR	NR	0.0 0.00	
1	0	100.00	4	NR	80.00	3.63 ± 0.41^{d}	Friable, green and harder callus observed when compared to callus formed on MS media supplemented with BAP and 2,4-D.
2		100.00	4	NR	70.00	3.60 ± 0.65^{e}	
3		100.00	4	NR	86.67	4.20 ± 0.50^{e}	
0	1	100.00	4	NR	60.00	1.30 ± 0.25^{a}	
	2	100.00	4	NR	76.67	0.83 ± 0.10^{cd}	
	3	100.00	4	NR	43.33	0.87 ± 0.21^{b}	
1	1	100.00	4	NR	46.67	1.67 ± 0.39^{b}	
	2	100.00	4	NR	33.33	1.10 ± 0.27^{f}	There is no shoot induced from the hormones tested.
	3	100.00	4	NR	10.00	0.27 ± 0.15^{f}	
2	1	100.00	4	NR	33.33	1.67 ± 0.57^{c}	
	2	100.00	4	NR	13.33	1.27 ± 0.41^{f}	
	3	100.00	4	NR	23.33	0.57 ± 0.22^{b}	White and fibrous root formation was observed.
3	1	100.00	4	NR	33.33	2.83 ± 0.90^{cd}	
	2	100.00	4	NR	46.67	1.20 ± 0.41^{f}	
	3	100.00	4	NR	23.33	0.97 ± 0.38^{f}	

Mean ± SE, n=30. Mean with different letters in the same column differ significantly at p=0.05

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Table 5.3: Callus induction of *Cucumis melo* L. from node explants on MS media supplemented with BAP (0-3 mg/L) and 2,4-D (0-3 mg/L) under 16 hours light for 10 weeks.

Hormone (mg/L)		Percentage of callus induced (%)	Callus proliferation score	Percentage of callus inducing shoots and roots (%)		Mean/Number of shoots induced per explant (Mean ±SE)
BAP	**2,4-D**			**S**	**R**	
0	0	NR	NR	NR	NR	0.00 ± 0.00
1	0	100.00	3	100.00	NR	5.00 ±0.27 ^{de}
2		100.00	3	100.00	NR	4.53 ±0.10 ^{e}
3		100.00	3	100.00	NR	3.27 ±0.23 ^{f}
0	1	100.00	3	63.33	NR	1.00 ±0.17 ^{gh}
	2	100.00	3	50.00	NR	0.80 ±0.17 ^{h}
	3	100.00	3	0.00	NR	0.00 ±0.00 ^{i}
1	1	100.00	4	100.00	NR	8.50 ±0.21 ^{c}
	2	100.00	4	100.00	NR	5.67 ±0.19 ^{d}
	3	100.00	4	100.00	NR	9.57 ±0.22 ^{b}
2	1	100.00	4	100.00	NR	13.10 ±0.36 ^{a}
	2	100.00	4	56.67	NR	3.20 ±0.53 ^{f}
	3	100.00	4	100.00	NR	7.83 ±0.14 ^{c}
3	1	100.00	4	46.67	NR	1.63 ±0.37 ^{gh}
	2	100.00	4	40.00	NR	0.90 ±0.24 ^{gh}
	3	100.00	3	16.67	NR	0.40 ±0.17 ^{gh}

Mean ± SE, n=30. Mean with different letters in the same column differ significantly at p=0.05

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Table 5.4: Callus induction of *Cucumis melo* L. from node explants on MS media supplemented with IAA (1 - 3 mg/L) and Kinetin (1 - 3 mg/L) under 16 hours light for 10 weeks.

Hormone (mg/L)		Percentage of callus induced (%)	Callus proliferation score	Percentage of callus inducing shoots and roots (%)		Mean/Number of shoots induced per explant (Mean ±SE)
IAA	Kin			S	R	
0	0	NR	NR	NR	NR	0.00 ± 0.00
1	0	100.00	4	100.00	NR	3.07 ±0.15 d
2		100.00	4	93.33	NR	1.93 ±0.18 e
3		100.00	4	80.00	NR	2.07 ±0.21e
0	1	100.00	4	100.00	NR	6.90 ±0.21 a
	2	100.00	4	100.00	NR	3.57 ±0.19 cd
	3	100.00	4	100.00	NR	4.90 ±0.21 b
1	1	100.00	4	100.00	NR	5.33 ±0.13 b
	2	100.00	4	33.33	NR	1.03 ±0.28 f
	3	100.00	4	46.67	NR	0.73 ±0.17 f
2	1	100.00	4	83.33	NR	4.03 ±0.36 c
	2	100.00	4	33.33	NR	0.80 ±0.22 f
	3	100.00	4	100.00	NR	5.50 ±0.12 b
3	1	100.00	4	76.67	NR	3.63 ±0.41 cd
	2	100.00	4	53.33	NR	1.00 ±0.20 f
	3	100.00	4	23.33	NR	0.70 ±0.24 f

Mean ± SE, n=30. Mean with different letters in the same column differ significantly at p=0.05

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Table 5.5: Callus induction of *Cucumis melo* L. from node explants on MS media supplemented with Mannitol (0.1- 0.3 M) and BAP (0-3 mg/L) under 16 hours light for 16 weeks.

Supplement		Explants	Percentage of callus induced (%)	Callus proliferation score	Percentage of callus inducing shoots (%)	Observations
Man (M)	BAP (mg/l)					
0	0	Hypocotyl	0	0	0.00	No Response
		Node	0	0	0.00	No Response
0.1	0	Hypocotyl	100.00	3	60.00	Green compact callus and shoot formation were observed
		Node	100.00	3	76.67	
0.2	0	Hypocotyl	100.00	3	0.00	
		Node	100.00	3	50.00	
0.3	0	Hypocotyl	100.00	3	0.00	
		Node	100.00	3	23.33	
0	1	Hypocotyl	100.00	3	0.00	Light green, friable, non embryogenic callus were observed
		Node	100.00	3	0.00	
0	2	Hypocotyl	100.00	3	0.00	
		Node	100.00	3	0.00	
0	3	Hypocotyl	100.00	3	0.00	
		Node	100.00	3	0.00	
0.1	1	Hypocotyl	100.00	3	0.00	
		Node	100.00	3	0.00	
0.1	2	Hypocotyl	100.00	3	0.00	
		Node	100.00	3	0.00	
0.1	3	Hypocotyl	100.00	3	0.00	
		Node	100.00	3	0.00	
0.2	1	Hypocotyl	100.00	3	0.00	
		Node	100.00	3	0.00	
0.2	2	Hypocotyl	100.00	3	0.00	
		Node	100.00	3	0.00	
0.2	3	Hypocotyl	100.00	3	0.00	
		Node	100.00	3	0.00	
0.3	1	Hypocotyl	100.00	3	0.00	
		Node	100.00	3	0.00	
0.3	2	Hypocotyl	100.00	3	0.00	
		Node	100.00	3	0.00	
0.3	3	Hypocotyl	100.00	3	0.00	
		Node	100.00	3	0.00	

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5.4 Summary of Results

1. Hypocotyl explants produced high rate of non-embryogenic callus on MS media supplemented with BAP and 2,4-D. The highest callus fresh weight was obtained from MS media supplemented with 1 mg/L of BAP with 1 mg/L 2, 4-D with callus fresh weight of 7.53 ± 0.18 g.

2. Hypocotyl explants induced high rate of callus with roots formation. The highest roots formation was exhibited by MS media supplemented with 3 mg/L IAA (86.67%) with the rate of roots formation of 4.20 ±0.50 per explant.

3. Node explants induced embryogenic callus where the highest shoots induced from MS media supplemented with 2 mg/L BAP with 1 mg/L 2,4-D (100.00 %) with the rate of shoots formation of 13.10 ±0.36 per explant.

4. Nodes cultured on MS media supplemented with 0.1M Mannitol induced callus with shoot formation. The node explants formed the highest shoots which was 76.67 % .

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CHAPTER 6: *IN VITRO* ROOTING AND ACCLIMATIZATION OF *Cucumis melo* L.

6.1 Experimental Aims

Acclimatization is the last phase in plant tissue culture studies. At this stage, the *in vitro* plantlets that have been established with well rooted and multiple shoots formation will be transferred to natural environment. The *in vitro* plantlets will be weaned and hardened gradually in order to make sure the *in vitro* plantlets adapt the environment successfully. The gradual exposure of the plants from culture room which have high humidity to natural environment which have lower humidity is very important to the plants. Relative humidity directly influences the water relations of plant and indirectly affects leaf growth and photosynthesis that will subsequently affect the growth of the plants. Light is an important factor counted in acclimatization phase. The plants will be exposed from low intensity of light in culture room to higher intensity of sunlight.

In acclimatization process, established plantlets will be transferred to an appropriate substrate and commonly will gradually harden the plants under green house with an observation.

This experiment aim is to obtain the most suitable substrate for acclimatization of *Cucumis melo* L. plantlets.

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6.2 Materials and Methods

6.2.1 *In vitro* rooting

The shoots formed from the previous treatments were isolated and cultured on two types of media which are MS media and half strength of MS media (1/2 MS) for rooting induction. The roots formation was observed and the frequencies of roots formation were recorded.

6.2.2 Sources of *in vitro* plantlets

Micropropagated plants of *Cucumis melo* L. were used in this study. The successful regenerated explants with shoots and roots via direct regeneration and somatic embryogenesis on MS medium previously were transferred for hardening in order to maintain and established the plant propagation *in vitro*.

6.2.3 Transfer of plantlets to *ex vitro* environment

After 3 months (12 weeks) *in vitro* plantlets were transferred to pots and polybags filled with different types of substrates. The plantlets were carefully taken out and the agar medium was carefully washed off the roots under running tap water. The roots were washed thoroughly in order to make sure there was no agar left on the roots. The plantlets then planted on three types of substrates which were organic soil, black soil and cocopeat.

All of the plantlets were covered with transparent plastic with small holes and placed in the culture room at 24 ± 1°C under 16 hours light and 8 hours dark + 1000 lux for better adaptation. The transparent plastic bag function was to reduce plants water loss. The plantlets were watered once in two days. After 15 days, the transparent plastic covers were removed and the plants were transferred to shade place. After 7 days in the

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shade place, the plantlets were transferred to bigger polybag and moved to a place with more sunlight and the plants were watered every day. Percentage of plantlets survival rate on different substrate was observed and recorded.

6.2.4 Data analysis

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

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6.3 Results

The shoots were transferred to rooting medium which was MS medium without plant growth regulators and half strength MS medium without plant growth regulators. Based on the results obtained, roots formation was observed on both media after 4 weeks of culture. The root formation was observed as white, thin and fibrous on both media. Results depicted, MS media exhibited higher frequencies of rooting success with 93.33% of the cultures showed root formation and ½ MS medium showed lower rooting success with only 66.67%.

For the acclimatization process, results portrayed the highest plant survival rate of the plantlets from both sources (direct regeneration and somatic embryogenesis) were those which were acclimatized on black soil followed by combination of black soils and red soils and the lowest plantlets survival were observed on cocopeat substrate (Table 6.2 and Table 6.3). In black soil, the plantlets were able to survive and showed healthy and normal growth, while in the combination of black soil and red soil, normal growth was observed but the growth was slower when compared to black soil. Cocopeat substrate exhibited the poorest respond of plantlets where the plantlets from both sources acclimatized in the substrate exhibited unhealthy and very slow growth. The plantlets acclimatized on black soil and the combinations of black and red soils showed high survival rate when compared to cocopeat where very low rate was observed. In addition, the plantlets acclimatized on cocopeat exhibited necrosis after 3 months of acclimatization and finally died.

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6.3.1 *In vitro* rooting on MS media and half strength MS media

Table 6.1: Root formation frequencies of excised shoots on MS media and on ½ MS media.

Media	**Frequencies of rooting (%)**
MS	93.33
½ MS	66.67

6.3.2 Survival rate of plantlets from direct regeneration.

Table 6.2: Survival rate of *Cucumis melo* L. plantlets obtained from direct regeneration.

Type of Soil	**Percentage of survival rate (%)**	**Observation**
Black soil	83.33	Plantlets survived and showed healthy growth.
Black soil with red soil (1:1)	50.00	Plantlets survived with slower growth
Cocopeat	33.33	Plantlets survived with unhealthy conditions and a very slow growth. The plantlets died after 3 months of acclimatization.

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6.3.3 Survival rate of plantlets obtained from somatic embryogenesis.

Table 6.3: Survival rate of *Cucumis melo* L. obtained from somatic embryogenesis.

Type of Soil	Percentage of survival rate (%)	Observations
Black soil	63.33	Plantlets survived and showed healthy growth.
Black soil with red soil (1:1)	30.00	Plantlets survived with slower growth
Cocopeat	16.67	Plantlets survived with unhealthy condition and a very slow growth. The plantlets died after 3 months of acclimatization.

Figure 6.1: *Cucumis melo* L. plantlet on black soil.

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Figure 6.2: Acclimatization of plantlet to natural environment after hardening stage**.**

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6.4 SUMMARY OF RESULTS

1. The highest percentage of root formation was obtained from cultures on full strength MS media (93.33%).

2. The black soil was recorded as the best substrate for acclimatization of plantlets from direct regeneration and somatic embryogenesis with the percentage of survival rate 83.33% and 63.33%, respectively.

3. Cocopeat substrate exhibited as the least suitable substrate for acclimatization of plantlets from direct regeneration and somatic embryogenesis with the percentage of survival rate of 33.33% and 16.67%, respectively.

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CHAPTER 7: PIGMENTS EXTRACTION OF *Cucumis melo* L.

7.1 Experimental Aims

This study aims to extract the most stable pigment to be applied in coating technology. The orange flesh of the fruit was chosen as a source of carotenoid compound and their green leaves as a source of chlorophyll pigment. The pigments of carotenoid and chlorophyll were extracted using different solvents. The best extracted pigments from the solvents then further tested with various pH in order to observe for the colour stability and to observe any colour changes. The colour changes due to the pH treatment then will be recorded and applied to the glass slides and tested as potential coating. The durability of the carotenoids and chlorophylls pigments was then tested by exposing to different heats in order to observe for the colour intensity.

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7.2 Materials and Methods

7.2.1 Plant materials

Fruits flesh and leaves of *Cucumis melo* L. were collected from Serdang, Selangor. The maturities of the fruits were determined by the day of harvest and the weight of the fruits. The fruits and the leaves were washed under tap water. The fruits were peeled and the seeds were separated from the pulp. Fruit pulps were cut into small pieces and freeze dried for 10 days. The freeze dried samples were then ground to powder form with dry blender and stored at -20°C in dark condition until further used.

7.2.2 Extraction of pigments

The freeze dried samples were taken out from freezer at -20°C. The samples were then weighed out for 1 g and mixed with the solvents for extraction. The solvents are 95% ethanol, pure methanol, and pure acetone. The ratio of the sample with solvents varied in order to determine the highest extraction results. The mixing ratios of samples to the extraction solvents are 1 g of samples in 50 ml of each solvent and 1 g of samples in 100 ml of each solvent. The extraction was performed at room temperature. The extraction was incubated for overnight. After the extraction, the solution was centrifuged at 10 000 rpm for 15 minutes. The supernatant liquid was filtered using No.1 Whatmann filter paper to remove any residuals and traces. The residues were discarded and the supernatant containing the compounds of interest were collected. The compound content extracted from each of the solvent and ratios were calculated. The best extracts were then used for subsequent experiments.

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7.2.3 Effects of pH variation test

The best extraction ratio of sample with the best solvents were tested by setting to pH 3,6,9, and 12 with 0.1 M HCL and 0.1M NaOH. Three replicates were used for each pH tested. The extracts were stored at room temperature and the color changes and color degradation of each extract was observed. The most stable extracts were then used in coating experiment. The effects of pH on the extracts were analysed with spectrophotometer in order to determine the amount of carotenoids and chlorophylls. The total amounts of these pigments were calculated according to the formula of Lichtentaler and Wellburn (2001). Table 7.1 below shows the types of solvents used with the specific equation formula in determining the carotenoids and chlorophyll contents of the samples.

Table 7.1: The different types of solvents and the specific equation used to calculate carotenoids and chlorophyll pigments.

Solvent	Equation
Ethanol with 5% of water	c_a (µg/ml) =13.36 $A_{664.1}$- 5.19 $A_{648.6}$ c_b (µg/ml) = 27.34 $A_{648.6}$- 8.12 $A_{664.1}$ $c_{(x+c)}$ (µg/ml) = (1000 A_{470}- 2.13 $_{Ca}$-97.64 $_{Cb}$)/209
Pure methanol	c_a (µg/ml) =16.72 $A_{665.2}$- 9.16 $A_{652.4}$ c_b (µg/ml) = 34.09 $A_{652.4}$- 15.28 $A_{665.2}$ $c_{(x+c)}$ (µg/ml) = (1000 A_{470}- 1.63 $_{Ca}$- 104.96$_{Cb}$)/221
Pure acetone	c_a (µg/ml) =11.24 $A_{661.6}$- 2.04 $A_{644.8}$ c_b (µg/ml) = 20.13 $A_{644.8}$- 4.19 $A_{661.6}$ $c_{(x+c)}$ (µg/ml) = (1000 A_{470}- 1.90 $_{Ca}$-63.14$_{Cb}$)/214

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7.2.4 Production of natural coating materials

The most stable extract from pH variation tests were used for the subsequent experiments. The extracts were mixed with poly methyl metacrylate (PMMA) resin in a glass beaker and slowly stirred until evenly colored and thick solution obtained. The mixing ratio of PMMA to the carotenoids and chlorophylls extracts was 1:5. The resultant mixture were then coated onto several glass slides and air dried for 1 hour. The surfaces of the coated slides were observed under microscope and the observations were recorded (Yaacob *et al*., 2011).

7.2.5 Effect of heat on coating materials

The heating test on the coating materials was done by exposing the coated slides to heat at 27°C (room temperature), 50 °C, and 100 °C. The experiments for higher temperatures were carried out in the oven. The microscope observations and absorbance measurement were carried out at 15 minutes time interval and the absorbance measurements were plotted as a graph. The colour durability against heat were analysed and compared.

7.2.6 Data analysis

Data was analysed using equation in Table 7.1 and the color durability was observed using microscope and spectrophotometer.

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7.3 Results

In the present study, two compounds were extracted from *Cucumis melo* L., which are carotenoids and chlorophyll using different types of solvent. The freeze dried fruit flesh of *C. melo* L. crude extract produced carotenoid compound with bright yellow pigment. The freeze dried leaves crude extract of *C. melo* L gave dark green pigment. Generally, the samples to be extracted must be homogenized in order to obtain the highest yield of the compound of interest.

The three types of solvents used for extraction of both pigments are ethanol with 5% of water, pure methanol and pure acetone. The results showed that the amount of extracted carotenoids and chlorophyll are different with the different types of solvents with different sample to solvent ratio. The results showed the amount of both pigments extracted was higher using acetone, followed by methanol and ethanol at different sample to solvent ratio.

Table 7.2: Carotenoid and chlorophyll content with different solvent extraction

Total carotenoid content (µg/ml)			
Ratio	Ethanol	Methanol	Acetone
1 g in 50 ml	1.884748	1.723987	9.377135
1 g in 100 ml	1.505324	1.241756	5.52318
Total chlorophyll content(µg/ml)			
Ratio	Ethanol	Methanol	Acetone
1 g in 50 ml	87.86563	79.3488	139.8706
1 g in 100 ml	53.00593	68.6355	100.299

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Based on the results obtained, both pigments were best extracted with acetone followed by ethanol and methanol. After an overnight extraction, acetone gave the highest amount of carotenoids and chlorophyll content which are 9.38 µg/ml and 139.87 (µg/ml) respectively, using ratio of 1 g of sample in 50 ml of acetone.

For the subsequent experiment, acetone was used as the extraction solvent for both samples. 1 g of the samples was extracted with 50 ml of acetone and stand overnight for the extraction. Sumanta *et al*. (2014) reported that acetone had rapid extraction during 24 hours and gradually declined after that for both carotenoids and chlorophylls. The filtered samples then treated with different pHs which are pH 3, 6, 9, 12. Figures 7.1 and 7.2 showed the colours of carotenoids and chlorophyll pigments which were subjected with different pH respectively.

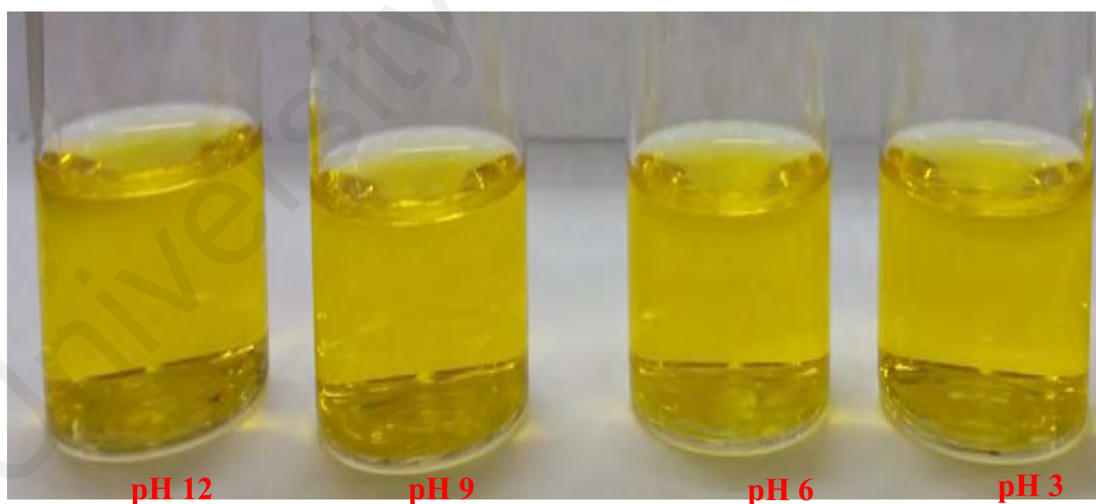

Figure 7.1: From left carotenoid crude extracts with acetone treated with pH 12, 9, 6, 3

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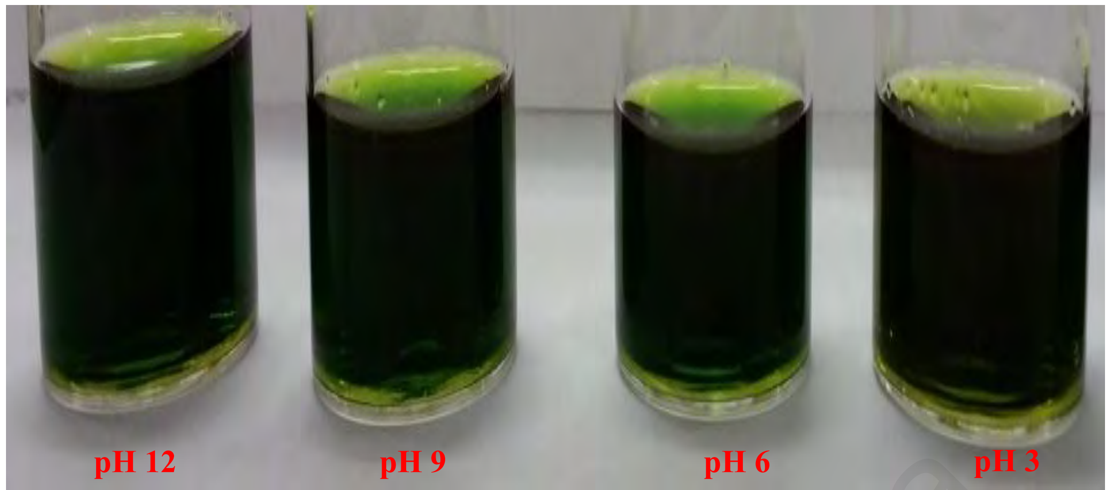

Figure 7.2: From left chlorophyll crude extract with acetone treated with pH 12, 9, 6, 3

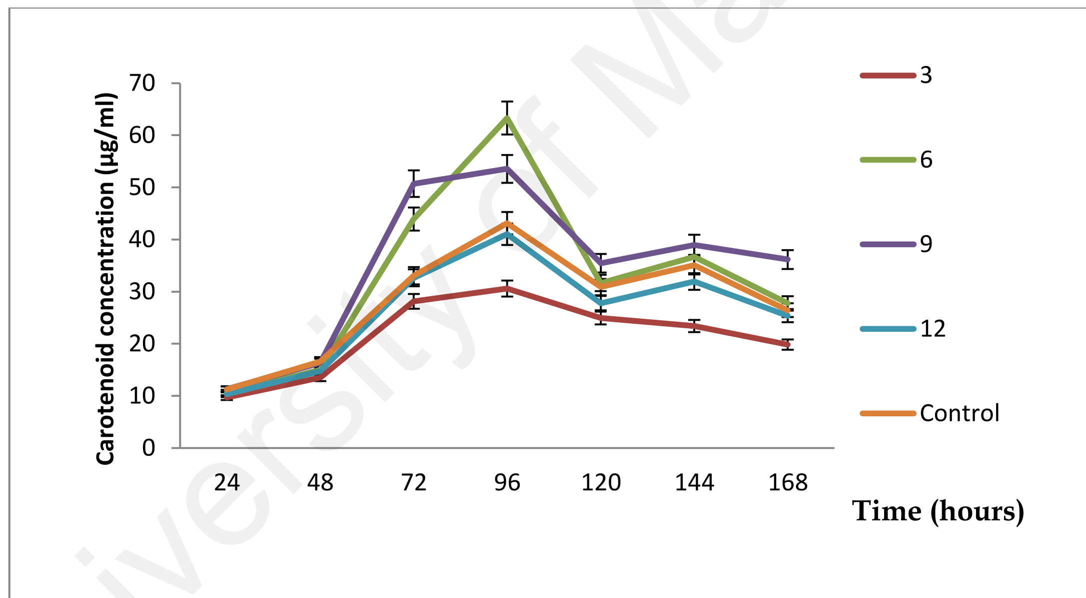

Figure 7.3: Total carotenoid in different pH

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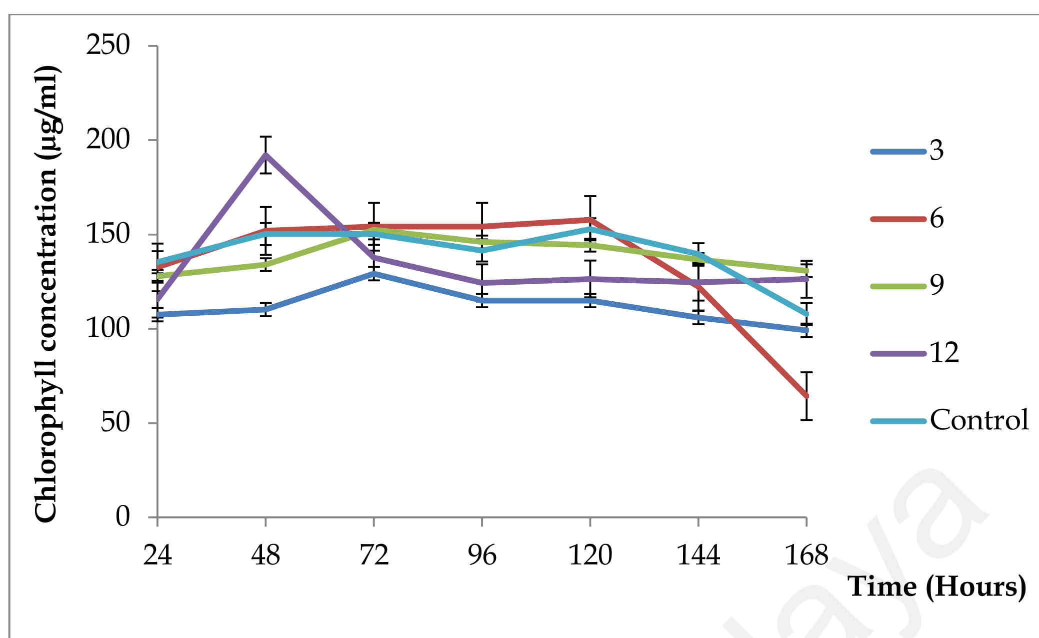

Figure 7.4: Total chlorophyll in different pH

Based on the resuts obtained, carotenoids gave clearer colors at lower pH which was pH 3 and more turbidity at highest pH which was pH 12 but no noticeable reaction at pH 6 and pH 9. The carotenoids were found to yield the highest content after 96 hours. Whilst, the longest carotenoids pigment durability obtained was with the pigments treated with pH 9, where it was found to be the last one to fade.

The current results also showed that there was no obvious or significant visible changes of the color when the chlorophylls extractants were treated at different pH which were pH 6, pH 9 and pH 12 except at pH 3, where the pH became more acidic, the dark green of chlorophyll color turned to olive green colour. Due to results of chlorophylls color durabilty, the pigments treated with pH 9 was the last to fade, followed by pH 12, pH 3 and pH 6.

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For the subsequence experiment, the best pigment treated with the best pH were chosen to be applied on the coated slides and then subjected to a weathering test which is heating test. This preliminary studies can provide additional information about the various extraction parameters to be considered.

Prior the heating test, the carotenoids and chlorophyll coated slides were even, shiny, neither crack nor peel off appeareance observed. The coated glass slides were exposed to different temperatures which were 27°C (room temperature), 50 °C, and 100 °C. The 50 °C and 100 °C temperatures were set up in oven. The coated slides were placed in an oven. There was no specific time limit applied. The colour fadedness and the condition of the coats were observed within 15 minutes interval. Based on the results obtained, the colour degradation was observed more at higher temperatures with obvious decrease in spectrophotometric readings. Specifically, the carotenoids and chlorophyll colours were most stable at room temperature, followed by exposure to 50 °C, and totally degraded at 100 °C.

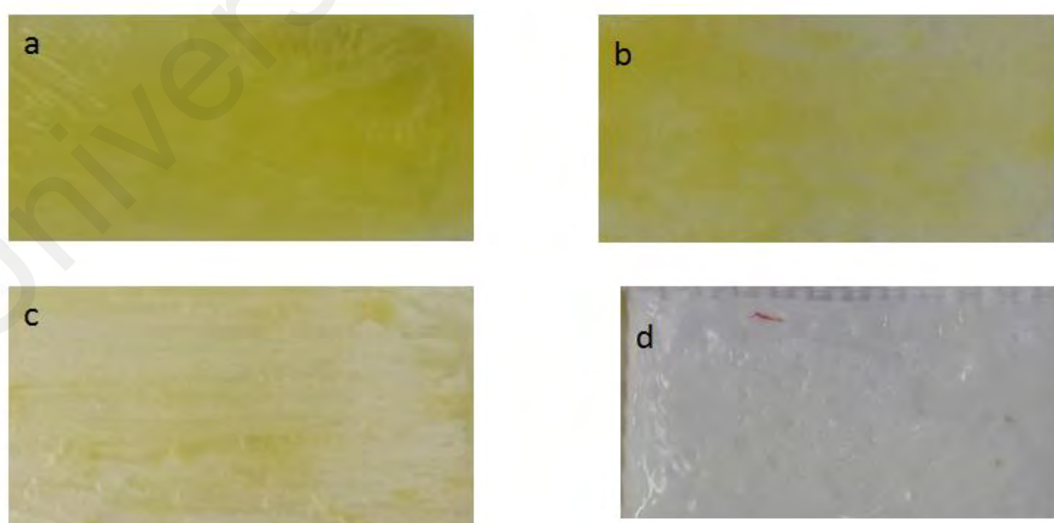

Figure 7.5: a. Coated slides with carotenoids before heat treatment; b. after an hour of heat at 27°C (room temperature); c. after an hour of heat at 50 °C; d. after an hour of heat at100 °C.

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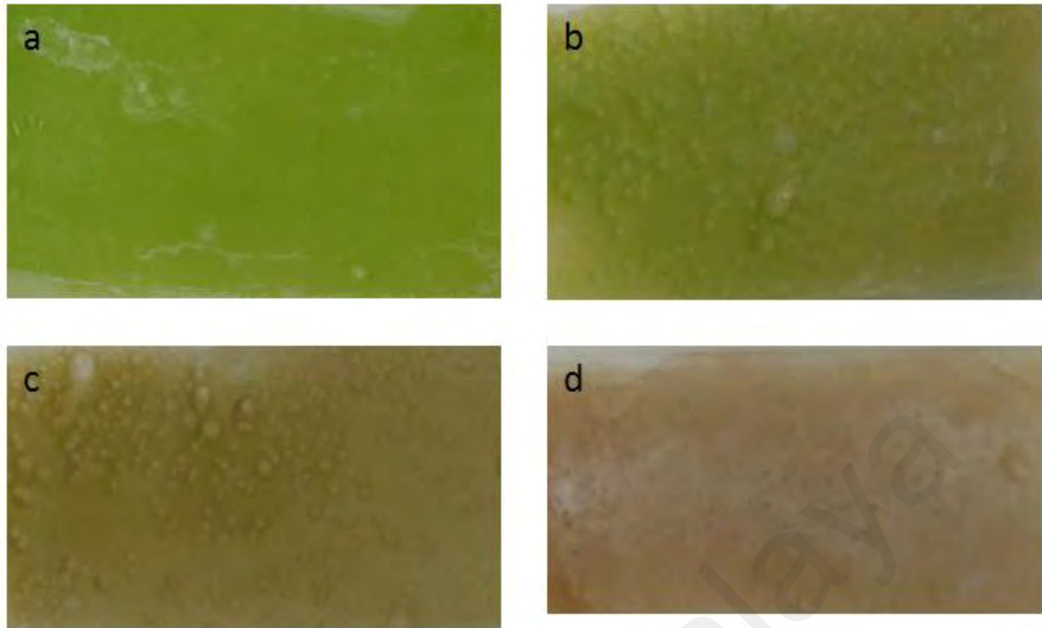

Figure 7.6: a.Coated slides with chlorophyll before heat treatment; b. after an hour of heat at 27°C (room temperature); c. after an hour of heat at 50 °C; d. after an hour of heat at100 °C.

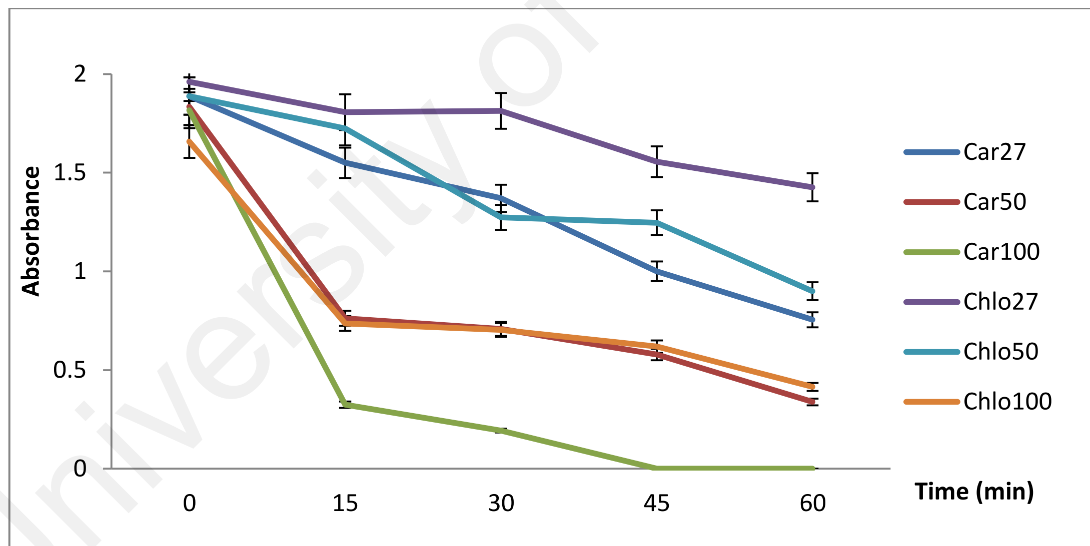

Figure 7.7: Colour degradation of the coated slides treated with carotenoid and chlorophyll pigments.

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7.4 Summary of Results

1. Acetone was identified as the best extraction solvent for carotenoids and chlorophylls pgiments.
2. pH 9 was the best pH to be applied due to the last for the colors to fade.
3. Although there is no significant visible color changes, pH played an important role since the color durability of the pigments affected when different pH was applied.
4. Carotenoids an dchlorophyll could mix well with PMMA when applied on glass slides.
5. During the heating test, carotenoids color faded from yellow to white color with increasing temperature. However, for chlorophyll, the color changed from green to light green, brown and orange at room temperature, 50°C and 100 °C, respectively.

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CHAPTER 8: ANTIMICROBIAL ACTIVITIES OF *Cucumis melo* L.

8.1 Experimental Aims

In this research, the investigation includes the screening of the best explants for rapid and massive callus production from *Cucumis melo* L. plant on MS media supplemented with different concentrations of hormones combination. The best responded explant which was then chosen and established on MS media supplemented from rapid and massive growth of callus as source for sample to be extracted. The best callus obtained were harvested after 6 weeks and extracted with methanol. The extracted fresh callus and *in vitro* hypocotyl were tested against *Escherichia coli, Bacillus subtillis*, and *Staphylococcus aureus, Aspergillus niger*, *Penicillium* sp, and *Fusarium* sp. The strongest antimicrobial activity was determined by disc diffusion method where the inhibition zones were observed.

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8.2 Materials And Methods

8.2.1 Plant materials

Young hypocotyls of *Cucumis melo* L. were used as explants to initiate callus culture. The hypocotyls were obtained from *in vitro* germinated seeds of *Cucumis melo* L. The seeds were sterilized and germinated on MS basal medium with no plant growth regulators. The sterilization of the seeds was done by soaking in water for 2 hours. The seed were then uncoated using scalpel blade. The uncoated seeds were soaked in 70% sodium hypocholrite mixed with 1mL of Dettol and 3 drops of Tween 20. The soaked seeds were agitated on shaker for 20 minutes. After agitated, the seeds were rinsed with distilled water for three times. The rinsed seeds were transferred into laminar flow chamber to prevent air contamination and maintained the aseptic conditions. In laminar flow, rinsed seeds were soaked again with 70% alcohol. Prior to inoculation, the seeds were rinsed again with sterile distilled water. The rinsed seed were dried by blotted dry with sterilized tissues. The seeds were cultured in horizontal position in order to obtain the best seed germination. The cultures were incubated in the dark for three to four days. After day 4, the germinated seeds were grown in the culture room at 25 ± 1°C with 16 hours light and 8 hours dark with light intensity of 1000 lux. After 7 days, the sterile hypocotyls were cut into 10 mm long and cultured on sterile MS media supplemented with different concentrations of BAP and 2,4-D combinations.

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8.2.2 Preparation of culture media for callus induction

In this study, MS solid media was used for callus induction. Solid media was prepared by adding 800 ml distilled water in 1000 ml conical flask. 30 g of sucrose was added to distilled water and stirred with magnetic stirrer. Then, 4.4 g of MS powder including vitamin was added and stirred until the solution become evenly dissolved.The pH of medium then adjusted to 5.8 using 1.0 N Hydrochloric acid (HCl) or 1.0 Sodium hydroxide (NaOH). After that, gelrite that was weighed at 2.8 g then added to the media. The gel-rite acts as a solidify agent to provide surface and support for the plants growth. Lastly, the volume of the medium then was filled up with distilled water to 1 L using graduated cylinder. Mixture was stirred and heat was applied to dissolve the medium completely. The medium was heated until the colour of the medium became transparent. Various concentrations of BAP, and 2,4-D, were pipetted into the culture medium. The combinations and concentrations of the hormones are:

1. 1mg/L BAP with 1 mg/L 2,4-D, 2.
2. 2mg/L BAP with 2 mg/L 2,4-D
3. 3 mg/L BAP with 3 mg/L 2,4-D.

The media was poured in to jam jar prior to autoclaved at 121 °C (15 psi) for 20 minutes.

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8.2.3 Preparation of callus culture

The sterile hypocotyls with the size of 10 mm long, leaves (10 mm x 10 mm) and cotyledons (10 mm x 10 mm) in size were cut, and then inoculated on to the media prepared in aseptic condition. Thirty replicates of explants were used in each treatment. All cultures were maintained in the culture room at 25 ±1 °C and 16 hours light and 8 hours dark with light intensity of 1000 lux. Callus induction percentage and scale of callus formation were observed and recorded. After 6 weeks of callus induction, the fresh callus was harvested and weighed out for extract preparation

8.2.4 Preparation of extracts

The fresh callus was taken and air dried to prior grinded with mortar and pestle. The ground sample then weighed 1 g and then dissolved in 10 ml methanol for solvent extraction process for 24 hours. After 24 hours, the extracts were centrifuged and the supernatant was taken and dried to remove the solvent. The extracts then dissolved in the same solvent which was used in the extraction.

8.2.5 Preparation of test microorganisms

Selected bacteria were obtained from Department of Microbiology, University of Malaya. Bacteria selected include: *Escherichia coli, Bacillus subtillis*, and *Staphylococcus aureus,* while the fungi selected include: *Aspergillus niger*, *Penicillium* sp, and *Fusarium* sp.

All of the test bacterial species were maintained on nutrient agar media. The bacterial culture were inoculated in nutrient broth, while the fungi were cultured in potato dextrose broth and incubated overnight in order to obtain homogenized suspension.

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8.2.6 Antimicrobial activity

The antimicrobial activity of *Cucumis melo* L. callus extraction was performed using disc diffusion method. The plates were incubated at 37°C for 24 hours and the antimicrobial activities were evaluated by measuring the diameter of the inhibition zones.

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8.3 Results

8.3.1 Callus induction from various explants

Callus inductions from various explants were examined in order to achieve the most optimum source for callus formation for subsequent experiment. There were three types of explants namely hypocotyls, cotyledons, and leaf used from *in vitro* seed germination of the plant in the present study. The explants were cut into desired size (10 mm x 10 mm) and cultured on MS basal media supplemented with BAP and 2,4-D at various concentrations. The screenings of the best explants were done in order to obtain the best explants and the most responsive for callus induction.

Based on the observations, all of the explants tested produced callus except for leaf explant and explants cultured on MS basal media (Table 8.1).The highest rate and the fastest of callus induction was achieved by hypocotyl explants with 100% with highest callus proliferation rate at a scale of 4 (started induced after day 7-9), followed cotyledons where callus induced percentage was 76.67% at the scale of 2-3. For leaf explant, it was observed that there was no callus induced and the response only showed that the explant was swollen after a few days of culture. The leaf explant and all of the explants cultured on MS basal media showed no active growth and become necrotic after 7-8 weeks of cultured (Table 8.1).

Different explants exhibited different types of callus. For hypocotyls and nodes, the callus induced was bright green and friable callus. The callus induced from cotyledon explants was green callus, friable and dry callus. Based on the results obtained, the best explants to be used for the next experiments are hypocotyls.

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Table 8.1: Callus induction of *Cucumis melo* L. from various explants cultured on MS media supplemented with BAP and 2,4-D.

Hormone (mg/L)		Explants	Percentage of callus induced (%)	Callus proliferation rate	Observation
BAP	2,4-D				
0	0	Hypocotyl	0	0	No response
		Cotyledon	0	0	No response
		Leaf	0	0	No response
1	1	Hypocotyl	100	4	Bright green and friable callus were observed after day 7-9 of culture
		Cotyledon	26.67	2	Bright green callus, friable,non-embryogenic
		Leaf	0	0	The explants were swollen, no callus induced
2	2	Hypocotyl	100	4	Bright green and friable callus were observed after day 7-9 of culture
		Cotyledon	60	3	Bright green callus, friable,non-embryogenic
		Leaf	0	0	The explants were swollen, no callus induced
3	3	Hypocotyl	100	4	Bright green and friable callus were observed after day 7-9 of culture
		Cotyledon	46.67	3	Bright green callus, friable, non-embryogenic
		Leaf	0	0	The explants were swollen, no callus induced

*0= no callus induced 1=little callus produced, 2=callus induced at the margin of injured area, 3= callus induced covering the explants, 4= callus induced covering surface media

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8.3.2 Callus proliferation after 6 weeks

Successful callus induction and callus proliferation were observed on MS media supplemented with different combinations and concentrations of BAP and 2, 4-D hormone. Based on the results obtained, after six weeks of callus induction and proliferation, the highest fresh weight of callus was obtained from hypocotyls explants cultured on MS media supplemented with 2 mg/L of BAP and 2 mg/L of 2,4-D followed by 3 mg/L BAP and 3 mg/L of 2,4-D and 1 mg/L of BAP and 1 mg/L of 2,4-D gave the lowest fresh weight (Table 8.2). The morphology of the callus depicted different morphology where, the callus from MS media supplemented with 1mg/L of BAP and 1 mg/L of 2,4-D (Figure 2) depicted green color, friable and soft callus, while the callus cultured on MS media supplemented with 2 mg/L of BAP and 2mg/L of 2,4-D (Figure 3) exhibited light green color, friable and more soft when compared to callus cultured on MS media supplemented with 1mg/L BAP and 1 mg/L of 2,4-D. However, the callus cultured on MS media supplemented with 3 mg/L of BAP and 3 mg/L of 2,4-D gave yellowish brown, very soft and very watery callus (Figure 2). After 8 weeks, all of the callus became brown and dead started with the cultured on MS media supplemented with 3 mg/L of BAP with combination of 3 mg/L of 2,4-D, followed by 2 mg/L of BAP with combination of 2 mg/L of 2,4-D and the last to became brownish was the culture from MS media supplemented with 1 mg/L BAP with combination of 1 mg/L 2,4-D. The best callus produced from 1 mg/L of BAP and 1 mg/L of 2,4-D (1.58g) was used as the sample for antimicrobial compound extraction and tested against six types of microrganisms which consists of 3 types of bacteria and 3 types of fungi.

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Table 8.2: Callus production of *Cucumis melo* L . from hypocotyl explants cultured on MS medium supplemented with different concentrations of BAP and 2,4-D at 24 ±1 °C and 16 hours light and 8 hours dark for 6 weeks of incubation.

PGR	1 BAP + 1 2,4-D	2 BAP + 2 2,4-D	3 BAP+32,4-D
Callus production	100%	86.67%	86.67%
Callus proliferation rate	100% of the callus proliferated at rate 4	86.67%of the callus proliferated at rate 4 13.33% of the callus proliferated at rate of 3	86.67% of the callus proliferated at rate 4 and 13.33% of the callus proliferated at rate 3
Callus color	Green	Bright green	Yellowish green
Callus morphology	Friable and soft	Friable and very soft	Very friable, very soft and watery
Callus fresh weight rate (g)	1.58	2.48	1.92

0= no callus induced 1=little callus produced, 2=callus induced at the margin of injured area, 3= callus induced covering the surface media

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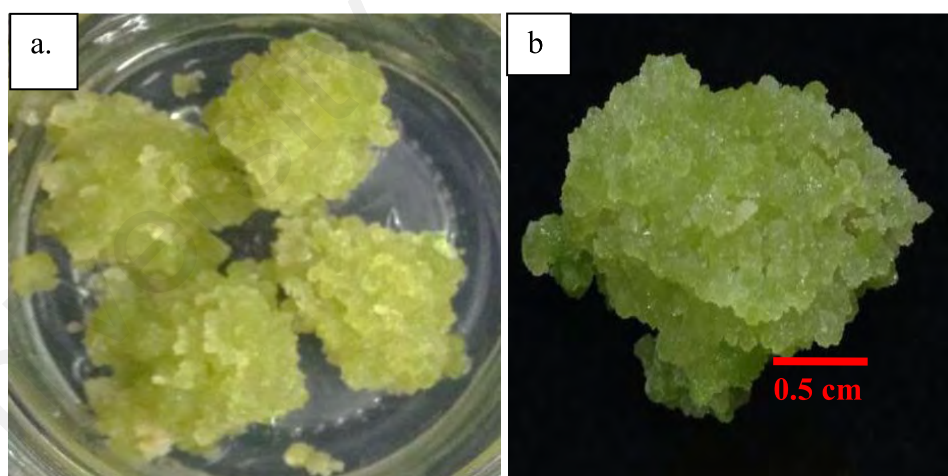

Figure 8.1: a. Callus proliferation on MS media supplemented with 1 mg/L BAP + 1mg/L 2,4-D on MS media and b. the close up of the callus.

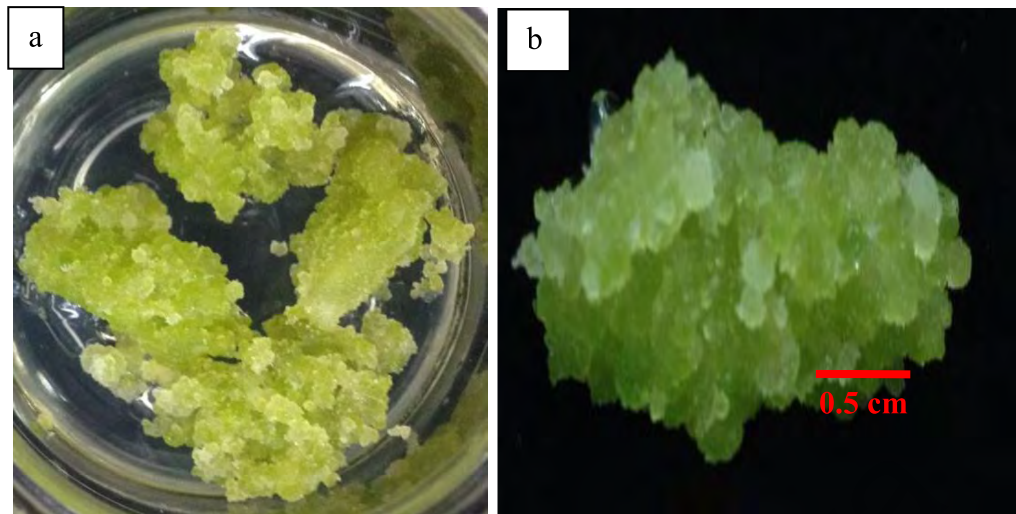

Figure 8.2: a. Callus proliferation on MS media supplemented with 2mg/L BAP + 2mg/L 2,4-D and b. the close up of the callus.

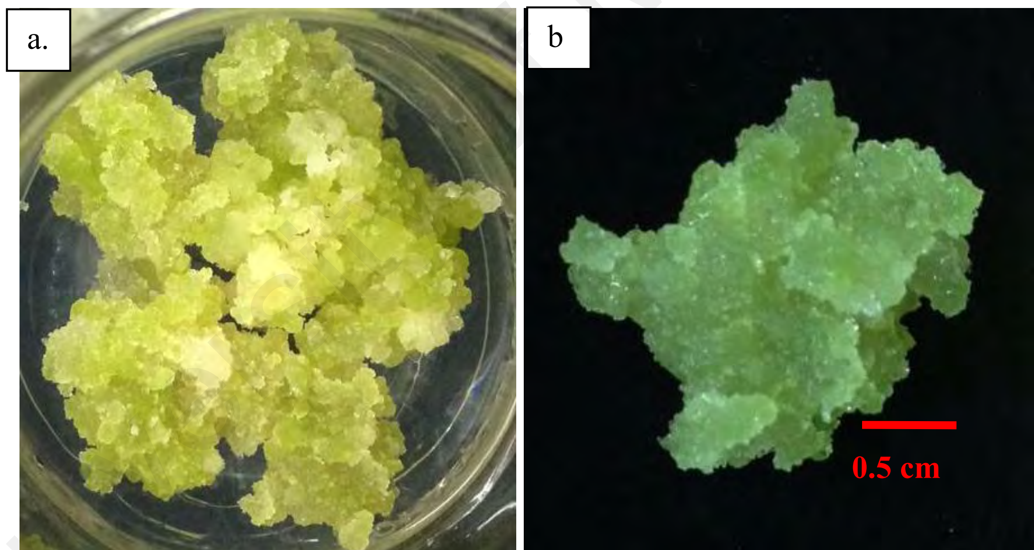

Figure 8.3: a. Callus proliferation on MS media supplemented with 3mg/L BAP + 3mg/L 2,4-D and b. the close up of the callus.

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8.3.3 Antimicrobial activity

Antimicrobial activity was done using disc diffusion method. All of the samples extracted were diluted again with the solvents used during extraction. All of the samples and controls were standardize to the standard concentration of 50 mg/mL. All of the works were done under aseptic control in order to avoid the contamination. All of the cultures with the diffusion disc were incubated in the incubator at the optimum temperature of 37°C for 24-48 hours observation of the results.

Results depicted, *Cucumis melo* L. callus methanolic extracts successfully showed inhibition zone against bacteria especially *Bacillus subtilis* (Table 8.3.). Callus methanolic extracts showed strongest activities due to large inhibition zone observed which is 14 mm same as the positive control (chloramphenicol). However, hypocotyl methanolic extracts and negative control (methanol) gave no inhibition zone against *Bacillus subtilis* (Figure 8.4 a.). For *Escherichia coli*, both methanolic extracts samples gave inhibition zone where the larger inhibition zone was expressed by callus methanolic extracts (13 mm) when compared to hypocotyl methanolic extracts (12 mm). The positive control effects on *E. coli* gave the same measurement of inhibition zone as the callus methanolic extract which was (Figure 8.4 b.) 13 mm. Effects of callus methanolic extracts of *Staphylococcus aureus* gave 12 mm of inhibition zone, followed by hypocotyl methanolic extract with 13 mm inhibition zone. For *Staphylococcus aureus,* the callus methanolic extract showed the same antibacterial activity results with positive control(Figure 8.4 c.). From the results, the callus methanolic extract activity was observed stronger against *Bacillus subtilis*, while the hypocotyl extracts activity was strong against *Staphylococcus aureus* (Table 3).

For antifungal activity (Table 8.3)*,* *Cucumis melo* L. callus and hypocotyl methanolic extracts exhibited a successful result against *Penicillium* sp. when the largest

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inhibition zone was observed from callus extracts with 11 mm inhibition zone followed by hypocotyl methanolic extracs with 10 mm (Figure 8.5). There is no inhibition zone observed on the plate tested with *Aspergillus niger* and *Fusarium* sp. from the sample extracted as shown in Table 3.

Table 8.3: Antimicrobial activity of *Cucumis melo* L. callus and *in vitro* hypocotyls extracts with positive and negative control.

Organism	Inhibition zone (mm)			
	Callus extracts	**Hypocotyl extracts**	**Positive control (Antibiotic)**	**Negative control (Methanol)**
Bacillus subtillis	**14.0**	**-**	**14.0**	**-**
Escherichia coli	**13.0**	**12.0**	**13.0**	**10.0**
Staphylococcus aureus	**12.0**	**13.0**	**12.0**	**9.0**
Aspergillus niger	**-**	**-**	**13.0**	**-**
Fusarium sp	**-**	**-**	**14.0**	**-**
Penicillium sp	**11.0**	**10**	**11**	**10**

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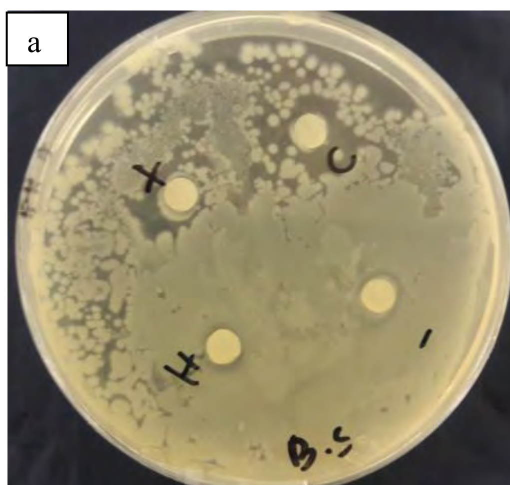

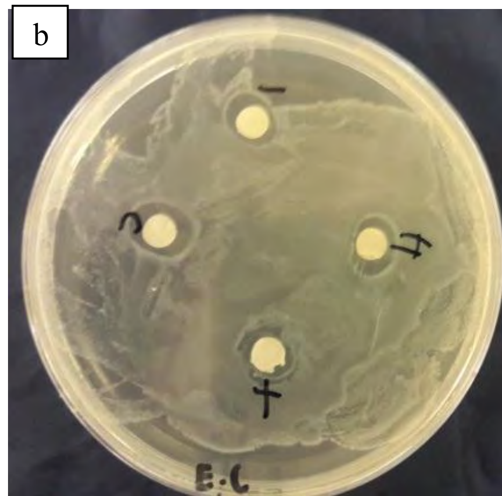

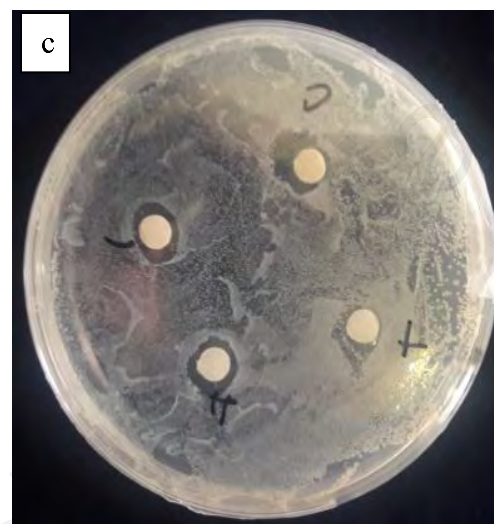

Figure 8.4: Antibacterial activity of a. *Bacillus subtillis,* b. *Escherichia coli, and* c. *Staphylococcus aureus*

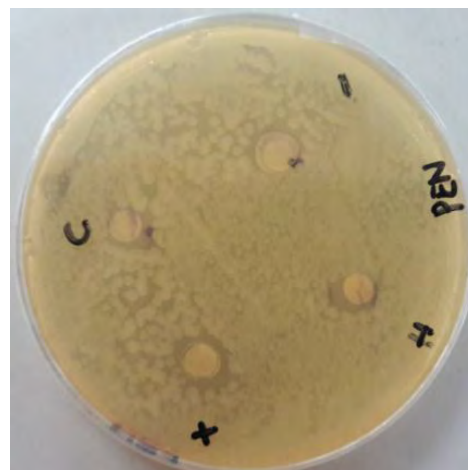

Figure 8.5: Antifungal activity of *Penicillium* sp.

8.4 Summary of Results

1. Hypocotyl was identified as the best explant for callus production.

2. MS media supplemented with 1mg/L of BAP + 1 mg/L of 2,4-D was identified as the best medium for the best callus production with desired morphology and characteristic.

3. Methanolic extract of callus gave highest inhibition zone against *Bacillus subtillis* (14 mm), followed by *Escherichia coli* (13 mm) and the least was *Staphylococcus aureus* (12 mm). Meanwhile for fungus, inhibition zone only observed on the plate with *Penicillium* sp. (11 mm)

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CHAPTER 9: DISCUSSION

Cucumis melo L. or also known as rockmelon or cantaloupe is a eudicot diploid (2n=2x=24) species which belongs to Cucurbitacea family. It is one of the contributors in horticultural crops grown worldwide due to its properties of short generation time and high phenotypic polymorphism, especially in vegetative and fruit morphology; melon is also an attractive model species for genetic and molecular studies (Garcia-Mas *et al*. 2012). Due to variable specialities and great economic importance of the plant, current studies deal with *in vitro* propagation of *Cucumis melo* L.. The research was divided into several parts which consist of the investigation on the reaction of various explants on callus induction, the somatic embryogenesis induction, regeneration of somatic embryogenesis, acclimatization of plantlets, and investigation on pigment extracted and antimicrobial activity of the *Cucumis melo* L.

Seeds act as the main explant source in this study. Time requirement for the seeds to germinate along with the conditions of germinated seeds was very important. Seeds germination rate depends on the seeds position when culture on media. In the early phase of the study, the best seeds position for seeds germination and direct regeneration of *Cucumis melo* L. from cotyledonary petioles as explant were investigated. The used of cotyledonary petioles from aseptic germinated seeds as the explant cultured on MS media supplemented with auxin (2,4-D) and cytokinin (coconut water) was tested. For aseptic seeds germination, media used was MS (Murashige and Skoog, 1962) as the basal media with gel-rite at 2.8 gm/L as solidifying agent and the pH of the media was standardized to pH 5.8. For aseptic seed germination studies, the cultures were incubated in the dark at room temperature for 3-4 days then transferred to culture room at 24 ± 1°C with photoperiod of 16 hours light and 8 hours dark for 8 weeks. While, for *in vitro* regeneration studies, the basal media used were all the same except the plant

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growth regulators added at the required concentration based on tested treatments. All of the cultures were incubated in the culture room at 24± 1°C with photoperiod of 16 hours light and 8 hours dark. In every treatment or experiment, thirty replicates were used in all experiments and observations were made once in 4 weeks interval.

The seeds were aseptically sterilized prior to culture on MS media. The seeds were soaked in water for two hours in order to soften the seed coats. The water also will be absorbed by the seeds and subsequently activate the embryo of the seeds. This will assist in the seeds to germinate easier and faster. Commonly, the seeds of *Cucumis melo* L. were soaked prior to germinating on soil. However based on observation in the study; the high temperature of water affected the seeds viability by killing the seeds embryo. The most suitable water to be used is tap water at room temperature. During the germination period, the seeds were incubated in the dark condition at early phase of growth. This is because; dark condition was justified to affect the seeds germination rate due to no effect to carbonic acid gas. The oxygen gas will not be disturbed thus will enhance the germination rate. It was found that, the light will react in decomposing the carbonic acid gas, then the oxygen expel will fix with the carbon that will eventually effect the vegetation by hardening the plant parts (Neff *et al*., 2009).

An efficient plant regeneration system is important because it is necessary for transformation and propagation at the most optimum rate. There are three stages of seeds germination as stated by Newman, (2009). The seeds start to germinate with activation stage. The uptake of water by the seed causes the seed to increase in volume. During the water uptake, the respiration increase and various enzymes plays their role to digest the endosperm and other function that work simultaneously. This will enhance the elongation of the embryo that resulting in the growing of the radical. The second stage starts when metabolic activity of the seeds increased dramatically, protein

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synthesis begins, and endosperm is metabolized. Enzymes involve will further soften and loosen the cell walls for cell elongation and volume increase. The growth area enhancement is supported by the nutrients supplied from endosperm. The last stage of germination occurred when there is rapid cell elongation and cell division. At last stage, the radical emerge from seed coat and embryo started to get contact with environment where water and nutrient are available for seeds growth.

The different positions of the seeds germinated on MS medium without hormone gave significant results. The current study results depicted the rate of seed germination was higher when the seeds were cultured on the agar media in horizontal position when compared to vertical position (Table 3.1). Based on examination of 30 replicates of culture for each treatment, horizontal cultured seeds give 100% of seeds germination after 7 days of cultured. The average height of the germinated seeds was about 10.12±0.38 cm with no leaf observed except 2 cotyledons. The white fibrous roots formed with an average of 10.70 ± 0.382 per plant. The heights of the germinated seeds were increased on the 14th days of observation to 17.16±0.36. The leaves formations were observed on day 14 with the average of 1.73±0.08 and the formation off roots are more than 20 counts per plant. The last observation showed that, the highest height rate of the seeds germinated was 20.28±0.35 cm and there were about 3.73 ±0.14 leaves observed per plant. The rates of roots formation also increased greatly (Table 3.1).

Vertical cultured seeds showed slower reaction of germination rate. The seeds started to germinate after day 7. The highest seeds germinated are 50% of the seeds culture after three weeks of observation. The rate of the germinated seeds also lower when compared to horizontal cultured seeds, where the height observed is about 9.26 ±0.65 cm. the leaf per explant is 1.46 ±0.380 and the roots formed at average of 9.40 ± 0.38per plant (Table 3.1).

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Commonly, *Cucumis melo* L. seeds were planted in soil in vertical position where the radicles were pointed down. However, based on the results obtained, horizontal position gave higher rate of germination. This might be due to reaction of the embryos with the nutrients and environments were more efficient. The germination ability of the melon seeds can be affected by both internal and external factors (Tekdal and Cetiner, 2013). Polarity of the seeds plays an important role where the seeds endogenous hormone reacted to the position of the seeds placed on growth media. The study by Bhat, (2011) on rattan species showed that different seeds orientation gave significant effect on the seeds germination time. *Pterocarpus marsupium* Roxb. seeds, which is one of the timber species inoculated in different horizontal position on half strengths of MS medium without any hormones was recorded to produce maximum regeneration when compared to those inoculated in vertical postion (Mishra *et al*., 2013). The findings suggested that seed orientation or seeds inoculation position gave significant effects on germination rate. Based on few research seeds sizes also can be considered as an important factor for better germination (Nerson 2007; Nerson, 2002; Vaughton and Ramsey, 2001)

In this study, *in vitro* regeneration of *Cucumis melo* L. was successfully obtained using cotyledonary petioles as the explants where coconut water was used as the cytokinin source for the plant growth. This is due to the incompatibility and several side effects like difficulties in rooting, hyperhydricity, stunted shoots and callus formation of the natural aromatic cytokinins like BAP that was usually included in the regeneration practice (Magyar-Tabori *et al*., 2010). Results portrayed, the highest frequencies of shoots and roots formation was obtained from MS media without hormone (100%) followed by MS media supplemented with 1 mg/L 2,4-D with the combination of 25% of coconut water (100.00 % shoots regeneration and 66.67% root formation) followed by 1 mg/L 2,4-D with combination of 45 % of coconut water exhibited 50.00 % of

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shoots regeneration and 50.00% of roots formation) (Table 3.2). All of the shoots regenerated and roots formed exhibited normal condition (Figure 3.2). The shoots formation with long and thin hypocotyls (Kageyama *et al*., 1991) and roots formation as white and fibrous roots indicated as the normal growth of *Cucumis melo* L. The shoots were regenerated after 7 days of culture and the roots formation was observed after 10 days of culture. Using coconut water as the source of *in vitro* propagation has been widely applied.

Several reports have stated that coconut water as a source of cytokinin yielded positive effects on *in vitro* direct regeneration of plants. For example, there were positive effects of coconut water on direct regeneration of *Elettaria cardamomum* Maton or also known as cardamom by Nadganda *et al*., (1983). Same results reported by the research findings by Shirgukar *et al*., (2003) on *in vitro* micorhizome production of turmeric. Other than that, this natural substance which contain high levels of cytokinin have been one of the important substances that have been discovered in production of some species like passion fruits (Hall *et al*., 2000), coffee (Ismail *et al*.,2003) and also flower like orchids (Santoz-Hernandez *et al*.,2005).

However, on MS media supplemented with higher concentration of coconut water, the shoots regenerated observed with abnormalities. The shoots regenerated with swollen stem were observed (Figure3.3). Reports obtained by Heide, (1969) on *Begonia* regeneration and (Kukreja and Mathur, 1985) on clonal propagation of *Duboisia myoporoides* where the addition of coconut water inhibited the shoot and bud differentiation. Meanwhile, at higher concentration of 2, 4-D as an auxin source, the formation of abnormal roots were observed,where the roots were less fibrous, short and thicker when compared to normal root formation. Tabei *et al*., (1991) verified, at higher

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concentration of auxin, the shoots formation will be inhibited and callus proliferation will be promoted.

There are many researches on *in vitro* regeneration of *Cucumis melo* L. Finding by Gnamien *et al*., (2010) on Cucurbits plants also supported that cotyledonary petioles is among of the best explants to be used. The results among the explants tested which were shoot tips, single node and cotyledon for adventitious shoot formation, the best response was found from cotyledon proximal part with the attachment of hypocotyl segment, where the multiple shoots formation was observed after 3 weeks of culture. This showed the potential of cotyledonary petioles to act as source of explants for *in vitro* regeneration of this species. Comparable results depicted by Zhao *et al*., (2015) during the *in vitro* plant regeneration of *Citrullus lanatus* L. which obtained the best from the basal region of cotyledon due to its ability to regenerate higher frequency of shoot formation. The active response of the explants to organogenesis was probably due to the properties of regenerative meristem in the junction point of the cotyledon and hypocotyl (Naderi *et al.,* 2011). It was reported that, the shoots buds were obtained in high rates in cotyledon explants of *Cucumis melo* L. cultivar Spanish (Moreno *et al*., 1985).

Contradict results were obtained when nodal parts could induce higher numbers of multiple shoots for organogenesis response. According to Ahmad and Anis, (2005) nodal segments used in the *in vitro* regeneration of the *Cucumis sativus* significantly increased the multiple shoots numbers of cultures when cultured on MS media with BAP with additional supplement of casein hydrolysate. According to Parvin *et al*., (2015), among the three explants of *Cucumis melo* L. tested (shoot tips, nodal segments and cotyledonary nodes) the highest response was obtained from nodal segments where 90% of the cultures induced multiple shoots on MS media supplemented with 2 mg/L of BAP followed by cotyledonary nodes, where the explant induced shoots on MS

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media with 1.5 mg/L BAP + 0.1 mg/L GA_3.Same results obtained by Margaret *et al*., (2014) when high frequency of multiple shoots regeneration of *Cucumis anguria* L. was obtained on MS medium supplemented with 1 mg/L of BAP + 0.2 mg/L NAA and 20 mg/L of L-glutamine. *In vitro* plantlets have been obtained from hypocotyls explants on culture medium with 1.0 and 2.0 mg/L BA for cultivar Gergana and in 1.0 and 3.0 mg/L K – line 15B. Induction of regeneration in cotyledons were established only in cultivar Gergana on culture medium supplemented with 3.0 mg/L BA and in combination of 0.5 mg/L IAA (Grozeva and Velkov, 2014). Similar finding by Curuk *et al*., (2002) on organogenesis response of *Cucumis melo* L. of melon hypocotyl found as the best explant that rapidly regenerate to multiple shoots when only attached to cotyledon fragment.

There were various parts of *Cucumis melo* L. plant can induce callus. However, best and successful callus induction depends on the qualities of explants. Thus, selection of the best explant for callus induction was done. Source of explants were obtained from the aseptic germinated seedlings of *Cucumis melo* L. Aseptic germinated seedlings gave a lot of advantages especially its lower the risk of culture contamination. The explants taken from aseptic germinated seeds have the best condition for culture because the seeds germinated are healthy, fresh and grow well when compared to the intact plants. The intact plants might have the possibilities of grow under nutritional, environmental and water stress or the potential of exhibiting disease symptoms. When aseptically germinated seeds were used as explants source, there will no requirement of sterilizing the explants again. This is an added advantage because the harm or over sterilized of the explants tissues will not occur.

Selection of the tissue or explants to be cultured is an important step in the studies because the potential and the degree of success with different tissues can lead to the

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production of different callus with different morphologies. Callus induction, embryo‘s growth and development of plantlet were different base on different explants and plant growth regulators concentrations. In *Cucumis melo* L. studies, there are various explants have been used either for direct regeneration or indirect regeneration. Earlier studies found that cotyledons have been a great source of explants for somatic embryogenesis. As the development in the tissue culture field studies well expand, the choices of explants to be used were greatly increased. In the recent study, the explants used include hypocotyls, nodes, leaves and cotyledons. In determination of the best explants response to callus, the explants were cultured on MS media supplemented with BAP and 2, 4-D either singly or in combinations.

Based on the observation of the current work, all of the explants induced callus at high rate except for leaves explant (Table 4.2). The highest rate and the fastest of callus induction was achieved by hypocotyls explants with 100% of callus induction at very high rate of proliferation (started induced after day 7-9), followed by node explants which also showed 100 % of callus induction at high rate of proliferation (started induced after day 9-12) and the cotyledon explants were less responsive when lower callus induction obtained which was 76.67% with lower rate of callus proliferation (started induced after day 15). The callus induced from cotyledons explant became yellow and necrosis after 8 weeks. Similar findings obtained by Zhao *et al*., (2015) when the cotyledon explants induced callus with no proliferation and became yellow and necrotic after one month. For leaf explants, it was observed that there was no callus formed and the explants only showed that the explants only swollen after 3-4 days of culture. The leaf explants and all types of explants cultured on MS basal media showed no active growth and become necrotic after 7-8 weeks of cultured. Different findings obtained by Ughandar *et al*., (2015) when a protocol for the production of somatic

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embryos from the seed derived callus of *Cucumis sativus. L*. (cucumber) used leaf as the explants and cultured showed successful callus formation was observed.

In this study, the callus started to appear from the wounded area or from the excised sides during the culture after 7 days. After 2 weeks, high callus proliferation were observed where the callus covered the explants. Callus formation is due to interaction between the endogenous plant hormones from the wounded surface with the hormones from surroundings of the culture (Haberlandt, 1902). The wound responses from the plants organ and plants tissues induced the callus. The injury of the explants or the wounds will intensify the endorsement of the nutrients and the growth regulators supplied on the media (Peirik and Steegmans, 1975). Other aspects of callus formation by wound are the callus formation was driven through the up regulation of cytokinin signalling (Iwase *et al*., 2011).

Naturally, wound-induced callus formation has long been observed and used in many situations like debarking the trees (Stobbe *et al*., 2002) and also in the propagation of horticultural practices (Cline and Neely, 1983). Callus usually stored phytoalexins and pathogen-related proteins (Bostock and Stermer, 1989) and thus are thought to prevent infection as well as water loss. Conversely, callus also can be produced without wounding by germinating some seeds on a medium containing a plant growth regulator like 2,4-D

There are various types of explants studied by previous researchers. Findings marked by Grozeva and Velkov, (2014) showed hypocotyls explants of *Cucumis sativum* L. induced callus in all medium where the percentage of successful callus induced was in ranged from 80.0% to 100% of the cultures. This suggested that hypocotyl explant was highly responsive due to their regenerative meristems found in the junction point of cotyledons and hypocotyls (Naderi *et al*., 2011). The finding of hypocotyl was very

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responsive as explant in line with the findings in the study of hypocotyls, cotyledons and true leaves of *in vitro* seedlings of 10 cucumber and melon genotypes cultured on several combinations of initiation and multiplication of media to produce callus where results obtained showed callus derived from hypocotyl explants provided higher number of protoplast after treated with several enzymatic solutions (Felner and Labeda, 1998). Homma *et al*., (1991) found that the best results of somatic embryo production was obtained from explants that comprised of radical, hypocotyls and proximal parts of cotyledon. Review article by Debeaujon and Branchard, (1993) on production of somatic embryogenesis of Cucurbitaceae also suggested that the best explants were from seed-derived parts, particularly cotyledon and hypocotyl explants. The findings also support the results obtained in this study where the seed-derived parts of *Cucumis melo* L. were used as source of explants and the explants that gave response were hypocotyl and cotyledon explants.

Other than hypocotyl, plant parts such as leaves, stems, roots and cotyledons had been used as sources of explants by for establishment of an efficient plant regeneration and callus induction in *Cucumis sativum* L. by Mazlan *et al*., (2014). Their results exhibited there were callus initiated from leaves, stems, roots and cotyledons after 4 weeks of culture. The highest percentage of callus induced was found from stem explant was 64.0 ± 0.50 %, followed by leaves explants which was 38.5 ± 2.27%, cotyledon at frequency of 13.33 ± 1.54 % and finally root explant at of 10.5 ± 1.50% (Mazlan *et al*., 2014). Findings by Tabei *et al*., (1991) on *Cucumis melo* L. results depicted adventitious shoots and somatic embryos were highly obtained from cotyledons of mature seeds, followed by hypocotyls of seedlings, and leaves and petioles of young plantlets. However, when compared to the current study contradict results obtained where all of the explants used for callus induction gave the results except for the leaves explant.

Explants comparison by Rhimi, (2006) on two Tunisian *Cucumis melo* L. cultivars Beji and Maazoun showed contradict results of callus induction response when the most responsive explant observed was cotyledon, followed by zygotic embryo and the less responsive was hypocotyl explant. In the current study, different report obtained where the best responsive callus induction was observed from hypocotyl explant whereby the cotyledon explant also induced callus but less responsive if compared to hypocotyl explant.

There are many factors affecting the rate of callus induction and somatic embryogenesis. One of the factors that contribute to the different reaction of the explants is the age of the explant. Theoretically, the younger tissue of the explant mark as very responsive due to physiological condition of the cell that are actively dividing. In addition, the younger tissues usually very fresh and the cells formed will make the culture grow easier and healthier to diseased free condition. Results obtained in the research of Zhao *et al*., (2015) on *Citrullus lanatus* L. showed that the most responsive explants were obtained from the 5-day-old seedlings. The same results also portrayed by Dong Jia, (1991), Choi *et al*., (1994) and Pirinc *et al*., 2003.

Other factors that can be included as the effect of different response are the explant sizes. Basically, the smaller explants will be harder to culture and grow *in vitro* condition when compared to larger explants. This is due to nutrient reserves in the explants where larger explants may exhibit higher nutrients and endogenous plant growth regulators reserved. The nutrients and plant growth regulators contain in the explants are very important because the compounds will reacts to the culture medium and hormones. A study by Van, (1977) on thin epidermal tobacco stem tissue taken from different part of the stem was cut in slices showed different reaction. The study revealed that plants have different hormonal distributions and hormonal balances that

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depend on the location of the explants. The different endogenous hormones and different levels of them in the tissues can result in different response of the *in vitro* culture. In this study, the size of the explant were standardize to 10 mm x 10 mm size and 10 mm long.

Different genotype also plays an important role in determining the response of the culture. Usually, there will be large differences of reaction and response of the *in vitro* explants due to differences in the genotypes, cultivars, or species. Some genotypes are not responsive in culture, or recalcitrant, while others easily respond to produce callus or shoots. Testing numerous genotypes of a crop or ornamental species is generally a major experimental parameter to identify those that will respond in culture (Yildiz, 2012).

Callus induction of *Cucumis melo* L. was carried out in order to identify the suitable types of plant growth regulators and concentration of plant growth optimum callus growth and production of somatic embryos. Exogenous application of auxin and cytokinin induces callus in various plant species (Ikeuchi *et al*., 2013). Callus induction, embryo's growth and development of plantlets were different due to the variety of explants and concentrations of plant growth regulators (Naderi *et al*., 2011). In the current study, there were 43 treatments were applied for somatic embryogenesis induction from two types of explants which are hypocotyls and nodes. The explants were chosen based previous investigation of reaction between different explants were used as starting material in the studies. Based on the results obtained from this study, explants of hypocotyls and nodes gave the best response with minimal time and high proliferation rate of callus. Other than supplied the MS media with different plant growth regulators, the effect of carbon source (by adding mannitol) singly and in combination with cytokinin (BAP) on induction of somatic embryogenesis from

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hypocotyls and nodes explants were also studied. The embryogenic and non-embryogenic callus was determined by using double staining method (Gupta *et al*., 1987).

Results depicted high callus formation in all treatments. There were three types of callus observed where the types of callus were classified based on their morphology, friability and colours. The first type of callus was light green with very loosely friable textured callus. The second type of callus was green with close friable textured callus. The third type of callus was dark green with hard textured callus. The different callus morphology was observed on different culture media of MS supplemented with different plant growth regulators. The first type of callus was observed on MS media supplemented with BAP, 2, 4-D and combination of both. The second types of callus morphology was observed on MS media supplemented with IAA, Kin or combination of both, while the last type of callus which was dark green and hard callus obtained from MS media supplemented with Mannitol applied singly.

Current study on the effects of different plant growth regulators on callus induction and somatic embryogenesis in *Cucumis melo* L. gave significant results. Hypocotyl and node explants used as starting materials in the studies, exhibited different reaction when cultured on MS media supplemented with different plant growth regulators. Hypocotyl explants cultured on MS media supplemented with BAP and 2, 4-D produced non-embryogenic callus (Table 5.7). However, the rate of callus proliferation observed was high whereby the highest callus fresh weight obtained from MS media supplemented with 1 mg/L of BAP in combination with 1mg/L 2, 4-D where the rate of callus fresh weight is 7.53 ± 0.18 g (Table 5.1). Similar results were obtained by Zhang *et al*., (2014) and Naderi *et al*., (2011) where non embryogenic callus induced on MS media supplemented with BAP and 2,4-D produced. Based on the observation of the study, the

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callus quality from somatic embryo reduced as the 2,4-D concentration increased. All of the callus induced observed was non embryogenic when the concentration of 2,4-D was higher than 1.0 mg/L. The callus inductivity was inhibited when higher concentration of 2,4-D was applied, where 2,4-D concentration reach 1.0 mg/L, most of induced callus was non-embryogenic. In addition, Zhang *et al*., (2014) also reported that the callus induced from hypocotyl explant were higher when compared with cotyledons and cotyledon nodes. It also showed that non embryogenic callus could not be induced somatic embryo. However, different findings were obtained from Moreno *et al*., (1985) on *Cucumis melo* L. cultivar Amarillo Oro, where the culture of hypocotyl as explant were formed somatic embryo but failed to form shoots on media containing auxin.

On MS media supplemented with IAA and Kin, and combination of both, hypocotyls exhibited different reaction where there were roots formation observed from the callus induced. The highest roots formation exhibited by MS media supplemented with 3 mg/L IAA with the percentage of callus inducing roots 86.67% with the rate of root formation was 4.20 ±0.50 per explant (Table 5.2). Similar results were obtained by Zhang *et al*., (2014) when the callus induced on MS media supplemented with 1.0 mg/L of NAA induced same types of callus with a large number of adventitious roots observed.

For node explants, embryogenic callus and shoots formation was observed from the callus. All treatments exhibited embryogenic callus which was green with close friable textured callus.. The callus induced exhibited the different stages of embryogenic callus. The highest shoot induced from the callus on MS media supplemented with 2 mg/L BAP in combination with 1 mg/L 2, 4-D at 100.00 % with the average rate of the shoot formation 13.10 ±0.36 per explant (Table 5.3).

Nodes and hypocotyls cultured on MS media supplemented with 0.1M Mannitol induced callus with shoot formation. The types of callus obtained were dark green with

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compact textured callus (Table 5.5.). Similar results were obtained by Jeyakumar *et al*., (2015) from *Cucumis anguria* when the green compact callus induced on MS media supplemented with 1 mg/L and 2 mg/L of 2,4-D was able to regenerate into multiple shoots when compared to yellow friable callus induced on MS media supplemented with higher concentration of 2,4-D which is at 3 mg/L. Other than that, the treatment of different concentrations of glucose also exhibited the similar results when callus induced have the same structure with the callus obtained in the study where the callus was green with compact structure. The nodes explants formed highest shoots at the frequencies of 76.67 % (Table 5.5). Callus induction in MS media supplemented with Mannitol induced later and required longer induction time. The callus induction and callus proliferation on MS media supplemented with Mannitol were very slow. It took about two months for the callus to form. The callus induced showed there were shoots formation after 14-16 weeks of culture. Other than 2,4-D, the somatic embryogenesis also controlled by osmotic shock with sucrose or sodium chloride (Loyola-Vargas *et al*., 2008). The increase of carbon source in term of sugar and mannitol gave the osmotic shock for the explants cultured. Lichtenthaler, (1998) proposed that the physiological response could depends on two main factors which are the physical state of the cells and the level of stress condition either based on time or intensity. Due to higher stress condition that can exceeds the cellular tolerance give dying effect to the cells but, when there is lower intensity of the stress, the cells will undergo adaptation mechanism (Lichtenthaler, 1998). Based on the concept mentioned, it was suggested that the cell proliferation undifferentiated stage could be inhibited and subsequently trigger the stimulation of embryo production (Lee *et al*., 2001).

Carbon functioned as the energy source for explants and it also balanced the osmotic pressure and plays an important role in plant somatic embryogenic (Ji *et al*., 2011). The study by Zeng *et al*., (2007) on *Cucumis melo* L. GT-1 found that indirect

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embryogenesis occurred when glucose was used as the carbon source, while direct embryogenesis was observed when the medium contained lactose. Similar findings by Yang *et al*., (2002) on cucumber species when cotyledons used as explant showed somatic embryogenic efficiency increases by combining mannitol with sucrose.

Callus formation can be different between explants and even within the replicates of the same medium (Loyola-Vargas *et al*., 2008). The formation can be referred to the growth rate and the friability of the callus (Thorpe, 1990). The differences in callus also can be seen through the varieties of callus in colours, morphology, structure, growth and metabolism. Callus can exhibit different ploidy and have different metabolic capability even observed under uniform or homogenous type of callus. The callus that can generate to became organized structure or undergo regeneration process are callus with totipotency expression (Loyola-Vargas *et al*., 2008). The totipotent callus can be identified by early visual selection (Smith, 1992). The early visual selection includes the morphology of the callus that exhibited the stages of callus growth. The stages include globular stage, heart shape stage, torpedo stage and cotyledonary stage in embryogenic callus. The types of callus formation are highly affected by the level (Loyola-Vargas *et al*., 2008) and the types of plant growth regulators supplemented to the media.

The effect of plant growth regulators is an important factor impacting somatic embryogenesis and plant regeneration. In most cases, successful plant somatic embryogenesis needs a mixture of the different concentration ratios of auxin and cytokinin , both of which are necessary for plant culture *in vitro* (Ji *et al.,* 2011). It was reported that callus induction and somatic embryogenesis induction of *Cucumis melo* L. and other Cucurbitaceae family required the plant growth regulators like auxin (2,4-D) and cytokinin (BAP). Embryogenic callus induction in melon has been widely reported using different types of auxin (Oridate *et al*., 1992; Gray *et al*., 1993; Guis *et al*., 1997;

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Nakagawa *et al*., 2001). Where in melon tissue culture, the most extensively used auxin was 2,4-D (Oridate and Oosawa, 1986; Debeaujon and Branchard, 1993).

Study of somatic embryogenesis of *Cucumis melo* L. using hypocotyls as explants by Ju *et al*., (2014) showed contradict findings when *Cucumis anguria* L. or also known as Gherkin exhibited high frequency of somatic embryos when culture on MS media salts plus B5 vitamins supplemented with 5% sucrose, 1.5 mg/L 2,4-D, 0.5 mg/L BAP and 150 M glutamine where the cultures were initially placed in the dark condition for two weeks and followed by four weeks under light condition. However in this study, the cultured explants were incubated under 16 hours light and 8 hours dark. Reports obtained from Comlekcioglu *et al*., (2009) gave different view on the ability of the medium on somatic embryogenesis of C*ucumis melo* var. *flexuosus* when hypocotyls explants showed somatic callus induced with the number of somatic embryos were 16.33 ± 3.06 per petri dish, when cultured on optimum concentration of MS medium supplemented with 2 mg/L 2,4-D with 0.5 mg/L BAP.

Bairwa *et al*.,(2013) also described the types of somatic embryogenic callus of *Cucumis melo* L. were influenced by the plant growth regulators concentration. Based on his studies, it was found that among the various medium tested, MS media supplemented with 1 mg/L of 2,4-D with combination of 0.5 mg/L of BA induced somatic embryogenesis while MS media supplemented with 2.0 mg/L 2,4-D with 0.5 mg/L BA was found the best concentration for somatic embryogenesis. Based on Tabei *et al*., 1991, all tissues of *Cucumis melo* L. that were cultured on MS media supplemented with with 2,4-D (0.25–1 mg/L) and BA (0.10–0.50 mg/L), produce callus with somatic embryos after 3 weeks of culture.

Naderi *et al*., (2011) reported that types of plant growth regulators combinations of BAP and 2,4-D at different concentration gave high production of somatic

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embryogenesis from cotyledonary petiole explants where the best results obtained in formation of numbers of embryos are from MS media supplemented with 0.1 mg/L BAP with 5 mg/L 2,4-D. Rhimi *et al*., (2006) study showed the best results for somatic embryogenic was best achieved on MS media supplemented with 0.25 mg/L 2,4-D with combination of 0.5 mg/L of BA for cultivars of Maazon and Beji. Based on Akasaka *et al*., (2004) on the studies of embryo induction in Vedrantais and Earl‘s Favourit cultivars showed 0.1 mg/L of 2,4-D with 0.1 mg/L of BAP as the best concentration for somatic embryogenesis induction.

Other than combination of BAP and 2,4-D, Mazlan *et al*., (2014) reported MS media supplemented with 0.5 mg/L BAP with 1.0 mg/L NAA plant growth regulators also induced callus from different types of explants like leave, stem, root and cotyledon. Combinations of BAP and NAA, and combinations of K and IAA also have been investigated by Grozeva and Velkov, (2014) on *Cucumis sativum*. The study revealed the callus induction from hypocotyl explants of cucumber 15 B cultivar and Gergana cultivar were highly responsive to MS media supplemented with plant growth regulators of BA and combination of BA with IAA and combinations of K with IAA. The callus induction reaction of hypocotyls explant for 15 B cultivar and Gergana cultivar showed 100.00% on MS media supplemented with 1 mg/L of BA, 3.0 mg/L of K and combinations of BA (1.0-3.0 mg/L) with 0.5 mg/L IAA, K (1.0 – 3.0 mg/L) with 0.5 mg/L of IAA. For 15B cultivar, the callus induction was decreased when cultured on MS media supplemented with 1 mg/L of Kin (90%) while, for Gergana cultivar callus induction also decreased on MS media supplemented with 1.0 mg/L of K, 2.0 mg/L of Kin and 1.0-2.0 mg/L of BA where the frequencies was 93.00%, 90.00% and 80.00% respectively. The results obtained showed similar effect on the hypocotyls explant tested in the current study where 100 % of the explants were observed highly responsive to the MS media supplemented with the same types of plant growth regulators which were

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BAP, 2,4-D, IAA and Kinetin. Moreno *et al*., (1985) reported the ability of the callus cultured from cotyledon and hypocotyl explants of *Cucumis melo* L. Spanish cultivar were able to undergo morphogenesis via organogenesis and embryogenesis where at the concentration of 1.5 mg/L IAA and 6.0 mg/L kinetin, more than 90% of the callus produced well-developed shoots.

The study by Tekdal and Cetiner, (2011), found that the MS media supplemented with 0.5 mg/L of NAA increased the callus formation. The findings also stated that the supplementation of NAA singly stimulated the formation of callus. Apart from using NAA as plant growth regulator, the researchers also used IAA and Kin in the studies. Results obtained showed the three combinations of plant growth regulator 0.5 mg/L NAA, 5 mg/L IAA and 5 mg/L Kin for Cinikiz and Hasanbey melon cultivars gave 100% and 87%, callus formation respectively. The medium containing 0,5 mg/LNAA, 5 mg/L IAA and 5 mg/L Kin stimulated callus growth for Hasanbey melon cultivar. On the contrary, the frequency of callus formation for Cinikiz melon genotype due to NAA concentration was significant. In all media containing 0,5 mg/L NAA, high callus formation (100%) from Cinikiz melons‘ cotyledons was obtained. The combination of IAA (1 mg/L) and Kinetin (5 mg/L) was seen very effective in inducing the regeneration of shoot buds from callus induced form epicotyl explants (Jain and More, 1992).

The formation of somatic embryogenesis occured when the somatic cells were induced under specific induction condition that will generate embryogenic cells and subsequently develop into plants after under of morphological and biochemical changes (Komamine *et al*., 1992; Zimmerman, 1993). The totipotency cell of the callus are formed by the somatic embryogenesis can be induced by the influences of components of culture media and plant growth regulators.

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There are many factors affected the somatic embryogenesis of the plants. One of the factors is explant type as discussed previously. Other than explants, the types and concentration of plant growth regulators and the genotypes of the plants also play important roles as the key factors affecting somatic embryogenesis. The efficiency of explant regeneration also depends on the genotype where not all of the cultivars of a genus or species will have comparable respond to the same specific procedure (Stipp *et al*., 2012). The interaction of genotype, explant types and the concentration of plant growth regulators play a major role in melon embryogenesis (Naderi *et al*., 2011). According to Mohiuddin *et al*. (2005) regeneration capability of cucumber strongly depends on explants type and genotype even the culture conditions were optimal and established that distal cotyledons as less responsive.

The genotype is the key factor in affecting plant somatic embryogenesis. The frequency of somatic embryogenesis has been shown to be quite diverse due to genotypic variation, although within the same genus. Not all species can be induced for somatic embryogenesis. Due to diverse genotypes, the extent and frequency of somatic embryogenesis of the same variety are distinct. The retardation of plantlets growth was observed after initiation where not all of the shoots were able to produce roots even on rooting medium (Bairwa *et al*., 2010).

The different ability of somatic embryogenesis induction among different genotypes was reported in other Cucurbitaceae plants. Nadolska-Orczyk and Malepszy (1989) suggested that a genetic determined the ability to regenerate somatic embryo derived plants from cucumber *Cucumis sativus L.* leaf explants. Gonsalves *et al*., (1995) studied the somatic embryogenesis of six squash cultivars; all cotyledons produced somatic embryos after 11 to 17 weeks on induction medium. However, the optimal culture time and the rate of plant regeneration were significantly different between the six cultivars.

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Significant differences of somatic embryogenesis were also observed among different cultivars in melon (Oridate *et al*., 1992). It proved that genotype was an important factor to affect somatic embryo induction. Most plants have potential to induce somatic embryo, but the sensitivity and difficulty of inducing is different; and the frequency of somatic embryogenesis is widely different as the difference of genotype in the same species (Zhang *et al*., 2014). Thus, the different rate of regeneration of somatic embryogenic callus in this study was highly influenced with the different genotypes of *Cucumis melo* L. when compared to the previous studies.

Acclimatization is the last step in tissue culture study. However, prior to acclimatize; the plantlets must be rooted. Rooting is a crucial step in order to prepare as a base for nutrient uptake of the plant. In this study, *in vitro* rooting had been applied. After the *in vitro* rooting phase the cultured plantlets of *Cucumis melo* L. were transferred to natural environment for further growth. The exposure of the plantlets to natural environment was done gradually. There were three types of substrates used for the transplanting process. The three substrates are black soil, black soil with red soil (2:1) and cocopeat.

Results depicted, full strength MS media showed higher percentage of rooting success with 93.33% while ½ strength MS medium showed lower rooting success with only 66.67% of roots formation observed (Table 6.1). Several cases showed that the MS medium marked as the ability of regenerated the roots of *Cucumis pepo* (Ananthakrishnan *et al*.,2003) and *Cucumis maxima* (Lee *et al*., 2003). However, there were many reports on the ability of MS medium with combination of plant growth regulators could effectively induced and increase the rooting capability of the shoots. The shoots of *Curcuma zedoaria* were also successfully rooted on MS media without plant growth regulators where the root formation was observed after 3 weeks of cultured (Chong *et al*., 2012). Chabukswar and Dheodhar, (2004) also stated that the *in vitro*

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rooting of *Garcinia indica* Chois gave almost 100% response of *in vitro* rooting were obtained from half strength MS medium with 2% sugar concentration and 0.6 % of agar.

Based on Krishna and Shashtree, (2015) rhizogenesis of *Citrullus colocynthesis* L. Shrad required the combination of IAA with IBA, and NAA with IBA which were capable for rooting ability. Margaret *et al*., (2014) reported that, the elongated shoots of the regenerated *Cucumis anguria* L were successfully rooted on MS medium supplemented with IBA at the concentration of 0.6 mg/L and can be transferred for hardening after 2 weeks of culture. Similar reports on the effectiveness of IBA also obtained from the investigation on *Momordica charantia* (Agarawal and Kamal, 2004) and *Mormordica dioica* (Haque *et al*., 2007). Kathal *et al*., (1988) also showed, there were significant results where elongated shoots were successfully rooted in high frequency (70%) on MS media supplemented with IAA at 0.5 µM. Mali and Chavan, (2016) also showed there were 100% of rooting response of microshoots on half MS medium supplemented with 1 mg/L of IBA.

In this study, *Cucumis melo* L. plantlets were found to be best grown on black soil with the percentage of survival rate of 83.33 % and 63.33% respectively (Table 6.2 and 6.3). This is followed by combination of black soil and red soil (2:1) as suitable substrate for acclimatization of *Cucumis melo* L. with the percentage of plantlets survived were 50% for direct regeneration and 30% indirect regeneration. The lowest percentage of survival rate was observed on cocopeat substrate where the survival rate for direct regeneration and indirect regeneration were of 33.33% and 16.67% respectively. On black soil substrate, the normal and healthy growths of plantlets were observed. Combination of black soil and red soil (2:1) gave normal but slower growths of plantlets were observed. While on cocopeat substrate, plantlets survived with very

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slow growth and the leaves produced were smaller when compared to other substrates. Same results also reported by Yaacob *et al*., (2014) when the rate of growth monitored was much better on regenerated plantlets of *Agapanthus praecox* ssp. Minimus was from direct organogenesis when compared to plantlets regenerated indirectly.

The same reports obtained in acclimatization process of *Lycium barbarum* L. where the rates of plantlets survived was higher on black soil followed by combination of black and red soil at 63±1.5 % and 50±1.3 % respectively (Taha *et al*., 2015). Yaacob *et al*., (2012) also reported that acclimatization of regenerated plantlets of *Agapanthus praecox* ssp. minimus (African blue lily) showed very high survival rate which was 86.67 ± 6.31 % on black peat soil substrate

However, study by Sidik *et al*., (2012) suggested that the aeration ability, the stability of the intense heat, and retain moisture and nutrients for roots have significant effects on the growth of the *Cucumis melo* L. It was found that, vermiculites portrayed as the best medium in hardening, followed by combination of top soils and vermiculites (1:1) and the least survivor plantlets were observed on those acclimatized on top soil (Sidik *et al*., 2012).

Findings by Rhimi *et al*., (2016) showed the highest percentage of survival plants of indirect regeneration of *Cucumis melo* cultivars Maazoun and Beji was 36.50%. The success of *in vitro* produced plantlets of *Cucumis sativus* L. by Ahmad and Anis (2005) showed 60% of survival in Soilrite and subsequently transferred to greenhouse exhibited no abnormal morphological changes. Contrast results obtained by Ahmed *et al*., (2012) when plantlets of *Gerbera jamesonii* maintained in garden soil (black soil : red soil, 2:1) gave the best results when there were 86 % of plantlets survived followed by vermiculite substrate with 73 % survival rates. However, there were 0% of plantlets survived in autoclaved garden soil (black soil:red soil, 2:1). Margaret *et al*., (2014)

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showed there were 70% of *Cucumis anguria* L. plantlets reported survived in combination of substrate sterile soil, sand and vermiculite (1:1:1 v/v/v) which is the similar results as reported by Compton *et al*,. (2001) for *Cucumis hystrix.*

Different results observed by Chabukswar and Deodhar, (2005) when the acclimatization of *Garcinia indica* Chois on different substrate where cocopeat showed the highest survival rate (76 %) followed by cocopeat and sand (1:1) with 75% of survival rate and the mixture of cocopeat, sand and soil (1:1:1) gave the lowest survival rate which are (67.5 %). Reports by Rodrigues *et al*., (2005) showed that among the three substrates tested for acclimatization process of *Heliconia bihai* plantlets, results revealed that the highest survival rate was found from substrates of washed sand and PlantMax® Horticultura had similar yields, with expressive survival rates (72.50% and 71.66%) substrates while the lowest was from vermiculites with the survival rate of plantlets was 32.50%. Jeyakumar *et al*., 2015 reported 75% of the acclimatized plantlets of *Cucumis anguria* was successfully established on substrates containing sterilized garden soil and sand mixture (1:1). Hydroponic technique was seen as a great method for grow the complete plantlets of *Cucumis Melo Var. Birdie* by Ismail (1989) where the plants were observed flowered and produced normal fruits. Findings by Goyary *et al*., (2010) on *Cucumis melo* L Var Karnal revealed that high percent of the regenerated plantlets were hardened successfully in mixture composition of three types of substrates which are sand: soil: vermicompost (1:1:1).

In the greenhouse where the plantlets were stabilized, the condition was different where the relative humidity was lower and light level was higher. Plants growing in natural environments must seek a balance between water uptake and the water loss from leaves due to evaporation (Wang *et al*., 2016). All of the conditions contributing to stressful environment to micro propagated plants when compared to *in vitro* conditions

(Hazarika, 2003). Thus, the transferred micro propagated plantlets were susceptible to various factors of stress that can reduce their survival rate. This is due to improper development of morphological and physiological features of the plants during in vitro cultivation (Chaves, 1994). In order to encounter the problems, gradual acclimatization of plantlets to *ex vitro* environment was made.

Consequently in this study, gradual exposure to the environmental humidity was performed. The acclimatization process must be carried out under controlled environment such as the greenhouse where the requirement for plant growth like temperature, light, and air humidity are adequate for a gradual hardening of the plantlets (Scaranari *et al*., 2009). It was done by keeping the plantlets in the pots containing substrates of interest in the culture room at 24 ± 1°C with photoperiod of 16 hours light and 8 hours dark for 15 days with the cover of transparent plastic with small holes punctured. After 15 days, the transparent plastic covers were removed and the plants were transferred to shade place. After 15 days, the transparent plastics were removed and the plants were transferred to shade place. After 7 days in the shade place, the plantlets were transferred to bigger polybags and moved to a place with more sunlight. Here, the plants were watered every day. This was done in order to structure of the of the plantlets to normal physiological condition of the plants in their natural environment.

There are many techniques that can be done for hardening process for *in vitro* plantlets. Among them using mist, fog, or high humidity combined with shading will reduce water loss thus increase the survival rate. Light levels can be gradually raised and relative humidity gradually lowered as the plantlets grow new leaves and their shoots elongate. Eventually, plants will thrive in ambient conditions. Soil additives like addition of mycorrhizal fungi also, one of the factors in suggesting the best way in

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aiding the acclimatization process since it can aid in sanitation and survival (Wang *et al*., 2016). Scaranari *et al*., (2009) used gradual exposure foe development of banana plantlets using cloth as the shades. The plantlets were exposed to the light condition gradually by covering the surface with different color of the shade cloth which are red (70%, 50%, and 30% shade) and black shade cloth at (50% shade), where both shades used transparent plastic film.

Cucumis melo L. have high carotenoid content in the fruit and have high chlorophyll content in the plant. In the current study, the pigments of interest extracted were carotenoids and chlorophyll with different types of solvent. The freeze dried fruit flesh of *Cucumis melo* L. crude extract produced carotenoid compound with bright yellow pigment. The freeze dried leaves crude extract of *Cucumis melo* L gave dark green pigment. Generally, the samples to be extracted must be homogenized in order to obtain the highest yield of the compound of interest. The freeze dried sample retain high levels of phenolic compound in plant samples when compared to air drying (Dai and Mumper, 2010).

There were three types of solvents used in order to extract both pigments which are ethanol with 5% of water, pure methanol and pure acetone. The results showed that the amount of carotenoids and chlorophyll extractant are different with the different types of solvents with different sample to solvent ratio. The results showed the amount of both pigments extracted was higher using acetone, followed by methanol and ethanol at different sample to solvent ratio (Table 7.2). Based on the results obtained (Table 7.2), both pigments were best extracted with acetone followed by ethanol and methanol. After an overnight of extraction, acetone gave the highest amount of carotenoids and chlorophyll extracted which are 9.38 µg/ml and 139.87 (µg/ml) respectively using ratio of 1 g of sample in 50 ml of acetone. This is in line with the findings by Costache *et al*.,

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(2012) where methanol and acetone were the best solvents to be used when extracting chlorophyll-a, chlorophyll-b and carotenoids of different types of vegetables when compared to diethyl ether. However, due to methanol toxicity, the extraction with acetone is preferred hence, providing reliable results that must be corrected with a suitable coefficient according to the vegetable species (Costache *et al*., 2012).

However, Sumanta *et al*., (2014) revealed that the best solvent for chlorophyll extraction was diethyl ether in their study compared to the chlorophylls and carotenoids extraction from fern species using different types of solvents. It was found that the extraction yields reaction depends on the chemical nature of biomolecules of chlorophyll-a and chlorophyll-b and carotenoids of the plants (Sumanta *et al*., 2014).

The solvents selection is an important factor to be considered because it determined the degree of affinity towards the compounds to be extracted (Henriques *et al*., 2007). Other than the dissolution ability factor of solvents towards the compound to be extracted, the solvent also play an important role in cell lysis (Henriques *et al*., 2007). Jamur and Oliver, (2010) stated that in the experiment to detect intracellular antigen, the organic solvent dissolved lipids from the cell membranes making them permeable to antibodies and because the organic solvents coagulate proteins, they can be used to fix and permeabilize cells at the same time. This would tend to suggest that the solvents used in this study had increase the permeability of the cells membranes and disrupted the protein in the phospholipid bilayer.

The ratio of the samples and solvents extraction also plays an important factor in order to get the highest content of the compound. The extraction using different chemical solvents play an important role because different chemical solvents have different polarities, extraction time temperature and sample to solvent ratio. Different samples or different plant materials may yield varying compounds (Dai and Mumper,

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2010). Therefore, the extraction procedure must be determined in order to gain the highest yield of interest compound. Other than that, natural pigments extraction with organic solvents generates a large amount of waste (Ribeiro and Olivera, 2013). This suggests finding the best solvents and the best sample to solvent ratio to be used that will minimize the amount of waste.

For the subsequent experiment, acetone was used as the extraction solvent for both samples. 1 g of the samples were extracted with 50 ml of acetone and left overnight for the extraction. Sumanta *et al*. (2014) reported that acetone have rapid extraction during 24 hours and gradually declined after that for both carotenoids and chlorophylls. The filtered samples then treated with different pHs which are pH 3, 6, 9, 12. Figures 7.3 and 7.4 show the colours of carotenoids and chlorophyll pigments were treated with different pH.

Based on the resuts obtained, carotenoids gave clearer color at lower pH which is pH 3 and more turbidity at highest pH which was pH 12 but no noticeable reaction at pH 6 and pH 9. Boon *et al*, (2010) revealed that the degradation of carotenoids by sulfuric acid occured at pH 3 - 3.5. The clear color of carotenoids at low pH have been found to produce ion pair that can dissociate carotenoid carbocation . The turbidity of the colors can be caused by the reaction of carotenoids towards the changes of *trans*-isomer to *cis*-isomer of plant where the isomers is very unstable and lead to color degradation . The carotenoids were found to yield the highest after 96 hours. Whilst, the longest carotenoids pigment durability obtained was with the pigments treated with pH 9 where it was found to be the last one to fade. The findings were in agreement with Sims *et al.* (1993) whereby the carrots juice were found to be acidified to pH 4 and 5 with citric acid solution. However heat treatment must be applied, and without heat treament color improvement was not significant.

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From Figure 7.1 and 7.2 there was no obvious or significant visible changes of the color when the chlorophyll extractants were treated with different pH which are pH 6, pH 9 and pH 12, except for the pH 3, when the pH become more acidic, the dark green of chlorophyll color turned to olive green. This is because, the chlorophyll was converted to pheophytins where the magnesium atom (Mg^{+2}) is lost. Hydrogen ion can transform the chlorophylls to their corresponding pheophytin and pheophorbid by the substitution of magnesium ion in the poryphytin ring. Conversion of the color from green to olive color or to dull color considered as low quality where the pigment structure was no more stable (Andres-Bello *et al*., 2013). Due to results of color durabilty, the pigments treated with pH 9 was the last to fade, followed by pH 12, pH 3 and control. At higher pH conditions the methyl and phytyl esters are removed, producing chlorophyllin which is a bright green color. This would suggest that pH can affect the color durability of both pigments. For the subsequence experiment, the best pigment treated with the best pH at the best extraction time were chosen to be applied on the coated slides and then subjected to a weathering test which was heating test. This preliminary studies can provide additional information about the various extraction parameters to be considered.

Before the heating test, the carotenoids and chlorophyll coated slides surfaces showed an even, shiny , neither crack nor peel off appeareance observed. This is in line with the results obtained by Yaacob *et al*., (2011). The coated glass slides were exposed at different temperature which are 27°C (room temperature), 50 °C, and 100 °C. The 50 °C and 100 °C temperatures were set up in an oven. The coated slides were placed in an oven. There was no specific time limit applied. The colour fadedness and the conditions of the coatings were observed with 15 minutes interval (Figure 7.5 and 7.6).

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Based on the results obtained, the colour fadedness was greater in increasing temperature, whereby obvious spectrophotometric reading decreased. Specifically, the carotenoids and chlorophyll colours were most stable at room temperature, followed by exposure to 50 °C, and totally degraded in 100 °C.

Other than natural sources of pigments, the extracts of higher plants also can be a very good source of antiobiotic (Cowan, 1999). Different plants gave different antimicrobial activities where different zone of inhibition were observed (Jadhav *et al*., 2013). It was found that the most effective solvent in extraction was methanol (Iranbakhsh *et al*., 2010) due to the ability to extract the substance or compound that took part in the bactericidal role. Previous research on antimicrobial activity from callus extracts showed many successful results in the reaction against the microorganisms. The callus extracts revealed antimicrobial activity against tested microorganism. Antimicrobial activity extracted from *Cucumis anguria* L. showed the *in vitro* plants extracts have higher activity against *S.aureus*, *E. coli*, and *P. aeruginosa* when compared to *in vivo* plants (Thiruvengadam and Chung, 2015). The extract solvent also playing an important role when Iranbaksh, (2010) found that methanol as the best solvent used for extracting the compound that have a role in as antibacterial. Study by Sasi *et al*., (2014) showed that ethanolic extract of *Cucumis melo* fruit contain high bioactive compound and there were antimicrobial activity observed against all tested microorganism tested which are *Salmonella typhi, Escherichia coli, Klebsiella pneumonia, Bacillus cereus, and Staphylococcus aureus.* Based on the current studies, the callus methanolic extracts was found to have antimicrobial activity against *Escherichia coli, Bacillus subtillis*, and *Staphylococcus aureus* and *Penicillium* sp, where inhibition zones were obviously observed (Table 8.2) . Bibi *et al*., (2011) reported the bioactive compound extracted from the callus of *Centella asiatica* was about the same when compared to plants grown *in vivo*. According to the recent finding

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by Ozdemir *et al*., (2016), *Scutellaria. orientalis* subsp. *bicolor* calli extracts shown highest antimicrobial activity on *Staphylococcus aureus* COWAN 1 and *Bacillus subtilis* var. *niger* ATCC 10.

It is hoped that the present study on micropropagation, pigments extraction and antimicrobial activity of *Cucumis melo* L. will contribute to the existing knowledge of this plant.

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CHAPTER 10: CONCLUSION

The present study dealt with *in vitro* regeneration, callus induction from various explants, effect of different hormones on callus induction and somatic embryogenesis, acclimatization, pigments extraction and antimicrobial activity of *Cucumis melo* L. The general aims of these studies were to investigate the micropropagation of *Cucumis melo* L. and the study of pigments and antimicrobial activity extracted from *Cucumis melo* L.

The seeds were obtained from one Ninetop Trading, a supplier of *Cucumis melo* L. seeds from Rawang, Selangor. The seeds were successfully germinated on MS media in both horizontal and vertical positions, however, the rate of germination on horizontal position was observed better since 100% of seeds were germinated in shorter time. *In vitro* regeneration of *Cucumis melo* L. was achieved using natural cytokinin, coconut water and 2,4-D as the source of auxin. Among the various concentration combinations of coconut water and 2,4-D, the best treatment was best observed when the MS medium supplemented with 25% of coconut water with 1 mg/L of 2,4-D where the normal growth of shoots, and normal white fibrous and thin roots were observed. At higher concentration of 2,4-D (2.0 - 3.0mg/L) and coconut water (35% - 45%), results obtained showed abnormal growth of *in vitro* regeneration where the stem became swollen and the roots were less fibrous. The ability of the explants to regenerate for rapid multiplication for pilot scale industry is of great benefit.

The explants screening is one of the most crucial steps. In the study, there were 4 types of explants screened which were, hypocotyls, cotyledons, nodes and leaves. All of the explants screened for callus induction and results obtained showed that all types of explants were able to induced callus except leaf explants. Callus production of different explants gave different proliferation rate whereby the highest callus induction frequencies were observed from hypocotyls (100%) and nodes explants (100%) and the

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least was cotyledon (76.67%). The time taken for callus production also different between the explants where the earliest callus induced from hypocotyls explant (day7-9 days after cultured) followed by nodes explants (day 9-12 after cultured) and cotyledon explant (after day 15).

Callus production was considered as the first step in somatic embryogenesis process. Thus, in order to induce embryogenic callus, the nodes and hypocotyls were used as explants and various combinations and concentrations of hormones were applied in this study. Among the various combinations and concentrations of hormones, Node explants successfully induced embryogenic callus where shoots formation was observed from the callus. The highest shoots formation was observed from the callus induced on MS media supplemented with 2 mg/L BAP in combination with 1 mg/L 2,4-D (100.00 %) with the average rate of the shoot formation 13.10 ±0.36 per explant. However, hypocotyls explants produced non-embryogenic callus on MS media supplemented with BAP and 2,4-D. High the rate of callus proliferation observed was high where the highest callus fresh weight obtained from MS media supplemented with 1 mg/L of BAP in combination with 1mg/L 2,4-D where the mean weight of callus fresh weight is 7.53 ± 0.18 g per explant. On MS medium supplemented with IAA and Kin, hypocotyls explants induced callus with roots formation observed where the highest roots formation exhibited by MS media supplemented with 3 mg/L IAA with the percentage of callus inducing roots 86.67% with the rate of root formation is 4.20 ±0.50 per explant. Nodes explants cultured on MS media supplemented with 0.1M Mannitol also induced callus with shoot formation where the highest shoots formation was 76.67 %. Due to the ability of the explants and treatments that gave high production of callus in the studies, there will be many potential research can be done for novel finding of the beneficial properties.

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Acclimatization is the last step of tissue culture and is very crucial step to ensure the success of micropropagation. After successful rooting (93.33%) of the plantlets on MS medium without plant growth regulators the plantlets were transferred to soil substrates for adaptation and further growth under natural environment. However, in this study due to a good care of the plantlets during the gradual exposure to the natural environment, the acclimatization of plantlets from *in vitro* direct regeneration and plantlets regenerated from somatic embryogenic callus were successfully done. In the study, there are three types of substrate which are, black soil, black soil with red soil (1:1), and cocopeat. The black soil was recorded as the best substrates followed by black soil with red soil (1:1) for acclimatization of plantlets from both sources. Plantlets from *in vitro* regenerated sources exhibited 83.33% survival percentage and plantlets from embryogenic callus portrayed 63.33 % of survival percentage. Cocopeat substrate exhibited as the least suitable substrate for acclimatization of plantlets from *in vitro* regenerated source 33.33% and regenerated plantlets from somatic embryogenesis 16.67%. Based on the studies, gradual exposure of plantlets to natural environment is very important during hardening in order to guarantee the higher rate of plants survival during acclimatization and further growth.

It is well known that pigments are found in C*ucumis melo* L. fruits and plants. The extractions of pigments in this study showed the potential of the pigments extracted from fruit flesh and leaves of *Cucumis melo* L. to be applied as a source of nature coating material. Based on this study, results showed that there were significant effects of types of solvent and the ratio of solvents to sample used in the extraction of both pigments. Among the three types of solvents used for extraction, it was found that acetone was the best extraction solvent for carotenoid and chlorophyll pigments. Further treatment on the extracted pigments at different pH in order to find the stability of the pigments extracted showed that pH 9 was the best pH to be applied due to the last

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for the colors to fade. Although there is no significant visible color changes, pH played an important role since the color durability of the pigments affected when different pH was applied. It was also found that carotenoids could mix well with PMMA when applied on glass slides. The application of coating on the glass slides exhibited the potential of the extracted pigments to be used as natural colorant source. The endurance test of the coated slides was done by exposing the coated slides to different heat treatment where carotenoids color faded from yellow to white with increasing temperature. However, for chlorophyll, the color changed from green to light green, brown and orange at room temperature, 50°C and 100 °C, respectively.

It was found that, callus induced have antimicrobial activity. The study on the antimicrobial activities from callus of *Cucumis melo* L. also revealed there is potential antimicrobial activity contained in the callus compound. The callus extracts exhibited the high ability against *Bacillus subtilis* and *Penicillium* sp. where the strength of the activity was similar with the standard positive control. This could be an added advantage when there is beneficial compound found in the callus because callus can be produced in high scale with minimum time. However, further studies are needed in order to find the best isolated compound and to evaluate extracts activity against more types of microorganisms.

There are future works to be suggested in order to make the research more beneficial of this species like, the studies on molecular biology, synthetic seeds, phytochemical identification and production of beneficial secondary metabolites products which will add a lot advantages in order to make the maximum of this plant.

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List of Publications and Papers Presented

Papers submitted/published

1. **The effects of different solvent extraction and pH on the stability of carotenoids and chlorophyll in *Cucumis melo* L. for potential coating technology.**

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   Accepted by Pigment & Resin Technology (ISI-Cited Publication)

Conferences attended

1. **ICFMD 2015: 5th INTERNATIONAL CONFERENCE ON FUNCTIONAL MATERIAL AND DEVICES 2015 (4th - 6th August 2015) NEW YORK HOTEL, JOHOR BAHRU, MALAYSIA**

   Poster entitile: Influence of various extraction parameters on pigments production from *Cucumis melo* L. for potential coating technology

2. **ICBT 2015: 17th International conference on Biotechnology- Madrid, Spain (12-13th November, 2015), 12 Nov 2015 to 13 Dec 2015, WASET (World Academy of Science Engineering and Technology, International Science Index, (International).**

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